CELLULAR MECHANISMS OF THE SYSTEMIC INFLAMMATORY RESPONSE FOLLOWING RESUSCITATED HEMORRHAGIC SHOCK: THE ROLE OF REACTIVE OXYGEN SPECIES AND TOLL-LIKE RECEPTOR 4

BY

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Institute of Medical Science, University of Toronto

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Acute Respiratory Distress Syndrome (ARDS) following hemorrhagic shock/resuscitation (S/R) is an important contributor to late morbidity and mortality in trauma patients. S/R promotes ARDS by inducing oxidative stress that primes cells of the innate immune system for excessive responsiveness to small inflammatory stimuli, termed the “two-hit” hypothesis. Activated alveolar macrophages (AM) play a central role and when recovered from S/R animals exhibit an exaggerated responsiveness to lipopolysaccharide (LPS) with increased activation of the proinflammatory transcription factor NF-κB, and augmented expression of cytokines. LPS triggers AM signalling through Toll like receptor 4 (TLR4), which resides in plasma membrane lipid rafts.

The objective of this work is to define cellular mechanisms of macrophage priming by oxidative stress following shock resuscitation. The main hypothesis investigated is that altered cellular distribution of TLR4 can lead to macrophage priming and antioxidant resuscitation strategies can diminish these effects.

AM of rodents, exposed in vivo to oxidant stress following S/R, increase their surface levels of TLR4, which in turn results in augmented NF-κB translocation in response to small doses of LPS. Furthermore, in vitro H$_2$O$_2$ treatment of RAW 264.7 macrophages results in similar TLR4 surface translocation. Depletion of intracellular calcium, disruption of the cytoskeleton or inhibition of the Src kinases prevents the H$_2$O$_2$-induced TLR4 translocation, suggesting the involvement of receptor exocytosis. Further, fluorescent resonance energy
transfer between TLR4 and lipid rafts as well as biochemical raft analysis demonstrated that oxidative stress redistributes TLR4 to surface lipid rafts. Preventing the oxidant-induced movement of TLR4 to lipid rafts using methyl-ß-cyclodextrin precluded the increased responsiveness of cells to LPS after H₂O₂ treatment. Further, AM priming by oxidative stress can be diminished by early exposure to resuscitation regimens with direct or indirect systemic antioxidant effects, such as 25% albumin, N-acetylcysteine and hypertonic saline. Hyperosmolarity was found to modulate AM TLR4 gene and protein expression.

Collectively, these studies suggest a novel mechanism whereby oxidative stress might prime the responsiveness of cells of the innate immune system. Targeting the TLR4 signalling pathway early during shock resuscitation may represent an anti-inflammatory strategy able to ameliorate late morbidity and mortality following S/R.
ACKNOWLEDGEMENTS

This thesis is a small tribute to my exceptional parents, Antonina and Wojciech, who continue to guide my life through their wisdom and love, to my son Elliott, who is my source of daily inspiration and belief in things seemingly impossible and to my sisters Dominika and Berenika who epitomize caring and friendship.

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Dr. Harvey Cushing, the pre-eminent Harvard Surgeon and researcher, once noted that the true potential of a student is only revealed when “under stress and responsibility, he breaks through his educational shell, and he may then be a splendid surprise to himself no less then to his teachers”. The last years have provided me with plenitude of stresses responsibilities and surprises all of which have helped me “break through my educational shell” and lead me to grow as a doctor, a scientist and as a person for which I am forever grateful.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SECTION I  TRAUMA AND THE SEQUELA OF HEMORRHAGIC SHOCK</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SECTION II  TRAUMA RESUSCITATION HISTORY, CURRENT PRACTICE AND ADVANCES</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>SECTION III  THE ROLE OF OXIDANT STRESS IN SIRS, MODS AND ARDS</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>SECTION IV  INNATE IMMUNITY AND TOLL-LIKE RECEPTORS: MOLECULAR MECHANISMS OF SEPSIS</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>SECTION V  PLASMA MEMBRANE COMPOSITION - IMPLICATIONS FOR HOST-PATHOGEN INTERACTIONS</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>SECTION VI  OXIDATIVE STRESS EFFECT ON RECEPTOR TRAFFICKING</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>OBJECTIVE, HYPOTHESIS, OVERVIEW OF THESIS CHAPTERS AND THEIR RELEVANCE TO THE HYPOTHESIS</td>
<td>93</td>
</tr>
<tr>
<td>3</td>
<td>OXIDATIVE STRESS GENERATED BY HEMORRHAGIC SHOCK RECRUITS TOLL-LIKE RECEPTOR 4 TO THE PLASMA MEMBRANE IN MACROPHAGES</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>SUMMARY</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>INTRODUCTION</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>MATERIALS AND METHODS</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>RESULTS</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>DISCUSSION</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>FIGURES</td>
<td>116</td>
</tr>
<tr>
<td>4</td>
<td>REACTIVE OXYGEN SPECIES INDUCE SRC KINASE DEPENDENT SURFACE UPREGULATION OF TOLL LIKE RECEPTOR 4 (TLR4) IN RAW 264.7 MACROPHAGES</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>SUMMARY</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>INTRODUCTION</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>MATERIALS AND METHODS</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>RESULTS</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>DISCUSSION</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>FIGURES</td>
<td>136</td>
</tr>
<tr>
<td>5</td>
<td>25% ALBUMIN, AS AN ANTIOXIDANT, PREVENTS LUNG INJURY FOLLOWING SHOCK RESUSCITATION</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>SUMMARY</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>INTRODUCTION</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>MATERIALS AND METHODS</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>RESULTS</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>DISCUSSION</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>FIGURES</td>
<td>160</td>
</tr>
<tr>
<td>6</td>
<td>HYPERTONIC RESUSCITATION OF HEMORRHAGIC SHOCK PREVENTS SYSTEMIC OXIDATIVE STRESS, UPREGULATES THE ANTI-INFLAMMATORY RESPONSE AND MODULATES TLR4 EXPRESSION IN MACROPHAGES</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>SUMMARY</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>INTRODUCTION</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>MATERIALS AND METHODS</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>RESULTS</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>DISCUSSION</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>FIGURES</td>
<td>197</td>
</tr>
</tbody>
</table>
GLOSSARY
µg: microgram
µl: microliter
AAA: Abdominal Aortic Aneurism
ACTH: Adrenocorticotropic hormone
ALI: Acute Lung Injury
AM: Alveolar Macrophages
ANOVA: Analysis of Variance
ARDS: Acute Respiratory Distress Syndrome
A25: 25% Albumin
A5: 5% Albumin
BAPTA/AM: 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester;
BAL: bronchoalveolar lavage
BALF: broncho-alveolar lavage fluid
C1: complement protein C1
C3: complement protein C3
C5a: complement protein C5a
C57BL: An Inbred Strain of Mouse
CARS: compensatory anti-inflammatory response syndrome
cDNA: complementary deoxyribonucleic acid
CHO: Chinese hamster ovary cells
CINC: Cytokine Induced neutrophil Chemoattractant
CNS: Central Nervous System
CM: Confocal Microscopy
CRP: C Reactive Protein
CXCR4: chemokine receptor 4
CTxB: cholera toxin B;
DMEM: Dulbecco’s Modified Eagle Medium
DNA: deoxyribonucleic acid
DO2: delivery of oxygen (ml O2/min)
DTT: DL-Dithiothreitol
EIA: eicosanoid immunoassay
ELISA: Enzyme-Linked Immunosorbent Assay
ERK: Extracellular signal-related kinase
ETs: Endothelins
FCS: Fetal calf serum
FITC: Fluorescein isothiocyanate
FiO2: inspired fraction of oxygen
FRAP: Fluorescence Recovery After Photobleaching
FRET: Fluorescence Resonance Energy Transfer
GDF5: growth differentiation factor 5
GLUT4: glucose transporter-4
GRO: Growth Related Oncogene
Hgb: Haemoglobin
HTS: hypertonic saline
HS: Resuscitated hemorrhagic shock
Hsp: Heat shock protein
HSP70: Heat Shock Protein 70
HSP90: Heat Shock Protein 70
H2O2: Hydrogen peroxide
ICAM-1: intracellular adhesion molecule-1
ICAM-2: intracellular adhesion molecule-2
ICU: Intensive Care Unit
IF: Immunofluorescence
IgG: Immunoglobulin G
IL-1: Interleukin 1
IL-1RA: Interleukin 1 receptor antagonist
IL-4: interleukin 4
IL-6: Interleukin 6
IL-10: Interleukin 10
IL-13: Interleukin 13
IL1-β: Interleukin 1 beta
IF: Immunofluorescence
IFN-α: Interferon-alpha
IKK: I kappa B kinase
IL-8: Interleukin 8
IP: Inducible Protein
IRAK: Interleukin-1 receptor-associated kinase 4
kD: kilo dalton
kg: kilogram
LPS: Lipopolysaccharide
LTA: Lipoteichoic acid
LTB4: Leukotriene-B4
IRAK4: IL-1 receptor-associated kinase 4
JASPA: Jasplakinolide
MAP: mean arterial pressure
mAb: monoclonal antibody
MβCD: methyl-β-cyclodextrin
MHC: major histocompatibility complex
MIP-2: Macrophage Inflammatory Protein-2
mL: millilitre
mmHg: millimetres of mercury
Mφ: Macrophage
MODS: multiple organ dysfunction syndrome
MOF: multiple organ failure
mOsm: mili Osmolar
mosmol: mili osmol
mRNA: messenger ribonucleic acid
MVC: Motor Vehicle Collisions
MyD88: Myeloid differentiation primary response gene 88
NAC: N-acetylcysteine
NAD⁺: Nicotinamide adenine dinucleotide
NADH: Reduced form of Nicotinamide adenine dinucleotide
NADP⁺: Nicotinamide adenine dinucleotide phosphate
NADPH: nicotinamide adenine dinucleotide phosphate-oxidase
NEM: N-ethylmaleimide
NF-κB: Nuclear Factor κ B
NIK: NF-κB inducing kinase
NO: Nitric Oxide
O₂: Oxygen
OXS: Oxydative Stress
PaCO₂: arterial partial pressure of carbon dioxide
PAF: Platelet-Activating Factor
PAI-1: Plasminogen Activator Inhibitor
PAMPS: Pathogen associated molecular patterns
PBS: Phosphate buffered saline
PE: Phycoerythrin
PEEP: Positive End Expiratory Pressure
PGA: Proteoglycan
PMNs: Polymorphonuclear cells, neutrophils
RAW 264.7: murine macrophage cell line established from ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV)
RL: Ringer's Lactate
ROS: Reactive Oxygen Species
sIL-6: Soluble IL-5 receptor
SIRS: Systemic Inflammatory Response Syndrome
ScvO₂: venous catheter oxygen saturation
SNARE: soluble N-ethylmaleimide sensitive factor attachment receptor
S/R: shock/resuscitation
SvO₂: venous oxygen saturation
TF: Tissue Factor
TFPI: Tissue Factory Pathway Inhibitor
TGFβ: Transforming growth factor β
T₇H1: T helper 1
TLR4: Toll Like Receptor 4
TM: Thrombomodulin
TNF: Tumour Necrosis Factor
TNFα: Tumour Necrosis Factor α
US: United States
VCAM-1: vascular cell adhesion molecule-1
VO₂: consumption of oxygen (ml CO₂/min)
V/Q: Ventilation-perfusion
XD: Xanthine dehydrogenase
XO: Xanthine oxidase
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Structure of TOLL-LIKE RECEPTORS (TLRs) and recognizing molecules</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>Toll-like receptor 4 (TLR4) signalling pathway</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>Domain-length scales and the biomembrane as</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>Redistribution of TLR4 in alveolar macrophages following resuscitated hemorrhagic shock</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Resuscitated hemorrhagic shock increases TLR4 surface levels</td>
<td>116</td>
</tr>
<tr>
<td>6</td>
<td>Oxidative stress induces increased TLR4 surface expression in RAW 264.7 cells</td>
<td>117</td>
</tr>
<tr>
<td>7</td>
<td>Oxidative stress induces clustering of TLR4 in plasma membrane lipid rafts</td>
<td>118</td>
</tr>
<tr>
<td>8</td>
<td>Recruitment of TLR4 into lipid rafts by oxidative stress</td>
<td>119</td>
</tr>
<tr>
<td>9</td>
<td>Fluorescence resonance energy transfer (FRET) verifies oxidant-induced molecular interaction between TLR4 and GM1</td>
<td>120</td>
</tr>
<tr>
<td>10</td>
<td>Role of exocytosis in TLR4 translocation to the plasma membrane following oxidativestress</td>
<td>121</td>
</tr>
<tr>
<td>11</td>
<td>Oxidative stress induces colocalization of TLR4 and MyD88 in the plasma membrane</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>The role of lipid rafts in NF-kB translocation</td>
<td>122</td>
</tr>
<tr>
<td>13</td>
<td>The effect of PP2 on the H2O2-induced increased cell surface expression</td>
<td>123</td>
</tr>
<tr>
<td>14</td>
<td>The effect of Csk expression on H2O2-induced increased cell surface expression</td>
<td>124</td>
</tr>
<tr>
<td>15</td>
<td>Effect of Src kinase inhibition on oxidant-induced TLR4 translocation into lipid rafts</td>
<td>125</td>
</tr>
<tr>
<td>16</td>
<td>Serum osmolarity and calcium concentrations</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Mean arterial blood pressure and urine output following shock/resuscitation</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Transpulmonary protein flux</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Bronchoalveolar lavage fluid neutrophil count</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Representative photomicrographs of lung histology after resuscitation</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>CINC mRNA expression in whole lung tissue following different resuscitation regimens</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Shock/LPS-induced NF-kB nuclear translocation in macrophages</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Effect of albumin resuscitation on CD11b expression on rat neutrophils</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Effect of albumin resuscitation on L-selectin expression on rat neutrophils</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>The effect of 25% on PMN CD11b expression</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Effect of albumin resuscitation on ICAM-1 expression in rat lungs</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Immunohistochemical staining of ICAM-1 on pulmonary endothelium after</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Plasma 8-isoprostanate levels following different resuscitation regimens</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Effect of NEM modified albumin on bronchoalveolar lavage fluid neutrophil count</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>The effect of Hypertonic Saline resuscitation on neutrophil sequestration in the lung as a measure of acute lung injury following shock, resuscitation, and lipopolysaccharide (LPS).</td>
<td>151</td>
</tr>
<tr>
<td>31</td>
<td>Representative photomicrographs of small intestine histology after resuscitation</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Plasma 8-isoprostanate levels following different resuscitation regimens</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>The effect of Hypertonic Saline or antioxidant NAC resuscitation on LPS induced</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>The effect of Hypertonic Saline or antioxidant NAC resuscitation on LPS induced</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>The effect of Hypertonic Saline Resuscitation on TNF-a production by rat alveolar macrophages with and without LPS stimulation <em>EX Vivo</em></td>
<td>197</td>
</tr>
<tr>
<td>36</td>
<td>The effect of Hypertonic Saline Resuscitation on IL-10 production by rat alveolar macrophages with and without LPS stimulation <em>EX Vivo</em></td>
<td>198</td>
</tr>
<tr>
<td>37</td>
<td>The effect of Hypertonic Saline Resuscitation on IL-10 production by rat alveolar macrophages with LPS stimulation in vivo</td>
<td>199</td>
</tr>
<tr>
<td>38</td>
<td>The effect of endotracheal anti IL-10 neutralizing antibody on neutrophil sequestration in the lungs as a measure of acute lung injury following shock, resuscitation and LPS.</td>
<td>200</td>
</tr>
<tr>
<td>39</td>
<td>The effect of HTS resuscitation on TLR4 distribution in alveolar macrophages <em>EX Vivo</em></td>
<td>201</td>
</tr>
</tbody>
</table>
DISSEMINATION OF THESIS CONTENTS

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<table>
<thead>
<tr>
<th>Number</th>
<th>Event Description</th>
<th>Location Details</th>
<th>Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.</td>
<td>Annual Assembly of Surgeons and Residents,</td>
<td>University of Toronto, April 2002 and 2003, podium.</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
Section I  Trauma and the Sequela of Hemorrhagic Shock

- Societal Burden of Trauma
- Mortality From Trauma
- Sequela of Trauma - an Evolution of Terms: SIRS, Sepsis and MODS
- Multiple Organ Dysfunction Syndrome (MODS)
- Acute Respiratory Distress Syndrome (ARDS)
- Pathogenesis of SIRS Leading to MODS and ARDS
  - The “Two-Hit” Theory of Immune Cell Priming
  - The Influence of Reactive Oxygen Species
  - The Influence of Gut Hypoperfusion
  - The Influence of Endothelial Cell–leukocyte Interaction
- Mediators of the Proinflammatory Response
  - Neutrophils
  - Macrophages
  - Endothelial Cells
  - Humoral pro and anti inflammatory mediators
  - Interleukin-1β, tumour necrosis factor - α and their counter-regulatory ligands and receptors
  - Interleukin-6
  - Transforming growth factor β
  - Chemokines
  - HMGB-1
  - Interleukin-10 (IL-10)
  - Other anti-inflammatory mediators
  - Nitric Oxide
- Cellular and Animal Models of MODS and ARDS
Societal Burden of Trauma

Despite active research on effective prevention strategies and appropriate treatment, trauma remains the number one cause of death in the North American population under 44 years of age and represents the most significant cause of morbidity responsible for a greater number of productive life years lost than cancer, stroke, and heart disease combined (1). Statistics Canada reports that 63% of all deaths under the age of 25 occur from trauma with motor vehicle collisions (MVC) (2;3). In addition to the enormous societal burden, the economical burden is also significant with injury treatment from motor vehicle accidents costing an estimated $7.3 billion in Ontario on 1990 and in the US in excess of 400 billion dollars per year (4;5).

Mortality from Trauma

Mortality following trauma occurs in four phases: prehospital deaths, acute (within 48 hours), early (within 3 to 7 days) and late (after 7 days). 34 to 45% of trauma victims die at the scene of the accident because of overwhelming injury and exsanguinations or hypoxia, hypovolemia or CNS injury. In the acute phase, exsanguination from injuries to the liver, heart, or great vessels was found to be the most common causes of death (51%) followed by Central nervous system (CNS) injuries (40%). In the early phase, CNS injuries were most frequent cause of early death (64%), mostly due to blunt trauma. The late phase mortality occurred due to CNS injuries (39%), sepsis and multiple organ dysfunction syndrome (MODS) (61%) including Acute Respiratory Distress Syndrome (ARDS) (6;7).

Recent studies demonstrate that the mortality from trauma has been significantly reduced in the last 25 years because of a decrease in deaths from MODS with the incidence of MODS staying the same. In 2001, Nast-Kolb et al. demonstrated in their study of 1361 patients, that the overall mortality from blunt multiple trauma decreased significantly by 48% with mortality due to organ failure or multiple organ failure decreased from 18.0% to 4.1% (8). 1999 Pape et al. reported a mortality of 5% due to MODS in 358 multiple trauma patients (9). These studies contrast earlier reports, which still describe a high incidence of MODS and associated mortality. For example, Sauaia et al. in 1998 in a prospective study of 411 multiple trauma patients demonstrated a 19% incidence of MODS with an associated 37% mortality (10). Part of the discrepancy between earlier studies and the more recent ones is the use of different scoring systems for MODS as well as different population studies. However, regardless of what the exact decrease in trauma mortality is, there is no doubt that the approach to trauma patient management has improved in the last 25 years. In the United States, the implementation of
comprehensive trauma systems was able to reduce the mortality rate by 9%, with the effects most evident on analysis of MVC deaths (5). Management guidelines implementation with the emphasis on the multidisciplinary approach has lead to an overall improved care for the trauma patient both in the emergency room and in the Intensive Care Units (ICU). There are several changes that have influenced the mortality from MODS (5). One of the changes includes the quantity and the quality of fluid resuscitation, which has come under scrutiny, as large volumes decrease lung function due to capillary leakage and multiple transfusions have been linked to increased mortality (11;12). However, despite all advances, trauma remains a major cause of morbidity and mortality in our population. Understanding the pathophysiology of hemorrhagic shock, resuscitation and its sequela is critical for further improvements and changes in the resuscitation strategies for the trauma patients. This thesis investigates some of the critical pathophysiology components of MODS and ARDS at a whole organism as well as at the cellular and molecular levels.

**Sequela of Trauma - an Evolution of Terms: SIRS, Sepsis, and MODS**

The current model of the inflammatory response following trauma is based on the concept of the systemic inflammatory response syndrome (SIRS). SIRS is considered an overly active systemic immuno-inflammatory response to various forms of insult to the organism such as resuscitated hemorrhagic shock, burns, severe pancreatitis, intra-abdominal catastrophes, or infection resulting in sepsis. A systemic immuno-inflammatory response is an evolutionarily conserved mechanism of host defence, however if the normal protective response is exaggerated or perpetuated by pathogens or other insults, it can evolve into SIRS that eventually may end in MODS. SIRS is a term for the same physiological response as that is observed during sepsis, except it relates to a host response independent of its cause. The Society of Critical Care Medicine and the American College of Chest Physicians, at the consensus conference in 1992, established a definition of SIRS. SIRS was defined as characterized by two or more of the following: (i) temperature, more than 38°C or less than 36°C; (ii) heart rate, more than 90 beats per minute; (iii) tachypnea: respiratory rate more than 20 or PaCO2 less than 32 mm Hg; (iv) white blood cell count, more than 12,000/mm3 or less than 4000/mm3, or more than 10% immature band forms. In various studies, only 50% or less of patients who are thought to meet criteria for a “sepsis” like syndrome or SIRS end up having documented infections (13). Therefore, “Sepsis” occurs when a documented infection is present and may or may not involve a SIRS response. If organs fail during “sepsis”, the condition becomes “severe sepsis” and
eventually “septic shock” if systolic hypotension occurs despite adequate volume resuscitation. SIRS, regardless if an infection is present or not, with progressive but reversible dysfunction in two or more organ systems is referred to as MODS (14). Dysfunction can occur in any number of organ systems including respiratory, cardiovascular and renal, affected most frequently and others such as hepatic, haematologic, gastrointestinal, endocrine, and CNS.

**Multiple Organ Dysfunction Syndrome (MODS)**

Multiple Organ Dysfunction Syndrome (MODS) occurs due to uncontrolled immuno-inflammatory response and is characterized by generalized and exaggerated inflammation. This process results in cellular damage at the level of the endothelium leading to aberration in endothelial permeability to fluid, protein, and cells as well as to gut bacteria. Coagulation and cytokine cascades are promoted resulting in microvascular thrombosis and infiltration of organs with various leukocytes. Cellular death occurs in local and regional environments secondary to hypoxia causing further release of toxic oxygen species and inflammatory mediators. Historically, the term MODS has evolved from a number of previously described and named phenomena. Tilney *et al.*, in the early 1970, studied patients who survived a ruptured abdominal aortic aneurysm and went on to develop what he termed sequential systems failure (SSF) (15). In 1977, Eiseman *et al* introduced the term “multiple organ failure” referring to sequela of intra-abdominal abscesses in 42 patients (16). Thus, both hemorrhagic as well as septic shock was recognized as the leading causes of MODS. In the 1980’s, however, MODS was thought to arise secondary to an overwhelming infectious process. Performing a laparotomy was common in patients with MODS to rule out intra-abdominal sources of sepsis. Fry and colleagues reviewed 553 patients requiring such emergency laparotomies and found that 7% of these patients, two thirds of whom were trauma victims, developed MOF. 90% of MOF in these patients was due to a septic source (17). Over the next twenty years, reports determined that organ failure may occur in the absence of sepsis (18-20) and may involve incremental organ dysfunction before overt failure occurs. Thus, the current concept of MODS was introduced by Marshall *et al.* (21) and investigation of the non-infectious models of SIRS and its progression to MODS ensued.

Several studies to date concluded that MODS after trauma represents a continuum from mild organ dysfunction to death, however the process itself begins early within 24 hours of the original insult. The temporal sequence of organ failure most commonly begins with ventilatory dysfunction and hypotension, which often leads to renal failure and liver failure. Coagulopathy ensues as well as dysfunction of the gut, endocrine system with hyperglycemia and adrenal
insufficiency followed by CNS dysfunction and eventual death (18). Recent studies demonstrated that it is the severity of organ dysfunction at the initial admission to the ICU that is the most important prognostic factor for the outcome of the patients and that MODS may not necessarily follow a course of progressive and sequential organ failure (22). Since the patients that deteriorate are ones that were most severely ill at the outset, targeting therapies at the initial injurious process represents a window of opportunity to aid these patients. This thesis focuses on the cellular and molecular mechanisms that occur during the early window following shock resuscitation in trauma with the aim of improving understanding and development new treatment strategies based on the pathophysiology of SIRS progression to MODS and death. Since the lung is one of the earliest and most commonly encountered organs involved in MODS, this thesis focuses on the pathogenesis of ARDS following trauma.

**Acute Respiratory Distress Syndrome (ARDS)**

The lungs are one of the initially affected and most frequently affected organs in MODS following hemorrhagic shock. Acute Respiratory Distress Syndrome (ARDS) occurs in up to 50% of MODS affected patients (23) and remains one of the leading causes of morbidity and mortality in all ICUs. ARDS has been estimated at 190,000 cases per year in the United States with 74,000 deaths annually (24). Despite sophisticated advances in the ICU care, the survival rates although improved from about 40% in the 1970s, still remains a low of 60% today (25).

ARDS was first described in 1967 by Ashbaugh et al. and initially the syndrome was called Adult Respiratory Distress Syndrome to distinguish it from neonatal disease (26). Since 1994, the American-European Consensus Conference Committee introduced a new definition and changed the name to Acute Respiratory Distress Syndrome. ARDS is a severe form of Acute Lung Injury (ALI) a continuum of pathological response of pulmonary parenchymal injury (27). Clinically ARDS is a form of acute infiltrative lung disease represented largely by interstitial pulmonary edema, with the resulting ventilation-perfusion (V/Q) mismatching, as alveoli are fluid filled and the interstitium edematous not allowing for oxygen diffusion. It develops within 12 to 72 hours after the initial insult and is characterized clinically by respiratory distress, hypoxemia and generalized bilateral pulmonary infiltrates on chest radiographs. Most ARDS definitions include hypoxemia despite increasing fraction of inspired oxygen (FiO₂) and express it in terms of a ratio between arterial and alveolar oxygen tensions (PaO₂/PAO₂ ratio). A PaO₂/FiO₂ ratio of 200 to 300 mmHg signifies ALI, whereas a ratio <200 mmHg is considered ARDS. When diagnosing ARDS, congestive heart failure with pulmonary
capillary hypertension must be ruled out and a pulmonary capillary pressure of <18 mmHg obtained with no evidence of elevated left heart filling pressures.

In general, the etiologies of ARDS can be divided into indirect causes and direct causes. Some of the indirect causes are hemorrhagic shock, sepsis, burns, multiple fractures, drug overdose, multiple transfusions, cardiopulmonary bypass, eclampsia, disseminated intravascular coagulation, acute pancreatitis, air, or amniotic fluid embolus. There are also direct causes of ARDS, which include aspiration pneumonia, pulmonary contusion, toxic inhalation, and near drowning. Some studies suggest that ARDS following trauma may differ from ARDS of all other etiologies in that it can occur in two forms: early and late onset. The early onset ARDS is associated with a less severe course and its severity depends on the magnitude of the initial insult i.e. hemorrhagic shock. Conversely, the late onset ARDS follows a more virulent course and is more commonly related to other causes then the initial insult, such as sepsis, pneumonia and MODS (28). Although ARDS may be initiated by a variety of insults, the histopathological picture and the clinical presentation of this disease are remarkably similar. These changes can be observed under the microscope at different stages of the disease (29). In the acute stage, inflammatory cells infiltrating the alveolar space release granular enzymes and oxidants injure sensitive Type I alveolar epithelial cells as well as pulmonary vascular endothelial cells. Damaged alveolar basement membrane and endothelium becomes more permeable. This process allows interstitial and alveolar edema to develop. Fibrin deposition occurs and the alveolar walls become lined with waxy hyaline membranes consisting of fibrin-rich edema fluid mixed with the cytoplasmic remnants of necrotic epithelial cells. Edema distorted alveoli lose their surfactant function and microatelectasis occurs leading to hypoxemia as well as to low lung compliance and reduced functional residual capacity.

In the proliferative phase, week 1 to 3 of the clinical ARDS alveolar type II cell that are more resistant and survive the acute phase begin regenerate the alveolar epithelium. Fibroblasts proliferate and migrate through the basement membrane and organization of the fibrin exudate occurs with resultant intra-alveolar fibrosis. Marked thickening of the alveolar septa ensues, caused by proliferation of interstitial cells and deposition of collagen.

In the fibrotic phase, there is loss of pulmonary microvasculature, intravascular thrombosis, and further distortion of septal architecture that is responsible for clinical pulmonary hypertension, increased dead-space ventilation, and a reduction in carbon monoxide diffusing capacity.
Although complete resolution of the fibrotic process is uncommon, sometimes, the fibrinous exudates may resolve and interstitial fibrosis of the lung is avoided. The recovery of normal lung functions is possible, but it is not known what factors determine the exact restoration of the normal pulmonary architecture after ALI/ARDS. The degree of fibrosis has been correlated with mortality as well as with prolonged ventilator dependence. Furthermore, it has come to light that the ventilator-induced lung injury that develops during the treatment of ARDS may also be contributing to the failure of ARDS resolution and failure of other organ systems in MODS (30).

**Pathogenesis of SIRS leading to MODS and ARDS**

The immuno-inflammatory response in SIRS is a complex interplay between cellular, molecular, and hormonal events. The self-protective homeostatic stress response of an organism becomes exaggerated in SIRS. Leukocytes (polymorphonuclear cells (PMNs), macrophages, and lymphocytes), the endothelium and cytokines and chemotactic agents that these cells produce mediate the immuno-inflammatory response. In addition, during SIRS, many stress hormones are released, such as catecholamines, ACTH, cortisol, growth hormone, glucagons, and insulin. Furthermore, coagulation, complement, and fibrinolytic cascades are also activated. On the molecular level, cytokines in the circulation can bind specific cellular receptors that result in activation of intracellular signalling pathways that regulate gene transcription and further activation or deactivation of various arms of the immune response. A compensatory anti-inflammatory response syndrome (CARS) occurs where activation of multiple anti-inflammatory cytokines, soluble cytokine receptors, and cytokine receptor antagonists keep the proinflammatory cascades in balance (31). By this mechanism immune cell activity, differentiation, proliferation and survival is controlled and results in production of other cytokines that either augment or attenuate the inflammatory response. Since multiple mechanisms are at play during the development of MODS and ARDS, they can be conceptually organized into several components. However, one must keep in mind that these components occur simultaneously and interactively and are part of one pathophysiological process. Some of the main components relevant to this thesis encompass: 1) the “Two-Hit” theory of immune cell priming, 2) the influence of reactive oxygen species, 3) the influence of gut hypoperfusion, 4) the influence of endothelial cell–leukocyte interaction.
**The “Two-Hit” Theory of Immune Cell Priming**

A concept of a “Two-Hit” hypothesis has emerged from various animal and clinical studies, suggesting that hemorrhagic shock renders trauma victims more susceptible to the development of SIRS and subsequently MODS (including ARDS) by priming neutrophils and macrophages for increased responsiveness to subsequent, even small, inflammatory stimuli, such as a line infection or a gastrointestinal bleed. Immune priming has been observed in animal models and patients sustaining ischemia-reperfusion from trauma or rupture of an abdominal aortic aneurysm (AAA). In the “Two-Hit” hypothesis animal models, primed leukocytes are characterized by excessive release of proinflammatory mediators (superoxidants, TNF-α, and IL-1β, IL-8 or CINC) which are thought to play an important role in the propagation of SIRS. In rodents, ischemia reperfusion was demonstrated to induce earlier and greater macrophage release of proinflammatory cytokines such as Cytokine-Induced Neutrophil Chemoattractant (CINC), which correlated with augmented and earlier signalling through the proinflammatory intracellular pathway via the transcription factor nuclear factor-kappa B (NF-κB). Studies also showed that ischemia-reperfusion could augment PMN-mediated tissue injury via the NADPH oxidase system. Lindsay and colleagues supported the notion of leukocyte priming in humans, by showing that priming of the phagocyte oxidant capacity occurs in patients with ruptured AAA and not in those undergoing an elective operative AAA repair. Although the exact mechanisms of the “Two-Hit” hypothesis are not yet fully defined, this construct allows a framework for modeling the clinical problem of organ dysfunction after shock/resuscitation in animals and cellular systems, which is exploited in this thesis.

**The Influence of Reactive Oxygen Species**

Molecular mechanisms of innate immune system priming remain subject of intense investigation, however, compelling evidence from animal and human studies indicates that reactive oxygen species (ROS) may be critical in mediating the enhanced responsiveness to inflammatory stimuli. Recent studies demonstrate that oxidant hydrogen peroxide and enzyme xanthine oxidase, are sufficient to prime macrophages in vitro where antecedent oxidant exposure results in greater and earlier NF-κB activation in response to subsequent small doses of inflammatory stimuli. Further ROS have been demonstrated to be necessary for immune priming as a broad range of antioxidants have been shown to abolish the pro-inflammatory responses. In addition, antioxidants such as N-acetylcysteine (NAC), vitamin E or pyrrolidinedithiocarbamic acid are able to prevent the release of proinflammatory mediators such as tumour necrosis factor alpha (TNF-α) and interleukin-8 (IL-8).
The Influence of Gut Hypoperfusion

Recent studies indicate that during hemorrhagic shock splanchnic ischemia reperfusion plays a central role in the pathogenesis of shock induced SIRS and MODS (48). The fundamental mechanism of this phenomenon has not been established yet, however substantial evidence exists from the works of Deitch and Alexander that bacterial translocation takes place through hypoperfused gut mucosa entering the portal and lymphatic circulation and providing the inflammatory stimulus that aggravates the already sensitized immune system (49-51). In studies by Zallen and colleagues it was demonstrated that mesenteric lymph from post shock animals was capable of priming PMNs for superoxide production as well as increased expression of adhesion molecules such as CD11b (52). Mesenteric lymph may be a critical link between splanchnic hypoperfusion and the development of MODS.

The Influence of Endothelial Cell–leukocyte Interaction

MODS is characterized by infiltration of tissues with activated PMNs that lead to further organ damage with the release of a variety of activators that stimulate further cellular and humoral cascades. A cellular defence mechanism that serves an important physiological role in controlling infectious threats to the organism becomes deregulated. Endothelial cells in organs remote from any site of insult or infection begin expressing chemotactic factors that attract leukocytes. There is a global increase in expression of adhesions molecules, a process that promotes leukocyte extravasation. Leukocytes adhere (roll and stick) to the endothelial surface, become activated and undergo a resultant respiratory burst. The release of toxic oxygen metabolites as well as a variety of neutrophil-derived proteases contributes to increased coagulation, diffuse capillary leakage with tissue edema, cell death, and eventual organ failure.

Mediators of the Proinflammatory Response

The pathogenesis of SIRS, MODS, and ARDS, although not completely understood, is thought to be mediated largely through inflammatory mediators produced in response to various pathological insults. Although many mediators have been identified, to date there are no specific laboratory tests that can predict the course or outcome of predisposed patients. Such mediators include cells of the innate immune system and various cytokines, oxidants, and growth factors, which form complex interactions resulting in altered patterns of gene expression and activation of various biochemical pathways. Although not used as predictive markers in patients, these various mediators are used as measures of lung injury, especially in various animal and cellular models, including ones studied here. New treatments and approaches to SIRS, MODS, and
ARDS may require a more in depth understanding of the processes governing the production and release of these immune reactants. Characterizing how some of these molecular events occur following hemorrhagic shock and how they lead to the proinflammatory response culminating in SIRS, MODS, and ARDS is critical for future prevention of these processes. Although relevant to many organs affected by MODS, a select group of mediators is reviewed here that are of particular importance to the model system of ARDS. They include: 1) leukocytes: neutrophils and macrophages 2) endothelial cells, 3) cytokines: tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), tumour necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6), and interleukin-6 (IL-6), TGFβ, interleukin (IL-10), HMGB1 and 4) chemokines.

**Neutrophils (polymorphonuclear leukocytes - PMN)**

The hallmark of MODS or ARDS is infiltration of neutrophils into the microvasculature of the organ involved such as the lung. PMNs can be isolated from the broncho-alveolar lavage fluid (BALF) of ARDS patients and are a measure of the severity of lung injury (53). Persistence of high PMN numbers in the BALF after the first week of ARDS is associated with higher mortality (54). Activated neutrophils are capable of releasing cytotoxic products, reactive oxygen species, cationic peptides, eicosanoids, proteolytic enzymes and cytokines and chemokines all important in mounting a defence against invading pathogens. PMNs accumulate quickly within the intravascular space and their appearance in organs precedes any clinically detectable evidence of injury in animal models of endotoxin, hypovolemic and/or ischemia/reperfusion mediated injury especially in the lung (36;55). When neutrophils are depleted, the organ injury has been shown to be less severe. For example, when neutropenic mice are exposed to hyperoxia they have a diminished lung injury (56) and inhibitors of neutrophil mediators prevent development of lung injury (57). However, the mere presence of neutrophils is not sufficient for ARDS to occur. Neutropenic patients can still develop ARDS secondary to ionizing radiation or anti-metabolite use (58). Further, when neutrophils are recruited into the normal lungs of healthy subjects with a Chemoattractant Leukotriene-B4 (LTB4), they do not cause lung injury (59). Therefore, the activation state of the neutrophils rather than their presence may be the most critical determinant of injury.

Since PMNs are neither necessary nor sufficient to cause lung injury in humans, it is likely that for PMNs to induce damage they need to interact with their environment in a particular way. It has been previously demonstrated that PMNs can adhere to endothelium, epithelium or extracellular matrix proteins in the interstitium, a step that leads to their
activation, transmigration into the tissues and release of cytotoxic agents (60-63). Neutrophils express adhesion molecules that enable them to adhere to the microvascular endothelium and sequester in vital organs. Various proinflammatory mediators released during the SIRS response are thought to increase the expression of adhesion molecules on the surface of both endothelial cells and neutrophils such as intracellular adhesion molecule-1 (ICAM-1), and β1-integrins CD11/CD18 (64). These changes, combined with a decrease in neutrophil deformability, are thought to contribute to neutrophil sequestration and trapping within pulmonary capillaries (60).

The initial attachment followed by the slow rolling of neutrophils along the vascular endothelium is mediated by the binding of leukocyte L-selectin (CD62L) to its endothelial ligands (65). Once this early contact has been achieved, L-selectin is shed from the cell surface by proteolytic cleavage (66), and firmer adhesion develops as a result of the ensuing up-regulation of another class of adhesion molecules, the β2-integrins (e.g. CD11b) (67). Upon activation of the β2 Integrins, their avidity is increased for their ligand ICAM-1 and ICAM-2 located on the endothelial surface. Integrins have been found to be critical for neutrophil migration and adhesion in phagocytosis and respiratory burst and therefore their activation/upregulation is an important step in the development of SIRS (60). Consequently, strategies that decrease integrin activation/upregulation are protective against lung injury in SIRS. Data that forms the groundwork for some of the studies in this thesis demonstrated, that hypertonicity, as an anti-inflammatory strategy, prevents the lipopolysaccharide (LPS)-induced up-regulation of CD11b. HTS also causes adhesion-independent shedding of L-selectin in vivo and in vitro (68;69). In experimental studies, blockage of L-selectin with monoclonal antibodies has been demonstrated to prevent neutrophil sequestration in the alveolar space. For example, in a rabbit endotoxemia model (70), there was reduced remote lung injury after infrarenal ischemia reperfusion injury with anti L-selectin antibody (71). Similarly, improved survival was noted in a baboon trauma model (72). Aselizumab, a recombinant humanized monoclonal antibody against L-selectin, was tested in a phase I and II trials, however, showed no significant benefit in treating MODS patients. Further studies are under way to evaluate other physiologic pathways and adhesion molecules that may be more effective in their anti-inflammatory effects.

The toxicity of PMNs stems not only from their adhesive and migratory properties, but also from their ability to generate ROS. The mechanism of ROS generation is thought to involve nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). Stimulation of neutrophils with TNF-α, IL1-β and LPS results in the assembly of the NADPH complex and production of the superoxide radical (73;74). NADPH is a membrane-bound enzyme that
catalyzes the formation of superoxide anion from molecular oxygen. Superoxide is subsequently converted to hydrogen peroxide and hypochlorous acid, all unstable and highly reactive oxygen species. ROS produced by neutrophils are capable of direct DNA damage, lipid peroxidation, and interference with actin metabolism leading to changes in cellular function and organ damage. The NADPH inhibition by apocyanin has been shown to decrease lung injury in guinea pigs (75). Other pathways such as the nitric oxide synthase pathway may also be involved in generation of free radicals by neutrophils. Knockout mice for nitric oxide have decreased lung injury when injected with LPS (76).

Neutrophils also contribute to SIRS generation and propagation by proteolytic enzyme production and deregulation of neutrophil apoptosis resulting in a profoundly increased neutrophil half-life. Both neutrophil elastase and metalloproteinase have been implicated in neutrophil mediated lung injury as elevated matrix metalloproteinases have been found in the BALF of patients with ARDS (77). In concert with this observation, administration of elastase and metalloproteinase inhibitors were shown to attenuate ARDS in pigs (78).

As to deregulation of apoptosis, under normal conditions neutrophils have a short half life of 8-20 hours, however in patients with SIRS their apoptosis is drastically decreased prolonging the life span of these activated cells and potentiating the injury (79). Although, the mechanisms regulating neutrophil apoptosis are not yet fully elucidated, in vitro suppression of apoptosis has been described to occur through activation of NF-κB and is associated with reduced activity of caspases-9 and -3 and maintenance of mitochondrial transmembrane potential (80). Deregulation of neutrophil apoptosis contributes to the detrimental effects of SIRS and sepsis on organ function.

In summary evidence that neutrophils are important in initiation and propagation of the SIRS cascades leading to MODS and lung injury is convincing. Elucidation of some of the key cellular and molecular processes involved will aide in development of therapeutic strategies for neutrophil mediated diseases, some of which will be addressed in this thesis.

**Macrophages**

Cells of monocyte/macrophage lineage are the cornerstone of the innate immune system, consisting of bone marrow monoblasts, promonocytes, blood monocytes, and tissue macrophages that play a crucial role in immune surveillance against microorganisms. Monocytes derived from bone marrow circulate in the blood for less then 48 hours and enter tissues to differentiate into macrophages. Macrophages express different biological activities depending on the type of tissue microenvironment. Compared with monocytes, macrophages
have a longer life span and more heterogeneous functions (81). Although the role of an innate immune reaction is to eradicate the invading microorganisms, a hyperinflammatory monocyte system contributes to the etiology and progression of SIRS.

Macrophages are critical in recognizing LPS, Lipoteichoic acid (LTA), or other bacterial components through various pattern recognition cellular receptors such as Toll receptors (82). The interaction between pathogens and macrophages, results in the initiation of inflammatory and coagulation cascades. These pathways yield soluble mediators that function in autocrine or paracrine loops; these further activate the proinflammatory cascades. Macrophages can release a series of inflammatory mediators such as TNF-\(\alpha\), IL-1, IL-6, eicosanoids, platelet activating factor (PAF), nitric oxide (NO), and ROS when stimulated with bacterial products (83-85). They are also a source of intracellular superoxide and hydrogen peroxide (\(\mathrm{H}_2\mathrm{O}_2\)), responsible for killing phagocytosed microorganisms (86). During SIRS, macrophages exist in an overactive state and they can release some of the inflammatory mediators and reactive oxygen species that contribute to tissue damage (87).

In their function as pathogen killers, alveolar macrophages (AM) are unique in that they possess Fc and complement receptors that respond to the Fc portion of IgG and C3 complement components. These receptors enable AM to participate in initial host defences through the production of chemokines such as IL-8 (88), monocyte Chemoattractant protein-1 (89) and growth factors, such as macrophage colony-stimulating factor, which augments monocyte survival (90). Further, activated macrophages increase their expression of CD40 and TNF-\(\alpha\) receptors, and secrete TNF-\(\alpha\). This autocrine stimulus synergizes with interferon-\(\gamma\) (IFN-\(\gamma\)) secreted by T helper 1 (TH1) cells to increase the antimicrobial action of the macrophage, in particular by inducing the production of nitric oxide (NO) (see description below) and reactive oxygen species (ROS) (87). In response to binding of CD40 ligands on T cells, macrophages increase their expression of MHC class II molecules, thus bridging the innate and adaptive immune responses and allowing further activation of resting CD4 T cells (87).

Since macrophages play such an important role in the development of the proinflammatory state, a thorough understanding of the molecular processes of macrophage activation and priming is a cornerstone of any MODS and SIRS therapies.

**Endothelial Cells**

During SIRS, endothelial cells are activated by inflammatory mediators or directly with microbial components via pattern recognition receptors. The endothelium has many functions. It maintains vasomotor tone, regulates cellular and nutrient trafficking, maintains blood fluidity,
and participates in generation of new blood vessels. During an insult such as microbial attack or ischemia reperfusion, damaged endothelium is one of the determinants of the balance in proinflammatory and anti-inflammatory responses (91).

Under insult conditions, the endothelial response is one, which tends to promote intravascular coagulation. The endothelium participates in the release of pro-thrombotic, pro-inflammatory, and vasoactive mediators including NO, endothelins (ETs) and products of cyclooxygenase metabolism. When activated, endothelial cells have been demonstrated to decrease synthesis of thrombomodulin (TM), tissue-type plasminogen activator, and heparin. Promoting the procoagulant state, damaged endothelium increases expression of tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) (92-94). Tissue factor in the circulation initiates the extrinsic pathway coagulation cascade, resulting in thrombin generation and fibrin deposition. With downregulation of the endogenous anticoagulation system (Activated Protein C, antithrombin, and tissue factor pathway inhibitor (TFPI)) during SIRS the coagulation balance is disrupted and microvascular thrombosis occurs (91). Studies demonstrate that Activated Protein C is depleted in patients with SIRS and this may correlated with increased mortality and morbidity in these patients (95;96). Human recombinant activated protein C is therefore used as a therapy in SIRS and MODS. Antithrombin and TFPI are both anticoagulants that are reduced in SIRS; however, administration of recombinant antithrombin and TFPI as therapy in patients with MODS has not proven successful.

In addition to its procoagulant properties, the endothelium increases expression of adhesion molecules during inflammation, including P-selectin, E-selectin, ICAM-1, and VCAM-1. These alterations result in increased rolling, strong adherence, and transmigration of leukocytes into underlying tissues as well as recruitment of platelets to the blood vessel wall (91). In trauma patients, increased levels of some of the adhesion molecules correlate with the development of MODS (97).

In summary, the endothelium plays an integral role in initiation and perpetuation of local proinflammatory and procoagulant state promoting cellular interactions, which ultimately lead to microvascular occlusion, hypoxia, and organ failure. Because the endothelium interacts with blood as well as underlying tissue, it can be a strategic target for sepsis therapy.

Humoral Pro and Anti Inflammatory Mediators

There are cellular and humoral mediators of the innate immune response in SIRS. From activated inflammatory cells a wide variety of chemical mediators are released into the systemic circulation. These mediators act in an autocrine, paracrine, and endocrine fashion and include
pro and anti-inflammatory peptides or cytokines, bioactive lipids including the eicosanoids and platelet-activating factor (PAF), toxic oxygen metabolites including Nitric Oxide, and neutrophil-derived tissue proteases. The discussion here focuses on those mediators that have been clearly implicated in the pathogenesis of ARDS. These include proinflammatory cytokines: IL-1β, IL-6, TNF-α, and chemokines CINC and IL-8. In addition, counter-inflammatory cytokines: IL-10, IL-4, IL-13 and IL-1 receptor antagonist (IL-1RA) are described.

*Interleukin-1β (IL-1β) and Tumour necrosis factor alpha (TNF-α) and their counter-regulatory ligands and receptors*

TNF-α and IL-1β are produced by activated macrophages. They are considered the major early mediators of septic shock and have been documented to contribute to the development of MODS in various conditions such as trauma, burns, or sepsis. They are both released within the first 30-60 minutes after exposure to LPS and are capable of initiating the proinflammatory cascade with up-regulation of adhesion molecules on neutrophils and stimulating the release of IL-6, eicosanoids, and PAF among others (98). As well, TNF-α stimulates IL-1β release and vice versa, thus initiating inflammatory cell migration into tissues. Both TNF-α and IL-1β are present in the BALF of patients at risk for and with established ARDS (99;100). Further, TNF-α upregulates phospholipase A2, cyclo-oxygenase, and nitric oxide synthase as well as a variety of inhibitors, which include plasminogen activator inhibitor 1 (101). Both TNF-α and IL-1β propagate the inflammatory response and are primary mediators of MODS.

Inhibitors of proinflammatory cytokines include soluble TNF receptors that compete with membrane receptors for binding of free TNF-α. Further, IL-1 receptor antagonist (IL-1ra) has been characterized and is structurally related to IL-1 and in *vitro* has been demonstrated to protect against endotoxin-induced lung injury in rats and improves survival after endotoxemia in mice (102). TNF-α antibodies and soluble TNF-α receptors as well as IL-1 receptor antagonists have been used in humans, however proved suboptimal in clinical settings (103;104).

*Interleukin-6 (IL-6)*

Interleukin 6 (IL-6) production is increased by macrophages/monocytes, endothelial cells and smooth muscle cells in response to endotoxin, TNF-α and IL-1β (98). IL-6 has been demonstrated to increase post burn and surgery (105). It is a cytokine integral in mediating the synthesis of acute phase proteins including C reactive protein (CRP) (106). IL-6 levels were
documented to be predictive of ARDS severity in patients with sepsis and pancreatitis (107;108).

**Transforming growth factor β (TGFβ)**

Transforming growth factor β (TGFβ) regulates cell growth and cell apoptosis, differentiation and extracellular matrix protein synthesis. It is therefore an integral cytokine mediating tissue fibrosis as well as in resolution of tissue injury. It is often elevated in later phases of disease such as ARDS contributing to pulmonary fibrosis (109).

**Chemokines**

Chemokines serve a function of recruiting and activating leukocyte subsets. In order for PMNs to be recruited from the bloodstream into tissues, such as the lungs, varieties of chemokines are released by macrophages and specifically alveolar macrophages. They are small proteins (8-10kD) characterized by their action on the superfamily of G-protein-coupled serpentine receptors (101). Chemokines are classified into 4 subfamilies on the basis of their cysteine residue positioning: 1) The CXC subfamily (α-subfamily), 2) The CC chemokine subfamily (β-subfamily) and 3) The CX3C chemokines (δ-subfamily). The murine homologs to the CXC chemokine family include macrophage inflammatory protein-2 (MIP-2) and monokine induced by IFN-γ (MIG) and are structurally homologous to human growth-related oncogene (GRO-α, -β, -γ), 10-kDa IFN-γ-inducible protein (IP)-10, and MIG, respectively (110;111). CXC chemokines, cytokine-induced neutrophil chemoattractants: CINC-1, -2 alpha, -2 beta and -3/MIP-2 are the homologs of the human chemokine GRO-α and are found to be increased in inflammatory conditions in rat models (112;113).

IL-8 is the predominant chemoattractant in the BALF of ARDS patients, it is rapidly synthesized and released (114). No murine or rat structural homolog exists for human IL-8, however CINC has been considered the orthologue of IL-8 (115;116). Other potent leukocyte chemoattractants exist, including the complement component C5a, and the low-molecular-weight lipids LTB₄ and PAF, however, the neutrophil chemotactic activity in BALF is due predominantly to IL-8 (117). Antibodies to IL-8 in animal models have been shown do decrease lung injury and mortality (118;119). IL-8 induces its action by binding to its receptor CXCR1 on PMNs which can be rapidly internalized and re-expressed for further stimulation (110).

In summary, there are >30 different chemokines and >20 different chemokine receptors with complex and overlapping functions. Targeting the chemokine system for pharmacological development of specific antagonists that interfere with leukocyte migration is a promising area in the treatment and prevention of SISR, MODS, and ARDS.
**HMGB-1**

High mobility group box 1 (HMGB1) is a transcription factor and a growth factor that has recently been implicated as a mediator of SIRS and ALI, especially after hemorrhagic shock (120;121) and after liver ischemia/reperfusion (122). It is released by activated macrophages, enterocytes and by necrotic cells (123). HMGB1 is capable of stimulating the release of proinflammatory cytokines, such as TNF and upregulating endothelial adhesion (124). Fan et al. recently reported that following shock resuscitation, HMGB1 contributes to the PMN NAD(P)H oxidase activation via a TLR4 signalling pathway (125). Immunization with anti-HMGB1 antibodies was shown to protect against sepsis and ALI in animal models. Strategies to inhibit HMGB1 can potentially lead to novel therapies of inflammatory processes.

**Interleukin-10 (IL-10)**

IL-10 is an anti-inflammatory cytokine, which is capable of inhibiting the release of IL-1β and TNF-α as well as IL-6 from monocytes and macrophages. It also stimulates the production of other anti-inflammatory agents: IL-1ra and soluble p75 TNF receptor (119). IL-10 levels have been identified to be higher in the lungs of patients with ARDS. In turn, higher levels of IL-10 in the BALF has been associated with improved survival (126). In a recent study of 46 patients, IL-10 has been demonstrated to peak on day 1 of ARDS and subsequently fall over 3 weeks to undetectable levels. At the onset of ARDS the molar ration of IL-10 and TNFα was low and increased with the progress and increasing severity of ARDS suggesting that an anti-inflammatory balance favours better outcomes in these patients (99). IL-10 administration in animal models of SIRS have been shown to protect from organ injury, however no human studies are available to date (127).

**Other Anti-inflammatory mediators**

In addition to IL-10, other anti-inflammatory cytokines have been noted to be elevated after severe injury. Park et al. measured pro- and anti-inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-10) and specific agonists and antagonists: IL-1ra, soluble IL-1 receptor II (sIL-1RII), soluble TNF receptor I (sTNF-RI), and soluble IL-6 receptor (sIL-6R) in patients at risk for ARDS and in patients with ARDS. They found that the anti-inflammatory: IL-1ra, IL-1RII, sTNF-Ri, sTNF-RiI, sIL-6R, and IL-10 reached maximal concentrations in BALF on Days 1 and 3 of ARDS, and returned to near normal values by Day 14 of ARDS (99). The anti-inflammatory response was greater than the proinflammatory response and provided a necessary mechanism for limiting the intensity of the inflammatory response, which may not be the case in patients with the most severe forms of the disease.
**Nitric Oxide**

Nitric oxide (NO) is a microbicidal product derived from L-arginine, secreted and rapidly converted to nitrate and nitrite (128). Besides its (beneficial) microbicidal and tumouricidal effects, NO causes vasodilation, cell damage and inhibition of acute-phase protein production, and increased leukocyte adhesion in liver and lungs (129). NO activates potassium channels and has been demonstrated to hyperpolarize plasma membrane of smooth muscle cells preventing vasoconstriction, even in response to vasopressors such as norepinephrine and angiotensin II (129). LPS was found to increase endothelial NO release further suggesting that NO may be responsible for hypotension in severe sepsis. NO is therefore a key contributory mediator of tissue damage in SIRS (129).

**Cellular and Animal Models of MODS/ARDS**

Human subjects offer some insight into the cellular and molecular processes that occur following shock resuscitation and in the development of SIRS/MODS and ARDS. However, data from human subjects is limited as there is marked heterogeneity in the extent and nature of the human injuries, variation in the time from injury that tissues can be obtained, significant differences in the medical treatments provided, and relatively limited access to tissue analysis. Further, the patient population may not be equivalent as they differ with respect to age, dietary status, sex, genetic background, and hereditary predispositions. Therefore, to explore the cellular and molecular mechanisms of SIRS/MODS and ARDS a number of animal and cell systems have been developed.

A body of literature exists supporting the observation that initial hemorrhagic shock primes the inflammatory cells including PMNs and macrophages, and makes patients more susceptible to a second, seemingly trivial inflammatory stimulus (130;131). This is called a “Two-Hit” hypothesis and has been described in the earlier sections.

Although there are several animal models of MODS and ARDS in the hemorrhagic shock context the most appropriate animal model continues to be debated as the pathophysiology of shock is complex and multifactorial. There are several rat, mouse, rabbit and pig models of combined hemorrhagic shock and subsequent inflammatory stimulus (132-134). Experiments have used controlled, uncontrolled, and hybrid hemorrhage models, with and without various types of sedation and anaesthesia. The type of shock, rate, and type of resuscitation fluid as well as the type of the inflammatory stimulus are variables that have been investigated in animal studies. LPS alone does not completely mimic the systemic or pulmonary
effects of bacteremia and ARDS, therefore a number of more complex models have been developed that combine hemorrhagic shock or other form of injury such as cecal ligation and puncture with endotoxin administration (135).

Studies in this thesis are based on a developed, highly reproducible rodent model of lung injury following hemorrhagic shock and subsequent endotracheal LPS administration (36). In this model, animals are bled and then resuscitated with shed blood plus an equivalent volume of LR solution. At end-resuscitation, one of two protocols is observed. In the first in vivo model, animals receive a small dose of LPS intratracheally. They are then studied at various time points. The second protocol is carried out ex vivo. At the end of the resuscitation period BALF is recovered and cells are cultured in vitro with or without addition of LPS.

Previous studies using the “Two-Hit” rat model protocol, demonstrated priming of alveolar macrophages. AM after shock resuscitation, exhibited increased release of Cytokine-Induced Neutrophil Chemoattractant (CINC), the rodent homologue of interleukin (IL-8), in response to LPS, compared to following hemorrhagic shock or LPS alone (36). The enhanced CINC expression correlated with augmented and earlier signalling through the proinflammatory transcription factor NF-κB. Further, TNF production was augmented in our model. Like CINC, TNF mRNA and protein were increased earlier and to a greater extent after LPS treatment in cells derived from animals exposed to antecedent shock/resuscitation compared with sham animals (136). The role of TNF in regulating lung injury in this model was examined using anti-TNF antibody. Although anti-TNF treatment markedly inhibited tissue factor expression and procoagulant activity in the lung, it did not affect lung neutrophil sequestration. However, neutrophil priming was inhibited as evidenced by reduced generation of oxidants. Thus, TNF was shown to affect neutrophil activation but not accumulation in the lung. In these and other studies, priming was reversed by adding antioxidants, such as N-acetyl cysteine (NAC), to the resuscitation fluid, suggesting an important role of oxidants in priming (36). Similarly, in other models and in cell culture systems antioxidants have been shown to abrogate LPS-induced proinflammatory responses (44;137). In critically ill patients, antioxidant therapy has been shown to lessen organ injury suggesting that oxidants play an integral role in the development of SIRS, MODS and ARDS (138;139). Based on these and other findings this thesis addresses the role of oxidants in the mechanisms of leukocyte priming in the developed and validated rodent model described.

The ex vivo protocol allowed for investigation of macrophage activation free of contamination by other cell types, particularly neutrophils, which sequester in large numbers at
later time points following resuscitation and LPS administration. Also an *in vitro* culture system was established for macrophages in order to examine single cells at the molecular level (41). Since oxidative stress during hemorrhagic shock is involved in cellular priming, murine macrophage cell line RAW 264.7 was stimulated *in vitro* with H$_2$O$_2$ followed by LPS. Others have demonstrated that in RAW 264.7 cells treated with xanthine oxidase or H$_2$O$_2$ there is increased CINC mRNA production, and priming for NF-κB translocation (140). Although an understanding of the role of oxidants in inflammation has provided a strong rationale for the use of antioxidants in the *in vitro* and *in vivo* settings, further elucidation of their mechanism of protective action is required and will be addressed in this thesis.

In the future, traditional genetic approaches may be used to understand the molecular pathways involved in priming of the innate immune response during shock resuscitation. Mouse phenotypic studies, forward genetics, chemical mutagenesis, gene mapping, and linkage studies may provide further clues to the complexities of cellular function under specific genetic backgrounds, which may dictate the variable responses observed with experimentally induced injury. Gene profiling and proteomic evaluation of tissues from affected animals models of MODS are other avenues that may aide in narrowing down the pathways involved in humans affected with MODS/ARDS and help design novel treatment modalities for this clinical condition.
Section II Trauma Resuscitation History, Current Practice and Advances

- Historical Perspective
- Current Management Strategies of MODS and ARDS
  - Fluid Resuscitation
  - Crystalloids Overview
    - Normal Saline and Ringer’s Lactate
    - Hypertonic Saline
  - Colloids Overview
    - Albumin, Properties and Clinical Use
    - Synthetic Colloids
  - Colloids Versus Crystalloids for Resuscitation
  - Cardiovascular Support
  - Respiratory Support
  - Nutritional Support
  - Renal Support
  - Hepatic Support
  - Endocrine Support
  - Antimediator Therapy
**Historical Perspective**

The history of resuscitation from trauma is as old as medicine itself (141). The first documentations of trauma resuscitation and treatment date back to ancient Egypt. Descriptions of amputations, foreign body extractions, and dressing of wounds can be found in ancient writings of the Edwin Smith Papyrus, written between 3000 and 1600 B.C.. The first hospitals are believed to have been developed by the Romans for the treatment of wounded soldiers. One of the earliest reports of respiratory resuscitation from trauma came from an alchemist Paracelsus (1493-1541) who was said to have attempted the resuscitation of a corpse using bellows, a trick he apparently learned from Arabic medical writings (141). Later Andreas Vesalius (1514-1564), the father of modern anatomy, successfully used bellows to resuscitate asphyxiated dogs. In the 1740s mouth-to-mouth resuscitation had been reported and in 1774 one of the first trauma organizations 'The Institute for Affording Immediate Relief for Persons Apparently Dead from Drowning' was established as described in English literature (141). The resuscitation practice at that time involved using a portable electrostatic generator to administer shocks through the chest as well as to dry and warm the body by applying friction to the skin, and to administer tobacco smoke enemas. In 1856, Marshall Hall added the control of airway and breathing to the initial steps in resuscitation. Since that time, the evolution in methods of trauma resuscitation paralleled advances necessitated by experience with a large number of patients during world military conflicts. During the American Civil War, shock was recognized to be a separate entity from hemorrhage. In the Army Surgeon General’s report written in 1876 it was stated, “The collapse of bleeding resembles syncope as distinguished from shock. Rest in bed, opium, and warm fomentation constitutes the treatment.” During World War I, the concept of “The Golden Hour” was introduced which stressed the importance of the time factor between wounding and adequate shock treatment. If the patient was treated within one hour, the mortality was 10 percent. This increased markedly with time, so that after eight hours, the mortality rate was 75 percent. However, fluid therapy was not used acutely in the trauma patients as it was thought to disrupt the clot tamponading any bleeding. The concept of what is now termed permissive hypotension emerged with Cannon, Fraser & Cowell:

“Injection of a fluid that will increase blood pressure has dangers in itself. Hemorrhage in a case of shock may not have occurred to a marked degree because pressure has been too low and the flow too scant to overcome the obstacle offered by a clot. If the pressure is raised before the surgeon is
ready to check any bleeding that may take place, blood that is sorely needed may be lost.” (142)

This concept is still debated to this day as a study by Bickell et al. in 1994 showed better survival in patients with penetrating chest injuries who received delayed fluid resuscitation until surgical repair of injuries (143). Further the most up to date meta-analysis of six trials from the Cochrane Database of Systematic Reviews failed to prove or disprove the merits of early and aggressive fluid resuscitation (144).

In addition, in 1918, it was recognized that traumatic shock was attributed to a toxin originating in dead or dying tissue. In the 1920’s, Blalock proved that shock after trauma was due to blood loss into the tissues and therefore no preoperative resuscitation was administered and many patients died. During World War II, colloid and banked blood became the mainstay of trauma care as haemoconcentration became important. Patient survival improved but so did the complications with a large percentage of people dying of acute renal failure (141). During the Vietnam War the use of crystalloids was popularized as the need for expansion of intravascular space became apparent (145). Replacement of blood loss was recommended in 3:1 ratio and as high as 8:1 for patients profoundly hypotensive and in severe shock (146). However About 3000 patients still died of shock despite being adequately treated with IV fluids and appropriate surgical procedures. Death was attributed to pulmonary failure and the concept of multiple organ failure (MOF) and especially acute respiratory distress syndrome (ARDS) emerged (26).

In the 1970’s and 1980’s the advent of intensive care units where advanced technology and intensive nursing care were used decreased patient mortality. However, mortality from MOF and ARDS remain the major cause of prolonged hospitalization and death after trauma. Recent reports indicate that the trajectory of patients who develop multiple organ failure is set within 6 hours of injury (32;147) and therefore reevaluation of the current approach to understanding the initial traumatic insult, resuscitation, inflammatory response and how these impact on the epidemiology of death from trauma is necessary to improve outcomes in the future.

**Current Management of MODS and ARDS**

The underlying clinical disorder in post traumatic MODS and ARDS is multifaceted and involves cellular and molecular responses to oxidative stress and infectious stimuli. Treatment of this underlying over exuberant proinflammatory response remains difficult. The aim of such treatment is prevention of the immune priming mechanisms and elimination of any secondary sources of inflammatory stimuli which can be intrapulmonary such as aspiration, inhalation, and
pulmonary contusion or extra pulmonary such as pancreatitis, focal tissue ischemia or other intra-abdominal catastrophes.

The principles of treatment for MODS and ARDS are the following: 1) careful nursing in the ICU, 2) treatment of the underlying clinical disorder, 3) fluid resuscitation and maintenance of hemodynamic stability, 4) treatment of infection, 5) nutritional support, 6) endocrine and immune support as well as 7) support to failing organs such as the kidney with haemodialysis or continuous venovenous haemofiltration or the lungs using ventilatory support to maintain adequate oxygen delivery. Some of the salient points in treatment of MODS and ARDS patients are outlined below with the focus on fluid resuscitation.

**Fluid Resuscitation**

Appropriate fluid resuscitation remains the cornerstone of care for trauma as over 30% of trauma patients die of bleeding complications. Although fluid resuscitation is essential, the choice of the appropriate fluid remains controversial. Conflicting results are presented in the literature and meta-analysis to date are inconclusive as studies considered do not have the same patient characteristics and may include old studies with differing resuscitation protocols. Besides hypo-, iso- and hypertonic crystalloids, such as Ringer’s Lactate (RL), normal saline (NS) and hypertonic saline (HTS) there are a number of colloids such as human albumin and other blood products as well as synthetic colloids including dextrans, gelatins, and hydroxyethylstarch. Combinations of the above are also available and currently clinical trials are under way testing two promising alternatives for prehospital resuscitation of traumatic hemorrhagic shock namely, hypertonic resuscitation (7.5% NS) with or without 6% Dextran 70 and polymerized haemoglobin blood substitutes. The objective of fluid resuscitation with any of the choices is to stabilize the hemodynamic status of the patient and improve microcirculation to vital organs in order to prevent the irreversible damage at the cellular level. Unfortunately, often the correction of the systemic hemodynamics do not correlate with microcirculatory improvements and oxygen availability to cells. This occurs because uneven distribution of the microcirculatory flow may predispose underperfused organs, such as the splanchnic bed or the kidney, to local hypoxia and cell death. The hypoxic capillary endothelium in these organs can be a source of inflammatory mediators and reactive oxygen species that further induce activation of various leukocytes involved in the propagation of the inflammatory response. Therefore, adequate volume restoration is only part of the overall goal of fluid resuscitation, as improving microcirculation involves not only improving the hemodynamics but also modulating
the inflammatory response induced by the initial hypoxia followed by tissue reperfusion and consequent exposure to proinflammatory reactive oxygen species.

**Crystalloids Overview**

Crystalloids can be broken down to Hypotonic (dextrose in water), Isotonic (Ringer’s Lactate and Normal Saline), and Hypertonic (7.5% saline, HTS). Crystalloid solutions permeate freely across any endothelial barriers, subject to Na⁺/K⁺-ATPase responsible for regulating intracellular Na⁺ concentration. Fluid exchange across the capillary wall is summarized by the Starling equation: \( Jv = K[(P_c - P_i) - \sigma(\pi_c - \pi_i)] \), where \( K \) is the permeability or filtration coefficient (flow rate per unit pressure gradient across the endothelium); \( Jv \) is the transcapillary fluid flux; \( P_c \) is the capillary hydrostatic pressure; \( P_i \) is the interstitial hydrostatic pressure; \( \pi_c \) is the intravascular osmotic pressure; \( \pi_i \) is the interstitial osmotic pressure; and \( \sigma \) is the reflection coefficient (148). In healthy tissues, the osmotic gradient between the capillary lumen and the interstitium is maintained by the difference in protein and colloid concentration on each side of the endothelial membrane. However, during inflammatory conditions such as SIRS, endothelial permeability increases and the coefficient \( \sigma \) tends to move toward zero thus increasing the transcapillary fluid flux (148).

Saline-based crystalloid solutions will theoretically expand the plasma volume by about 200 mL per liter infused. Therefore, only 10-20% of the infused crystalloid solution remains in the intravascular space and 75% extravasates into the interstitium. Consequently, in animal and human studies crystalloids consistently demonstrate large amounts of edema. The sugar containing crystalloids behave as free water, distributing equally across all compartments. Glucose-based solutions will therefore expand the plasma volume by about 60 to 70 mL per liter infused. Colloid osmotic pressure (COP) or the oncotic pressure is considered the osmotic pressure exerted by macromolecules such as plasma proteins and represents the pressure required to keep movement of water and ions across semi permeable membranes such as the endothelium. With large crystalloid infusion volumes, plasma proteins become diluted causing reduced plasma COP. That further contributes to tissue edema.

**Normal Saline and Ringer’s Lactate Solutions**

Isotonic NS and RL are most commonly used crystalloids for resuscitation. 0.9% NS contains 9 g/L Sodium Chloride (NaCl) with an osmolarity of 308 mOsmol/L, which is actually slightly hypertonic. It contains 154 mEq/L sodium and 154 mEq/L chloride. Its high chloride concentration poses a risk of metabolic acidosis when infused in large quantities (over 2L).
RL Chloride content is less than in NS but still higher than in plasma chloride (115 mM), and the Na+ content is 131 mM. RL is slightly hypotonic at 273 mOsm/l which is thought to be associated with cerebral edema if high volumes are used or the blood–brain barrier has been disrupted (150). In addition, LR solution may be responsible for a proinflammatory response contributing to the development of SIRS. LR was reported to be a racemic mixture of D-isomer and L-isomer of lactate that may contribute to the proinflammatory response (151). For that reason, LR is currently also available as a pure L-isomer solution (152;153). Despite some differences, NS and RL resuscitation were shown equivalent in aortic aneurysm surgery with only a mildly higher perioperative blood loss in the NS group (154).

**Hypertonic Saline, experimental and clinical studies**

To avoid large volume requirements the clinical use of hypertonic saline (HTS) in small volume resuscitation protocols has been under intense investigation for a number of years. A number of clinical studies to date have proven HTS safe and effective at improving trauma patient hemodynamics with the severe head injury group having the most beneficial effects. Historically In 1980 Velasco and colleagues studied dogs resuscitated from hemorrhagic shock with 7.5% NaCl with only 10% of the shed blood volume (155). He found the hemodynamics improved significantly with this treatment and the dogs had an improved survival. Later on, Nakayama et al. published their results on 7.5% NaCl use in sheep and similar to Velasco reported improved hemodynamics in their treatment group over isotonic saline alone (156). Other groups demonstrated similar rapid, however transient hemodynamic improvements with a number of hyperosmolar solutions of 2400 mOsm concentrations (157) with NaCl and NaCl-acetate having the longest lasting effects. In humans, the first studies were performed by Felippe and colleagues who demonstrated that resuscitation of terminally hypovolemic patients with hyperosmotic (7.5%) sodium chloride (100-400 ml), who had not responded to vigorous volume replacement and corticosteroids and dopamine infusions, caused an immediate increase in arterial pressure, the resumption of urine flow, and recovery of consciousness (158). In his studies, nine out of twelve patients survived.

In the last 15 years various randomized controlled trials have been performed comparing HTS alone or mixed with a colloid versus isotonic saline resuscitation. Although randomized studies have demonstrated safety and efficacy of HTS no clear survival advantage over standard isotonic therapy was demonstrated (159-167). A meta-analysis by Wade et al. from 1997 evaluated HTS with Dextran (HSD) for hypovolemic shock in six trials (168). They found that overall survival rates were better with HDS than crystalloids alone, especially in head injury
patients, and in patients who required blood transfusion or immediate surgical intervention for bleeding. Another analysis by Wade et al. from 2003 showed that patients with penetrating chest injuries have an improved survival when the HSD is the initial resuscitation fluid, especially if surgery is subsequently required for these patients (169). A more recent study by Cooper et al. from 2004 showed that hypotensive patients with severe head injury who received pre-hospital resuscitation with HTS had almost identical neurological function six months after injury as patients who received conventional fluid (170). Bulger et al, also reports that in their 2006 trial of HSD versus LR solution for the initial resuscitation of blunt trauma patients, no significant difference was observed in 28-day ARDS-free survival and the trial had to be stopped early for futility reasons (171).

No consensus exists as to the benefits in outcomes from HTS or HSD resuscitation as compared to conventional trauma fluid resuscitation protocols. Therefore, the ideal resuscitation strategy continues to be subject of intense investigation. Most recently, The Resuscitation Outcome Consortium founded by the National Institutes of Health and the US Department of Defence is conducting two multi-center trials of hypertonic resuscitation in patients with blunt or penetrating trauma in hypovolemic shock and in patients with severe traumatic brain injury. The results of these large multi-center trials are eagerly anticipated (172).

Despite no clear survival advantage, HTS resuscitation, especially mixed with colloids, has consistently shown an advantage in improving microcirculatory environments where it is immunomodulatory. Recent animal and human investigations revealed that HTS is capable of diminishing neutrophil oxidative burst and activation and adhesive properties. Exposure to HTS was demonstrated to suppressed neutrophil adhesion molecule expression and functions, which is thought to occur through the modulation of chemoattractant receptor signalling pathways (68;173;174). Also HTS was found to stimulate T cell proliferation and function (175-177). For example, costimulation of suppressed T cells with HTS restored their IL-2 production by up to 80% of the control response, suggesting that HTS resuscitation of trauma patients could potentially reduce posttraumatic sepsis. HTS was also found to increase anti-inflammatory cytokine production such as IL-10 (178). In humans, hypertonic HSD resuscitation has also been shown to mitigate the development of inflammation. In a recent study by Rizoli et al. 27 adult blunt trauma patients received 250-mL of either HSD (7.5% NaCl, 6% dextran-70) or placebo (0.9% NaCl) in hemorrhagic shock (179). They found that the immunologic/anti-inflammatory effects of HSD persisted for 24 hours with decreased neutrophil activation and proinflammatory TNF-α production. Neutrophils’ adhesive properties were reduced with decreased CD11b up-
regulation and increased CD62L shedding. Further, HSD reduced the drop in “classic” CD14++ and the expansion of the “pro-inflammatory” CD14+CD16+ subsets. In contrast, the anti-inflammatory response was unregulated with increased IL-1ra and IL-10 production. Therefore, with the hemodynamic advantage and the more recently discovered anti-inflammatory properties, HTS has become an attractive alternative to the currently accepted resuscitation fluids. However, more clinical trials still need to be conducted for it to be accepted as a standard of care.

**Colloids Overview**

Colloids can be defined as “homogenous non-crystalline substances consisting of large molecules or ultramicroscopic particles of one substance dispersed through a second substance” (148). Colloids can be divided into natural and synthetic. Natural colloids include human albumin, plasma protein fraction, fresh frozen plasma, and immunoglobulin solutions. Semisynthetic colloids include gelatins, dextrans, and starches, which are then usually suspended in isotonic, hypertonic saline or balanced salt solutions. Colloids as a group possess higher molecular weights than crystalloids and therefore are more efficient at remaining in the intravascular space. However, in severe hemorrhagic shock as in septic shock, the permeability of capillary endothelium increases. This allows for greater extravasation of proteins from the intravascular space. Leaky endothelium can allow colloids to enter the interstitial space and contribute to the formation of edema to a similar degree as crystalloids.

**Albumin, Properties and Clinical Use**

Albumin is a plasma protein of 69,000 Daltons that is synthesized in the liver and is the most abundant protein in the human plasma (4.5 to 5.0 g/kg) exerting 75% to 80% of the normal colloid osmotic pressure (180;181). 50% of the interstitial albumin is in the skin. Albumin serves a number of physiological functions. It is able to reversibly bind lipids and lipid-soluble substances: fatty acids, hormones, enzymes, dyes trace metals, and drugs. It can serve as a carrier molecule for those substances and regulate their extracellular concentrations as well as their biological activity. The transcapillary escape rate for albumin is about 5% per hour, however 90% of this returns into the circulation in healthy individuals as opposed to during sepsis where the transcapillary escape rate is markedly increased (182). Albumin’s ability to imbibe fluid into the intravascular space makes it an excellent plasma volume expander.

The commercially available human albumin solutions come in 4% hypooncotic, 5% isooncotic, 20 and 25% hyperoncotic forms. Albumin is a blood product, prepared from human
plasma. Donors are screamed for high-risk behaviour and plasma is screened for hepatitis B, C and HIV. The pooled albumin is pasteurized by heating to 60°C for 10 hours and then observed for 2-4 weeks. To date, albumin has not been known to transmit any viruses, although cases of bacterial contamination were reported. The adverse reactions quoted with the administration of human albumin can be divided into cardiovascular: CHF precipitation, edema, hyper-/hypotension, hypervolemia, tachycardia; central nervous system: chills, fever, headache; dermatologic: pruritus, rash, urticaria; gastrointestinal: nausea, vomiting; respiratory: bronchospasm, pulmonary edema; and miscellaneous: anaphylaxis. Concerns have also been raised in the United Kingdom about the transmission of variant Creutzfeld-Jacob disease associated with bovine spongiform encephalopathy, as the prion particles implicated cannot be removed by heating or by ultrafiltration. Nevertheless, risks of administering human albumin remain minimal.

One unique aspect of albumin’s biological function is its antioxidant properties exerted primarily through the presence of 17 disulphide bonds as well as single free thiol at the C-34 position (183). Indeed, much of the antioxidant activity of serum can be attributed to the presence of albumin (180;181). Albumin can act as a direct scavenger of reactive oxygen species, a so-called sacrificial antioxidant, via its thiol group (184). In addition, albumin has been shown to bind iron and prevent iron-dependent oxidizing events such as lipid peroxidation (185). Because albumin resides both intravascularly and extravascularly it is capable of scavenging free radicals in those areas. In cell culture systems and in vivo, albumin has been shown to augment intracellular and tissue reduced glutathione stores, respectively (186). In cell culture, the increase was sufficient to prevent hydrogen peroxide-induced cytotoxicity and TNF-induced NF-κB translocation (187). These latter properties appear to be independent of the redox status of Cys-34 residue, since its oxidation does not alter the increase in cellular reduced glutathione. Albumin may also possess anti-inflammatory properties as is has been previously demonstrated to decrease the neutrophil oxidative burst activity in in vitro studies (153). Together, these findings suggest a potential role for albumin as not only plasma expanding resuscitation fluid but also an immunomodulatory agent based upon its unique antioxidant properties. These properties of albumin will be explored in this thesis.

**Synthetic Colloids and Blood Substitutes**

There is a wide choice of resuscitation fluids other than crystalloids or albumin. These include gelatins, dextrans and hydroxyethylstarches as well as hemoglobin-based blood substitute oxygen carriers. Gelatins are derived from animal (bovine) gelatin, a derivative of
collagen and contain urea-linked gelatins (polygeline, Haemaccel 3.5%) or succinylated gelatin (modified fluid gelatin, Gelofusine 4%) components (148). They are excellent short-term volume expanders but have a short intravascular persistence and redistribute. Dextrans are made of branched polysaccharides of 200 000 glucose units and range in MW of 40 kDa – dextran 40 and, MW 70 kDa – dextran 70. On their own, dextrans may impair platelet adhesiveness and factor VIII activity. Dextrans have also been shown to obstruct the renal tubules after filtration and produce renal impairment. Therefore on their own they are not suitable for resuscitation of hemorrhagic shock (148). Hydroxyethylstarches are D-glucose polymers modified by partial hydrolysis and then reacted with ethylene oxide in the presence of an alkaline catalyst, resulting in hydroxyethyl substitution. The greater the number of substitutions the more soluble this colloid becomes (148). The starch solutions available in the United States are high-MW hetastarches in different solvents such as Hespan or Hextend. In Europe the use of medium-MW starches prevails such as Haesteril, Elohes and low-MW tetra starches such as Voluven (148). One of the disadvantages to resuscitation with starches is their potential for inducing a coagulopathy when used in high volumes especially with the high-MW HES solutions. Despite some of the disadvantages to the synthetic colloids, they have immunomodulatory properties. Both Dextrans and Hydroxyethyl starch have been described to diminish adhesive properties of leukocytes and platelets. Pentastarch with a MW range of 100 to 1000 kDa was described to inhibit endothelial cell activation and neutrophil adhesion (148).

Hemoglobin-based blood substitute oxygen carriers have recently emerged as another group of potential resuscitation fluids and are currently studied in the context of clinical trials. Products available to date are Polyheme and Hemolink, which are human forms of polymerized hemoglobin, and Hemopure, which is a bovine-based hemoglobin solution (188). Their effects on mortality or morbidity in patients are still not fully known.

**Colloid versus Crystalloid for Resuscitation**

A number of studies examined the effects of crystalloids versus colloids for fluid resuscitation. Despite potentially beneficial properties of colloids and especially albumin, randomized controlled trials and systematic meta-analyses have failed to demonstrate these theoretical benefits to date. Important to note, however, is the fact that the use of 25% Albumin intensively studied in this thesis for its antioxidant properties has not yet been thoroughly evaluated in the clinical setting.
There is a number of meta-analysis available and they review the use of albumin or other colloids versus crystalloids for resuscitation in trauma and critical care patients. One of the initial and well know Cochrane meta-analyses from 1998 quantified the effect of albumin administration in the critically ill on mortality (189). In reviewing 30 trials, the study concluded that that the relative risk of death after albumin administration was 1.68 (95% confidence interval 1.26–2.23) in all patients and 1.69 (1.07–2.67) in hypoalbuminemic patients and that for every 17 critically ill patients treated with albumin there is one additional death. The study concluded that there is a strong suggestion that albumin resuscitation may increase mortality and that albumin should not be used outside the context of rigorously conducted randomized controlled trials. This conclusion was further supported by the findings of Alderson et al. in the 2000 meta-analysis comparing randomized and quasi-random trials of different colloids compared to crystalloids, in patients requiring volume replacement. Overall, they reported the risk of death in patients receiving albumin was 14% compared with 9% in the control groups, suggesting that for every 20 critically ill patients treated with albumin there is 1 additional death (190). These two studies were widely criticized for inclusion of studies with very wide indications for albumin administration and widely differing fluid administration strategies and treatment goals. The studies analyzed did not have mortality as an endpoint, and the studies did not analyze the different types of colloids separately. In contrast a meta-analysis by Wilkes and colleagues included a greater number of studies, 55 trials with 3504 people, demonstrating that there is no increase in the risk of death when albumin containing fluids are used regardless of indications (191).

Although the controversy continues, the most recent and the largest double-blind, randomized study from Australia, called the SAFE trial, included 6997 patients from a heterogeneous population of critically ill ICU patients (192). The Investigators randomly assigned patients who had been admitted to the ICU to receive either 4% albumin or NS for intravascular-fluid resuscitation during the next 28 days. The primary outcome measure was death from any cause during the 28-day period after randomization. The study demonstrated that there is no difference in 28-day all-cause mortality when 4% albumin is compared with 0.9% saline administration. The secondary outcomes, such as requirements for mechanical ventilation, renal replacement therapy, time in the ICU, time in hospital during the study period were all equivalent in both the albumin and NS group. This well-designed study supports the safety of albumin administration and is a landmark in critical care medicine. Since the SAFE study, any subsequent meta-analysis updates from the Cochrane Injuries Group Albumin Reviewers are
heavily influenced by its results. In a most recent meta-analysis from 2006, the SAFE trial contributed 91% of the information (based on the weights in the meta-analysis). In this most recent update, 32 randomized controlled trials were reviewed comparing albumin with crystalloid solutions use in critically ill patients with hypovolemic, burns or hypoalbuminemia with mortality as an outcome in the studies (193). The results showed that for hypovolemic patients or patients with burns and/or hypoalbuminemia there was no decreased mortality with albumin as compared to saline administration. The authors point out that there is a possibility that there may be highly selected populations of critically ill patients in which albumin may be indicated. However, based on their findings and the high cost of human albumin, their major recommendation is that albumin should only be used within the context of well-concealed and adequately powered randomized controlled trials.

In summary, critically ill patients needing fluid resuscitation need to be resuscitated to achieve an optimal hemodynamic status within temporal constrains. Currently, isotonic crystalloids are a mainstay of therapy for hypovolemia secondary to bleeding; they have been shown to be as safe, as effective as colloid solutions, and much less expensive. Specifically albumin has been demonstrated to be safe and efficacious in resuscitation by the SAFE study. The main criticism of the SAFE trial is that it was underpowered to detect small but important differences in mortality among some specific subgroups. For example among patients with sepsis albumin use was associated with a trend toward decreased mortality, with RR of 0.87 (95% CI 0.74-1.02). In contrast, a trend toward increased mortality was observed in subgroups with trauma, particularly without head injury. The next generation of studies may be able to better answer whether different types of shock require different types of fluid resuscitation. Fluids differ in their effect on blood flow hemodynamics, coagulation and their immunomodulatory properties. Therefore, the choice of fluid and the volume of resuscitation may need to be tailored to particular patient’s needs, according to the various properties of the particular solution. To date, no clear advantage was documented as both colloids and crystalloids have been shown to be equivalent.

Cardiovascular Support

MODS is characterized by cardiovascular dysfunction even in the absence of pre-existing cardiac/vascular conditions that peaks early and resolves by 7 to 10 days in those that survive. Cardiac output is high, vascular tone low with arterial hypotension, and decreased
oxygen extraction. Fluid resuscitation to maintain the patients in a euvolemic state, however difficult to achieve, is the cornerstone of therapy. Recent evidence from Rivers et al. indicates that early goal-directed resuscitation to normalized end points in septic patients improves survival (194). The main goal for patients with MODS is to normalize the oxygen delivery (DO2) to oxygen consumption (VO2) ratios that are measured by the mixed-venous oxyhemoglobin saturation SvO2. A central venous catheter oxygen saturation (ScvO2) higher than 70% within 6 hours of presentation was demonstrated to prevented organ dysfunction (194). The basic endpoints of fluid resuscitation and the ones used in our animal studies remain the response of blood pressure, heart rate, and urine output to volume loading.

**Respiratory Support**

When ARDS develops, the noncardiogenic pulmonary edema is related to the damaged and leaky lung capillary endothelium. Diuresis therefore cannot pull water from the lung, and ventilatory support increases the end-expiratory pressures, which in turn decreases venous return. Subsequent decreased preload results in more fluid requirements by the patient. Therefore the principles of fluid resuscitation are aimed at maintaining euvolesia, limiting the intrathoracic pressures, facilitating venous return with increased cardiac output using pressors if necessary, limiting oxygen consumption with sedation, analgesia and temperature control and judicious use of packed red blood cells for increased oxygen carrying capacity. In a recent study, Finfer et al. demonstrated that fluid replacement with crystalloids and albumin was equivocal in mortality rates in 7000 critically ill patients and there are ongoing trial with respect to the use of synthetic colloids (195). Vasopressors dopamine or norepinephrine are the mainstay of therapy (196).

A higher incidence of ARDS has been detected in patients whose sepsis is caused by a pulmonary rather than a nonpulmonary source (197). On the other hand, nosocomial pneumonia is found in 15% of ARDS patients (198). Therefore, immune surveillance with frequent BAL is important to detect treatable causes of sepsis.

Ventilatory support strategies that have been shown to decrease ventilator-induced lung injury and decrease mortality are based on decreased volume and pressure ventilation, where optimal oxygen delivery is achieved with lowest levels of inspired oxygen, tidal volume, plateau pressure, and PEEP. Ventilator management is recommended at tidal volumes of 6–7 ml/kg of ideal body weight (199) and when combined with pulmonary toilet and recruitment manoeuvres, this approach limits atelectasis and prevents shunting. Other modes of ventilation such as
HFOV although extensively studied and shown to decrease the incidence of ventilatory induced lung injury in ARDS animal models, has failed to show any significant difference in mortality in human studies (200).

**Nutritional Support**

Nutritional support using full protein and calorie enteral nutrition is the standard of care. In a study by Heyland DK and colleagues (201) twenty-two randomized trials compared the use of immunonutrition with standard enteral nutrition in surgical and critically ill patients. They found that studies using commercial formulas with high arginine content were associated with a significant reduction in infectious complications and a trend toward a lower mortality rate compared with other immune-enhancing diets. However decreasing infectious complication rates was not associated with an overall mortality advantage.

**Renal Support**

In the context of MODS, renal failure carries a mortality of 50% to 90%. Therefore, it must be addressed aggressively with a trial of loop diuretics followed by continuous venovenous haemofiltration or intermittent haemodialysis.

**Hepatic Support**

Hepatic dysfunction from a hypotensive crisis in MODS results in ischemic hepatitis or shock liver and mimics acute fulminant hepatic failure. Secondly, a more mild form of hepatic dysfunction can occur, termed “ICU jaundice”. This takes place secondary to microcirculatory collapse, total parenteral nutrition-induced cholestasis, and/or drug toxicity. At present, hepatic dysfunction (coagulopathy, hypoproteinemia, thrombocytopenia, ascites, and encephalopathy) are treated symptomatically.

**Endocrine Support**

Endocrine and immune supports are important in management of MODS and ARDS. Critically ill patients are often hyperglycemic due to insulin resistance. To decrease the risk of infection, polyneuropathy, MODS, and death blood glucose needs to be maintained at concentrations between 4 and 6 mmol/L (80 to 110 mg/dL). Further, adrenal insufficiency should be considered in all patients with MOF and sepsis on vasopressors. Adrenal insufficiency is confirmed if corticotropin stimulation test results in a rise in serum cortisol level to less than 9
Therapy with hydrocortisone and fludrocortisone is recommended and continued for up to 7 days if the cortisol level remains less than 9 µgm/dL (202). A recent meta-analysis confirmed that although short courses of high dose corticosteroids do not affect mortality from severe sepsis and septic shock, long courses of low dose corticosteroids improve systemic hemodynamic, reduce the time on vasopressor treatment as well as reduce mortality at 28 days in ICU (203). Thyroid dysfunction can also occur and however, treatment has not been shown to be beneficial.

**Antimediator Therapy**

Antimediator therapy has received attention as potential “magic bullets” for the intermediaries of the proinflammatory response. One of the promising agents that have been studied extensively for their use in patients with septic shock and organ failure is Recombinant Activated protein C (drotrecogin alfa). It is an endogenous fibrinolytic whose production is impaired during the inflammatory response. In a multicenter, randomized clinical trial (PROWESS trial), drotrecogin alfa (24 µg/kg per h for 96 h) reduced mortality rate at day 28, from 30.8% in the placebo group to 24.7% in the treatment group. An absolute reduction in mortality by 6.1% occurred in all patients and by 13% in patients with severe septic shock, defined by APACHE II scores of 25 or more (204).

Other agents such as monoclonal antibodies (HA-1A, E5) targeting lipopolysaccharide were studied. Although randomized trials suggested a benefit when the studies were pooled the overall effect was not statistically significant (205). A number of other LPS-targeting drugs are currently under investigation (cationic antimicrobial protein 18, synthetic analogues of lipid A, E5564, human lipoproteins that also exert anti-inflammatory effects, recombinant monoclonal antibody to CD14) and TLR4 antagonist – Eritoran (206). Their effectiveness in treating sepsis is yet to be determined.

In MODS and ARDS proinflammatory cytokines such as tumour necrosis-alpha, IL-1β, and IL-6, exert many deleterious effects, including activation of coagulation and enhanced formation of thrombin and fibrin clot. Most drugs designed to block these factors in the inflammatory cascade have so far failed to improve survival. Many of the most avidly studied ones are the following: TNF alpha soluble receptor p55, IL-1 receptor antagonist, platelet activating factor receptor antagonist, anti adhesion molecule antibodies, arachidonic acid metabolite inhibitors, antioxidants, bradykinin inhibitors, phosphodiesterase and C1 esterase inhibitors, inhibition of nitric oxide with l-N-monomethyl arginine, polyclonal
immunoglobulins, interferon gamma and granulocyte macrophage colony stimulating factor. However, a recent meta-analysis of 10 trials of neutralization of the TNF-α with monoclonal therapy showed an absolute reduction in mortality of 3-5% (205), which makes this agent a possible adjunct to accepted therapy.

In summary treatment of MODS/ARDS and sepsis remains a challenging task. Although progress has been made in understanding some of the cellular and molecular processes that govern the host response to tissue injury, designing effective clinical treatment strategies for ARDS and MODS based on current knowledge are still in their infancy.
Section III  The Role of Oxidant Stress in SIRS, MODS and ARDS

- Production and Natural Function of Oxidants
- Oxidant-Antioxidant Balance
- Oxidants as Mediators of Ischemia/Reperfusion Injury
- Cellular Targets of Oxidative stress
  - Proteins
  - Phospholipids
  - Nucleic Acids
  - Cellular Redox State
  - Calcium Homeostasis
  - Signalling Pathways
- Oxidant Induced Ligand Independent Signalling
- Oxidative stress and NF-κB Activation
- Antioxidant Therapy in Animal and Human Trials
  - Inhibitors of lipid peroxidation
  - Inhibitors of xanthine oxidase
  - Mimetics of superoxide dismutases (SOD) and catalases
  - Glutathione peroxidase (GSHPx) and its mimetics
  - Inhibitors of nitric oxide synthase (NOS)
  - Metal chelators
  - Dietary free-radical scavengers
  - Poly(ADP-ribose) polymerase (PARP) inhibitors
  - Mitochondrial permeability transition inhibitors
  - Spin traps and peroxynitrite scavengers
Production and Natural Function of Oxidants

Oxygen is the most abundant element in the earth’s crust and although necessary for life of all aerobic organisms, it poses a danger to them. This is the “Oxygen Paradox” as coined by JM McCord (207). Aerobic energy metabolism occurs through oxidative phosphorylation, a process by which the energy released during oxygen reduction is converted into high-energy phosphate bonds of ATP. This process occurs in the mitochondria of eukaryotes in the electron transport chain driven by a multicomponent nicotinamide adenine dinucleotide (NADH) dehydrogenase enzymatic complex (208).

Oxygen is an oxidizing agent as it is capable of taking on four additional electrons to enter a lower free energy state. The product of oxygen that is completely reduced is water. Incomplete reduction of oxygen results in the formation of reactive oxygen species (ROS) commonly referred to as free radicals, which is not a correct term since not all ROS are free radicals (209). A free radical is defined as “any atomic or molecular species capable of independent existence that contains one or more unpaired electrons in one of its molecular orbitals” (210). Molecular O$_2$ although stable, is itself a free radical since it has two unpaired electrons (208). According to Thannickal et al., a one-electron reduction of O$_2$ results in the formation of superoxide anion (O$_2^-$), and a two-electron reduction of oxygen results in hydrogen peroxide (H$_2$O$_2$) formation. Both are formed by either enzymatic catalysis or from "electron leaks" in the mitochondrial electron transfer mechanism (208). In 1969 McCord and Fridovich discovered an enzyme superoxide dismutase (SOD) that catalyzes the formation of hydrogen peroxide from superoxide ions according to the following equation:

$$\text{SOD} \quad \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

Hydrogen peroxide is not a free radical and a weaker oxidizing agent then O$_2^-$. It is stable and capable of diffusing across biological membranes. However, by a Fenton reaction, it can react with ferrous iron or cuprous copper to form the reactive hydroxyl radical (OH·) which is the most potent oxidant known. Other ROS derived from molecular oxygen include: singlet oxygen, hypochlorous acid, peroxynitrite and nitric oxide (208).

Mitochondria are the source of O$_2^-$ and H$_2$O$_2$ formation, the latter being the most ubiquitous ROS in living tissues. Cytochrome-c oxidase in the mitochondria is responsible for transferring four electrons to oxygen to produce two molecules of water, however the electron-transport chain is not fool proof, and electrons may leak resulting in ROS formation. There are two leakage sites in mitochondria: one at Complex I and another one at ubisemiquinone (211). Approximately 1-2% of electrons that are involved in the mitochondrial electron transduction...
pathway can leak to form superoxide. The electron leak rate is directly proportional to the oxygen tension in the atmosphere; therefore, an atmosphere of 100% oxygen is toxic to most living organisms. Other locations of ROS production are the endoplasmic reticulum (ER), nuclear membranes, peroxisomes, and intracellular membrane-associated oxidases. The most extensively studied membrane oxidase that is involved in $O_2^{-}$ production is xanthine oxidase (XO). XO can be formed from xanthine dehydrogenase after tissue exposure to hypoxia (212).

While ROS are harmful to living organisms, evolution paradoxically has turned this phenomenon to a survival advantage. ROS when generated by specialized plasma membrane oxidases function in normal physiological signalling by growth factors and cytokines (208). In eukaryotes, ROS are part of a conserved defence mechanism against invading microbes. Indeed, phagocytic cells are capable of generating $O_2^{-}$ required for killing invaders and protecting the whole organism from demise. For reasons that are not yet fully elucidated, pro and anti oxidant checks and balances can fail in living organisms. According to McCord, the immune defence system tends to overreact to any challenge, as too timid a response may be fatal (211). Therefore, in the instances where the defence systems become overzealous at protecting against the enemy damage to the host itself can occur. An example of such a deleterious process is the exaggerated inflammatory and response seen following ischemia reperfusion where oxidative stress plays a critical pathological role. This thesis addresses some of the cellular mechanisms at play during the oxidative stress during ischemia-reperfusion states.

**Oxidant-Antioxidant Balance**

Since oxygen radicals are a normal by-product of aerobic metabolism, all aerobic organisms developed mechanisms to achieve an oxidation/antioxidation balance in order to prevent death by oxidation. Antioxidant systems work to prevent oxidation of lipids, proteins, and nucleic acids by ROS. Mechanisms also exist for either repair or removal of any damaged species. Oxidative stress occurs when antioxidants cannot prevent oxidative injury by overabundant oxidant production. The burden of oxidative stress occurs in various disease processes such as during resuscitation of a massive hemorrhagic shock, atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, as well as in a natural process of aging (207).

The antioxidant systems can be divided into enzymatic and non-enzymatic. The non-enzymatic systems include the water-soluble ascorbic acid, and the lipid soluble alpha tocopherol, glutathione and cysteine containing proteins such as albumin. Another way of
removing ROS is by catalysis with enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase which can break down hydrogen peroxide to oxygen and water and prevent harmful effects of ROS (211).

**Oxidants as Mediators of Ischemia/Reperfusion Injury**

Ischemia/Reperfusion injury refers to tissue damage when blood flow is restored after an ischemic period and is common to pathophysiology of many clinical conditions including myocardial infarcts, peripheral vascular insufficiency, stroke, trauma and hypovolemic shock. Despite its vital function in restoring the body’s hemodynamics, reperfusion leads to ROS generation and results in activation of the proinflammatory signalling pathways. Specifically, following resuscitation from hemorrhagic shock, production of ROS contributes to the development of SIRS and later to MODS and ARDS. ARDS patients have been shown to have increased levels of ROS such as H$_2$O$_2$ in their expired air (213). In addition, alveolar epithelial lining fluid in ARDS patients is deficient in glutathione and contains high levels of peroxynitrite (214). Markers of oxidative stress, such as malondialdehyde and 4-hydroxynonenal, nitrites and nitrates, as well as lipid peroxidation products - F2 isoprostanes have been found in increased amounts in ARDS subjects especially in those with higher APACHE scores (215). The imbalance of the redox state has been observed in SIRS patients and the process of continued oxidative stress in SIRS is thought to promote the development of MODS and ARDS in ICU patients (216).

There are multiple sources of ROS during ischemia-reperfusion. The most well studied source is the reactions catalyzed by the enzymes xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. These superoxide-generating reactions occur in many cells, macrophages, PMNs and the endothelium being some of them. However, any cell is equipped with potential to produce ROS if injury to the mitochondria or intracellular membranes occurs. The mechanism of ROS generation during ischemia-reperfusion has been studied by Granger *et al.* in the 1980s (217). They concluded that ROS are mediators of reperfusion injury and that it is the enzyme xanthine oxidase, which is known to be present in high concentrations in the intestinal mucosa that is the initial triggering agent of ROS formation at reperfusion (218). In his partial mesenteric ischemia feline model, Granger demonstrated that intestinal mucosal necrosis could be ameliorated by such antioxidant means as: 1) administration of SOD prior to reperfusion, 2) scavenging of hydrogen peroxide with catalase or 3) scavenging hydroxyl radicals with DMSO or mannitol or 4) by chelating iron with
deferroxamine or transferin. Further, by feeding animals with allopurinol, pterin aldehyde or tungsten, superoxide generation by xanthine oxidase was prevented (219).

Granger et al. described that XO is generated during ischemia from its precursor Xanthine dehydrogenase (XD), a constitutively expressed 150 kDa protein. An important site of this conversion has been shown to be the intestinal microvasculature as XD/XO are especially abundant in the small intestine, where it is expressed predominantly in the villous epithelium (220). During ischemia, catabolic processes were found to generate increased levels of purine metabolites: hypoxanthine and xanthine, from catabolism of ATP. XD is converted to XO, and once oxygen is reintroduced during reperfusion, superoxide is generated. Superoxide triggers a free radical chain reaction that results in further ROS generation such as hydrogen peroxide (H$_2$O$_2$), the hydroxyl radical, peroxynitrite, hypochlorous acid, and the chloramines. Systemic oxidative stress ensues through a direct release of oxidants as well as xanthine oxidase into the systemic circulation (221) with resulting concomitant tissue damage. Accordingly, increased plasma levels of XO have been reported after hypovolemic shock, released from organs rich in XO, such as the liver and intestine, into the systemic circulation (222).

The second important source of the “respiratory burst” in both phagocytic and nonphagocytic cells is the NADPH oxidase system that catalyses one-electron reduction of oxygen to form superoxide using NADPH as an electron donor (223). When a macrophage or PMN is activated via a number of signals, the NADPH enzyme complex assembles in the cytosol in a membrane-bound vesicle, once the vesicle fuses with the plasma membrane superoxide is released. NADPH oxidase can be regulated by inflammatory cytokines such as TNF-α, IL1-β and LPS, although its expression and regulation differs in different cell types. NADPH is also structurally different in different cell types especially in the endothelium (224). Recent studies suggest that it may serve not only as an ROS generator but also as a sensor of oxygen tension and an iron uptake system (225). The superoxide generation by the XO or NADPH systems is not the final path to tissue injury. As outlined above, superoxide can trigger further generation of free radicals from H$_2$O$_2$. In addition, in phagocytes another enzyme - myeloperoxidase can also react with H$_2$O$_2$ to form hypochlorous acid.

Finally, another free radical in ischemia/reperfusion injury is nitric oxide (NO$\cdot$). NO$\cdot$ is a microbicidal product, a diatomic free radical, derived from L-arginine. The synthesis of NO$\cdot$ is catalyzed by a family of enzymes called nitric oxide synthases (NOS) comprised of endothelial NOS (ecNOS), neuronal cell NOS (neuronal NOS) and a third inducible form of NOS (iNOS) which is induced in response to inflammatory-like stimuli (128). NO$\cdot$ production is part of the
host defence mechanism against inflammatory insults and it serves to regulate vascular tone, maintain vessel patency by helping to prevent platelet aggregation and to down-regulate adhesion molecule expression. Under normal conditions, NO acts to regulate normal vascular permeability and it also scavenges ROS thus preventing formation of H₂O₂. Excessive production of NO⁻, however, can potentially induce tissue damage via formation of ROS such as peroxynitrite when NO reacts with superoxide (226).

Therefore, NO⁻ can be protective or harmful following ischemia/reperfusion a balance that is not yet fully elucidated. i-NOS inhibition has been shown to prevent endothelial dysfunction, suggesting that NO may be cytotoxic in certain circumstances (227). Contrasting studies indicate the therapeutic potential of administering exogenous NO⁻. During ischemia reperfusion, the dysfunctional endothelium decreases its production of NO⁻ resulting in superoxide accumulation, H₂O₂ formation and decreased vasodilation contributing to increased leukocyte adhesion and no-reflow phenomenon (228;229), NO⁻ may protect against it (230).

In summary, any insult capable of activating leukocytes is capable of triggering the release of reactive oxygen species. The imbalance in the redox state of an organism, therefore, represents a common pathway for many life-threatening conditions including the proinflammatory state observed following shock resuscitation.

**Cellular Targets of Oxidative stress**

Traditionally, oxidants were considered to exert their toxic effects in inflammation by directly damaging cells (231). Recently, however, there has been considerable interest in the potential role of oxidants in the regulation and disregulation of cell activation. Several cellular signalling pathways are known to be redox sensitive (232-235). Relevant to inflammation, oxidants have been shown to participate in signalling cascades which culminate in nuclear translocation of the proinflammatory transcription factor NF-κB and the induction of several proinflammatory genes (46;47;236). They have also been implicated in priming of the immune system and specifically macrophages for an exaggerated response following ischemia reperfusion injury (36;41).

Although all the cellular effects initiated by oxidative stress have not yet been fully elucidated, they can be broadly classified into four categories: 1) oxidation and reduction of proteins at sulphydryl groups or iron–sulphur clusters that affect enzyme, receptor or other activity 2) Direct lipid peroxidation especially in cell membranes 3) Direct nucleic acid damage 4) Alterations in the cellular redox state. By their effect on proteins, lipids, nucleic acids or
intracellular redox state, ROS transform different signalling pathways to affect cells and organisms in five overlapping categories: 1) effect on cytokine, growth factor, and hormone action and secretion; 2) effect on ion transport; 3) regulation of transcription; 4) neuromodulation; and 5) apoptosis. Mediators of the signalling pathways in these five categories are either activated or inactivated by ROS. This results in generation of different downstream physiological responses, some beneficial and some detrimental to the organism as a whole as is in the case of SIRS (208).

Proteins

Changes in the local redox state can lead to conformational changes in protein shape affecting their function. As outlined in a comprehensive review by Thannickal et al., there are a number of ways proteins are affected by oxidative stress: 1) the sulphydryl group (-SH) on cysteine residues of proteins can be oxidized to form sulfenic (-SOH), sulfenic (-SO2H), sulfonic (-SO3H), or S-glutathionylated (-SSG) derivatives, 2) the sulphydryl groups on cysteine residues can form intra or inter-molecular disulfide bonds within the same protein or two different proteins 3) oxidants can induce protein cross-linking by H2O2 - or peroxidase-catalyzed dityrosine formation or 4) transitional metal-containing proteins may be targets of site-directed, metal-catalyzed oxidation by ROS produced by certain mixed-function oxidases (MFO), which “targets” them for ubiquitination and degradation by proteases. Consequently, conformational changes in proteins or dimerization of proteins can increase or decrease their DNA binding, activate or inactivate their enzymatic activity or increase or decrease their ion channeling abilities at the cell surface, thus regulating signal transduction. For example, after H2O2 exposure there is a reduced state of critical cysteines in some transcription factors like egr-1 (237) or NF-κB (238), which promotes their DNA binding. Disulfide linkages can also lead to dissociation of a regulatory protein from an inactive complex resulting in activation (239;240). Release of inhibitory subunits such as the iron regulatory protein has been shown to occur following oxidative stress (241). Further, protein tyrosine phosphatases contain highly conserved regions of 11 amino acids with cysteine in their catalytic domains (242). Oxidation of the cysteine by ROS inactivates these enzymes in many cases leading to increased protein tyrosine phosphorylation. In addition, as described below, ROS can induce ligand-independent receptor signalling through dimerization or oligomerization of proteins.

Phospholipids

Phospholipids are another important target of ROS. Since polyunsaturated fatty acids are building blocks of cell membranes, the oxidative damage to lipids affects many cellular
functions. The products of unsaturated fatty acid oxidation are short-lived lipid hydroperoxides that can react with metals and produce a number of reactive products (e.g. aldehydes and epoxides) (243). Some of those products are diffusible and can spread the damage far beyond the site of the original free radical attack. The primary damaging effect of lipid peroxidation products is exerted at the level of the DNA and results in mutagenic or cancerous effects. An example of such an aldehyde product that reacts with DNA bases is Malondialdehyde (MDA) (244), shown to be both mutagenic and carcinogenic on rodents. In addition, hydroperoxides interfere with the regulation of several metabolic pathways such as oxidation of arachidonic acid by cyclooxygenase and lipoxygenase in eicosanoids. Consequently, one of the most widely used biomarkers of lipid peroxidation is a set of arachidonic acid oxidation products termed F2-Isoprostanes (243). F2-Isoprostanes are prostaglandin-like compounds that are derived nonenzymatically. A substantial body of evidence now exists in support of measuring F2-Isoprostane levels in body fluids, such as plasma, to gauge levels of oxidative stress and lipid peroxidation \textit{in vivo} (245). Several methods have been developed to quantify the F2-Isoprostanes, such as gas chromatographic/negative ion chemical ionization mass spectrometric (GC/NICI-MS) assays as well as immunological assays used here (245).

**Nucleic Acids**

In addition to protein and lipid damage, ROS have the potential to cause direct damage to DNA bases or the deoxyribosyl backbone of DNA to produce strand breaks. Therefore, oxidative DNA damage can result in genetic mutations that modulate gene expression and chromosomal rearrangements. DNA mutagenesis is directly proportional to the number of oxidative DNA hits that are not repaired (243). The lesions that escape repair can be promutagenic and therefore oxidative damage is proposed to play a role in cancer development. An example, is the nitric oxide induced inhibition of a DNA repair enzyme FAPY glycosylase that removes 8-oxo-dG (246). Oxidative DNA damage can results in a wide range of chromosomal abnormalities, causing a blockage of DNA replication and resultant cytotoxicity (247). It has been demonstrated that the hydroxyl radical is able to activate certain oncogenes, such as K-\textit{ras} and \textit{C-Raf-1} (247). Thus ROS-induced DNA damage can be due to chemical or structural alterations of this molecule.

**Cellular Redox State**

ROS not only induce their effect on proteins, lipids and nucleic acids but also, not surprisingly, regulate the intracellular redox status and are therefore critical in regulation of various redox sensitive enzyme and gene transcription activities. Under homeostatic
Physiological conditions, the intracellular redox status of the cell is maintained by thiols, specifically the tripeptide \( \gamma \)-glutamyl-\( \gamma \)-cysteinyl-glycine, or glutathione (GSH) and thioredoxin (TRX). GSH and TRX both reduce \( \text{H}_2\text{O}_2 \) and lipid peroxides to maintain strong “reducing” conditions within the cell (208). Evidence suggests that, in addition to their “antioxidant” functions, GSH and TRX also regulate cell signalling. GSH has been reported to regulate redox signalling by alterations in both the level of total GSH (248) and in the ratio of its oxidized (GSSG) to reduced (GSH) forms (208). ROS have been demonstrated to alter the regulation of the transcription of IL-4, IL-6, IL-8, and TNF-\( \alpha \) through a thiol-dependent mechanism (249;250). As evidence of its gene regulatory properties GSH has also been implicated in regulating DNA binding of transcription factors Sp-1 and NF-\( \kappa \)B (208). TRX, on the other hand, can regulate the activity of some proteins by directly binding to them, for example binding of TNF receptor-associated factor-2 (TRAF-2) to ASK-1 and consequent ASK-1 multimerization (251). A similar effect of TRX on DNA binding of NF-\( \kappa \)B has also been demonstrated (252).

**Calcium Homeostasis**

The redox status induced by exogenous oxidants has been shown to induce changes in intracellular \( \text{Ca}^{2+} \) homeostasis which potentially has consequences on cell signalling mechanisms including activation of endonucleases, and apoptosis (32;253;254). \( \text{H}_2\text{O}_2 \) stimulation has been able to induce a rapid increase in \( \text{Ca}^{2+} \) from the sarcoplasmic reticulum stores followed by a slower recruitment of \( \text{Ca}^{2+} \) from the extracellular space (255). Recent studies demonstrated that ROS can modulate vesicular traffic and receptor expression in various cell types mainly by altering normal \( \text{Ca}^{2+} \) homeostasis within plasma membranes, mitochondria and sarcoplasmic reticulum and result in intracellular \( \text{Ca}^{2+} \) increases causing actin filament remodelling (256;257). In pancreatic acinar cells, ROS generated by the hypoxanthine/xanthine oxidase system, similar to that observed in ischemia/reperfusion, was able to induce increased exocytosis of amylase. Together these studies demonstrate that ROS are capable of mobilizing different intracellular calcium stores and play an integral part in complex temporal and spatial modifications of the actin cytoskeleton leading to altered exocytosis.

**Signalling Pathways**

With its effect on proteins, lipids, nucleic acids, the intracellular redox state and with calcium homeostasis, ROS have widespread effects on many signalling pathways, some with proinflammatory effects. The most studied signalling pathways affected by ROS are 1) the MAPKs pathway comprising of a large family of protein kinases that include ERK1
(p44MAPK)/ERK2 (p42MAPK), JNKs (also known as the stress-activated protein kinases), and p38 MAPKs, 2) the activation of the Activator protein-1 (AP-1) transcriptional complex formed by the dimerization of Fos-Jun or Jun-Jun and 3) the activation of the NF-κB proinflammatory transcription factor pathway (208). Activation of NF-κB has been implicated in a number of proinflammatory states as well as in SIRS and is most relevant to this thesis. Therefore, the role of oxidants in NF-κB activation will be discussed in more detail.

**Oxidant Induced Ligand Independent Signalling**

A growing body of evidence points to multiple steps in the signalling pathways that are regulated by ROS; however, the specific signalling molecules that are targeted by ROS are not as well defined. ROS can influence gene expression as well as post-translational modification of proteins that can induce downstream signalling through ligand-independent receptor activation (130;258). Growth factor receptors, are such an example, where under normal conditions they are activated by ligand-induced dimerization or oligomerization that autophosphorylates their cytoplasmic kinase domains (259). In response to UV light, ligand-independent clustering and activation occurs (260), and this effect appears to be mediated by ROS (261). PDGF-α, PDGF-β, and EGF receptors have also been responsive to exogenous ROS, exhibiting tyrosine phosphorylation with H₂O₂, O₂- or NO· exposure (262-264). These observations raise a possibility that, when produced in excess, ROS may directly activate cell surface receptors independent of their ligands.

**Oxidative stress and NF-κB Activation**

The NF-κB family of transcription factors is composed of homodimers or heterodimers of Rel proteins that are involved in regulating a large number of genes related to immune function, inflammation, apoptosis, and cell proliferation (265). The mechanism(s) by which ROS regulate this activity have been studied extensively (137) and remain under investigation. What has been documented thus far, is that ROS, such as XO and H₂O₂, can induce NF-κB activation and DNA binding and antioxidants prevent or diminish it (137;266). For example, hyperoxia has been reported to upregulate the nitric oxide-sensitive pathway in vitro and similarly activate AP-1 and NF-κB to induce transcription of genes (267). In addition, an impaired pulmonary NF-κB activation has been observed in response to LPS in NADPH oxidase-deficient mice (268). In line with these observations exposure of cells to antioxidants
such as NAC decreased NF-κB activation in *in vivo* as well as in *in vitro* models of oxidative stress (36;137;269). Another antioxidant, a phenyl-tertbutynitrone, a spin trapping agent, has been shown to inhibit the NF-κB-dependent signalling pathway in pancreatic β cells. Phenyltertbutynitrone prevented β cell death and the development of insulin-dependent diabetes mellitus by inhibiting the proinflammatory state (270). Interestingly, NF-κB itself must be in a reduced form to exhibit DNA binding activity and reducing agents such as dithiothreitol or mercaptoethanol enhance its DNA binding activity, while oxidizing agents such as diamide inhibit this activity (268).

Many studies attempt to characterize which signalling event in NF-κB activation is affected by oxidative stress. Under nonactivated conditions, the NF-κB heterodimer is composed of subunits p50 (NF-κB1) and p65 (RelA) and associates with an inhibitory protein, IκBα (271). One common step in the NF-κB signalling pathway induced by LPS, TNF-α and IL-1 is the phosphorylation of the inhibitory IκB. The phosphorylation of IκB on serine residues S32 and S36 results in recognition of IκB by a subunit of a specialized E3 ubiquitin ligase complex, known as E3IκB (271). The polyubiquitinated IκBs are targeted for rapid degradation by the 26S proteosome (272). Once free from its inhibitory protein, NF-κB translocates to the nucleus to bind DNA and activate transcription. Therefore, NF-κB activation can be blocked by interference with any of multiple steps: IκBα phosphorylation, binding of E3IκB to phospho-IκB, the polyubiquitination reaction, and proteosome mediated degradation. Antioxidants, such as PDTC and NAC, or overexpression of peroxidases, have been demonstrated to block IκBα ubiquitination however not phosphorylation (273;274) making it a likely candidate for oxidative stress sensitivity. Since the specificity of protein ubiquitination is dependent on ubiquitin ligase E3, the interaction of E3 with phosphorylated IκB may be a step affected by oxidative stress. Alternatively, some evidence suggests, that oxidative stress can lead to phosphorylation of IκBα on another site, namely on tyrosine-42. This phosphorylation also results in displacement of IκBα from NF-κB, however not its degradation (275). The tyrosine-42 residue lies within a consensus sequence for binding the regulatory subunit of PI3K: p85. Tyrosine-42-phosphorylated IκB has been shown to stably interact with p85 following pervanadate treatment of Jurkat cells (276), therefore oxidative stress may alter the signalling pathway from NF-κB to PI3K activation.

A number of different kinases have been reported to phosphorylate IκB and be sensitive to H₂O₂. These include Iκ-kinase (IKK) complex, NF-κβ-inducing kinase (NIK), double-stranded RNA-activated serine-threonine protein kinase (PKR), MEKK1, Akt, Syk, p38 and
ERK just to name a few (275;277;278). Akt has been studied extensively and demonstrated to be activated by H$_2$O$_2$ in several cell types (279). Akt is a potential downstream mediator of PI3 kinase. Recent evidence indicates that activation of PI 3-kinase is linked to activation of Src kinases which in turn are involved in macrophage priming by oxidants (41). Oxidative stress was also demonstrated to reprogram LPS induced NF-κB signalling such that it changes from a Src-independent pathway to one that is Src-dependent. During its activation, PI3 kinases are phosphorylated on tyrosine residues, and thus might serve as a possible substrate for the Src kinases (280). This is consistent with other’s findings that the PI3-kinase pathway may be a downstream effector of cellular response to oxidative stress (281).

NF-κB activation can be influenced by ROS at any of multiple steps leading to its nuclear translocation. Most relevant to this thesis and the role of TLR4 receptors in oxidant mediated NF-κB activation. Recent evidence suggests that IRAK-1 and IRAK-4 activation is diminished with antioxidants after exposure of neutrophils to LPS (282). In addition, recruitment of IRAK-1 to the IL-1R in a T cell line was shown to be regulated by intracellular oxidant balance (283). Since the IRAK molecules are critical players in the LPS-TLR4 signalling pathway (284), these findings suggest that proximal events in TLR4 signalling are affected by ROS can modulate NF-κB activation.

As demonstrated by many, virtually every step of the NF-κB signalling cascade is comprised of redox-sensitive proteins whose activities are modulated upon changes in ROS. Despite much investigation however, the role of upstream components in oxidative stress mediated regulation of NF-κB activation remain ill defined. Oxidant induced signalling via the TLR4 receptors and their role in oxidant mediated macrophage priming are virtually not known and are therefore a subject of this thesis.

**Antioxidant Therapy in Animal and Human Trials**

Studying antioxidants in animals as well as in humans has aided not only in the search for pharmaceutical interventions, but also in dissecting the role of oxidative stress in ischemia/reperfusion injury. Innumerable pharmacological agents have been studied and their impact on ischemia-induced intracellular cascades described. Selected strategies used in animal studies relevant to this thesis will be described as well as clinical trials reviewed.

Antioxidants treatment strategies can be broadly divided into those supplementing endogenous antioxidant enzymatic activity and those supplementing non-enzymatic oxidant scavengers. The strategies that supplement endogenous antioxidant enzymatic activity can be
further categorized as: (1) inhibitors of lipid peroxidation; (2) inhibitors of xanthine oxidase; (3) mimetics of superoxide dismutases (SOD) and catalases, (4) glutathione peroxidase (GSHPx) and its mimetics; and (5) inhibitors of nitric oxide synthase (NOS).

The strategies that work by supplementing non-enzymatic oxidant scavengers can be further categorized as: (1) metal chelators; (2) dietary free-radical scavengers; (3) poly(ADP-ribose) polymerase (PARP) inhibitors; (4) mitochondrial permeability transition inhibitors; (5) spin traps and peroxynitrite scavengers. Individual categories and their contribution to treatment of MODS and ARDS are discussed.

**Inhibition of lipid peroxidation**

Lipid peroxidation induced by neutrophil-derived and other oxygen radicals has been implicated as a common pathway in endothelial cell injury that is responsible for the increase in capillary permeability observed in ARDS (285;286). Numerous pharmacological inhibitors of lipid peroxidation have been tested in animals and humans following ischemia reperfusion injury. The lipid peroxidation inhibitor H290/51 was demonstrated to attenuate cardiac injury induced by ischemia-reperfusion (287). Another compound a lipid peroxidation inhibitor U-101033E was examined and found to protect hippocampal CA1 and cortical neurons after 15 minutes of global cerebral ischemia (288). Further, in heart preservation studies administration of a lipid peroxidation inhibitor lazaroid (U74500A) resulted in an improvement of functional recovery after the 24-hour preservation period. Lazaroids were also shown to ameliorate smoke-induced lung injury by attenuating alveolar macrophage TNF-α release (289). The most extensively studied lazaroid is tirilazad. It is a 21-aminosteroid designed to localize and act within cell membranes and inhibit lipid peroxidation. Tirilazad mesylate (U-74006F), was selected for clinical development and has been extensively studied (290). Despite abundant preclinical evidence that tirilazad improves ischemic outcomes by inhibiting lipid peroxidation, no positive outcome effects have been concluded from human studies (291).

**Inhibitors of xanthine oxidase**

Several xanthine oxidase inhibitors have been tested in both animal and human studies of ischemia reperfusion injury. Tungsten and allopurinol are competitive inhibitors of XO and have been tested and shown effective against ischemia reperfusion injury when included in animal diets (292). Allopurinol is commonly used in humans as a standard treatment for gout and hyperuricemia (293). At high doses (>1000 mg/d), allopurinol becomes a non-competitive inhibitor of xanthine oxidase and is oxidized by xanthine oxidase to oxypurinol. Oxypurinol occupies the active site of xanthine oxidase effectively inhibiting its action (293). Other xanthine
derivatives: pentoxifylline and lisofylline have been used in studies and were shown to decrease neutrophil adherence, improve microvascular circulation by increasing RBC flexibility and decrease cytokine release and oxidant release in animal models (294-296). Furthermore, Tungsten and Allopurinol were shown to reduced ARDS induced by hemorrhage and resuscitation (292). In a dog study performed to assess the effect of xanthine oxidase inhibition (allopurinol 50 mg/kg/day), on the 24-hr survival of dogs subjected to irreversible hemorrhagic shock, beneficial effect of xanthine oxidase inhibition on survival was demonstrated. In humans, allopurinol has been used prophylactically in patients undergoing cardiac surgery. Studies indicate that prophylactic allopurinol may prevent arrhythmias occurring following CABG, decrease pressor/inotrope utilization, and decrease mortality (293).

**Mimetics of Superoxide Dismutase and Catalase**

During states of ischemia reperfusion the production of superoxide is increased to such an extent that the capacity of the endogenous SOD to clear ROS is overwhelmed. When an imbalance between oxidant and antioxidant systems occurs superoxide-mediated damage ensues. Evidence from studies indicates that it is possible to protect organisms from ROS damage through overexpressing the antioxidant SOD (297). Through extensive pharmacological research several SOD mimetics have been developed and tested in animal systems (298). Some of the ones available to date are: Mn(II) cyclic polyamines, Mn(III) salen derivatives, Mn(III) porphyrins and stable cyclic nitroxides such as tempol. Tempol has been tested and shown effective preclinical of ischaemia-reperfusion injury, shock and inflammation (299). Other SOD mimetics have also shown effective protection against ischemia reperfusion injury in disease models of stroke, Parkinson's disease, experimental allergic encephalomyelitis (EAE) and LPS-induced adult respiratory distress syndrome (ARDS) (299). These data implicate an important role for SOD mimetics in the treatment of oxidative stress induced diseases, however human studies have yet to be undertaken.

**Glutathione (GSH) peroxidase and GSH mimetics**

While catalase is localized mainly in peroxisomes glutathione peroxidase is present in the cytosol. As a result, the ubiquitous presence of glutathione peroxidase predicts it to be the more important enzyme in responding to increased hydrogen peroxide. Glutathione peroxidase catalyzes peroxide detoxification by glutathione and it needs selenium to be active. Selenium replacement in patients with SIRS has been studied and was shown to decrease the rate of renal failure, a more rapid resolution of organ dysfunction, and a trend toward a decreased mortality rate (300).
Glutathione is an essential intracellular antioxidant and substances such as glutamine and N-acetylcysteine (NAC) that provide new substrates for glutathione may enhance natural antioxidant activities of cells. While the beneficial effects of glutamine in critically ill patients are not completely understood, NAC has been used extensively in animal and human studies. Clinically, NAC is used for the treatment of acetaminophen overdose and as a mucolytic agent in obstructive pulmonary disease. The use of NAC in ischemia reperfusion states has been beneficial in extremity ischemia resuscitation and during cold preservation of the donor liver for transplantation (301;302). NAC has also been proven beneficial in animal models of ischemia and reperfusion injury as well as ARDS (303-307).

Human studies on intravenous NAC administration have been performed in patients with septic shock. Results have been encouraging with NAC inducing a decrease in systemic vascular resistance and an increase in cardiac index as well as a decreased interleukin-8, TNF-α levels and improved phagocytosis activity in septic patients (308;309). However, a distinct mortality improvement has not been documented with NAC, although survivors who received NAC had fewer ventilator days and were discharged earlier from the ICU (139;310);(311;312).

Together, these studies suggest that treatment with the antioxidant NAC may improve lung function and shorten ICU stay in patients with early septic shock. However, further trials, perhaps with larger sample sizes or different modes of administration of NAC, are needed to determine whether patients at an earlier stage in the disease process or those with specific risk factors will benefit from NAC treatment.

**Inhibitors of nitric oxide synthase (NOS)**

Several NOS inhibitors have been developed and are base on L-arginine analogues, which nonspecifically inhibit all NO− production although the development of isoform-selective NOS inhibitors is an area of active research. In a recent study by Paul-Clark et al., NOS inhibitors administered locally exacerbated inflammation suggesting that the local production of NO− is protective. By contrast, however, giving NOS inhibitors systemically ameliorated inflammation, thereby showing differential anti-inflammatory properties of NOS inhibitors depending on their route of administration (226). In humans, NOS inhibitors have recently been studied in patients with septic shock with a surprising result. Even though treatment with NG-methyl-L-arginine hydrochloride (546C88) was associated with improved hemodynamic parameters, a subsequent international, randomized, double-blind, placebo-controlled phase III study in patients with septic shock had to be discontinued due to the emergence of increased mortality in the 546C88-treated group (313). Clearly, although there is little doubt that nitric
oxide plays a pivotal role in mediating tissue damage in ischemia reperfusion, its regulation and development of pharmacological inhibitors need further investigation.

**Metal Chelators**

Metals, such as iron and copper, participate as substrates for the hydroxyl radical formation from hydrogen peroxide. Metal chelators serve as antioxidants through binding of the transitional metal ions as well as by scavenging ROS themselves. These may include transferrin, ceruloplasmin, hemopexin, or synthetic metal chelators available commercially. One of the best-studied metal chelators is Deferoxamine (DFO), which has been used in animal models of human disease with promising results. However, its use in humans is limited since it causes severe hypotension. It has been coupled to hetastarch, and in initial animal studies of burn resuscitation or trauma, it has demonstrated an improvement in hemodynamics and decreased oxidant production. This product is still under investigation (314). 21-aminosteroids (eg. compound U74006F) are another group of antioxidants that chelate iron and act as direct oxidant scavengers. They have been shown, in animals, to decrease TNF-α release following reperfusion injury or endotoxemia (315). Their role in clinical use is currently being defined. Because metal ions are critical in many enzymatic reactions that keep the overall homeostasis, such interventions may be limited in their clinical applications by their toxicity to the organism as a whole.

**Dietary free-radical scavengers**

The most active dietary free-radical scavengers are α-tocopherol (Vitamin E), ascorbic acid (Vitamin C) and β-carotene (Vitamin A). Oxidative stress during critical illness overwhelms the endogenous antioxidant mechanisms and depletes dietary free-radical scavengers (316). Plasma concentrations of antioxidant vitamins have been shown to predict the development of multiple organ failure in patients at risk (317). These substances have been administered exogenously and studied in animals and humans to determine if such treatments can improve outcomes in ICU patients. For example, in a rat model of hypoxia-induced lung injury, Vitamin E administration protected tissues from lipid peroxidation, improved oxygenation and inhibited the proinflammatory protein kinase C (318;319).

In humans, several clinical trials have been performed. In a prospective, randomized, double-blind, placebo-controlled trial Crimi et al. demonstrated that vitamins C and E in enteral feeding diminished oxidative stress and improved 28-day mortality by approximately 20% (320). In another randomized, prospective study, Nathens et al. analyzed the effects of prophylactic administration of vitamin C (1000 mg i.v.) and α-tocopherol (3,000 IU/day
enterally) in 595 critically ill patients (321). Antioxidant supplementation was shown to reduce the rate of pulmonary morbidity and organ dysfunction. Patients randomized to antioxidant supplementation also had a shorter duration of mechanical ventilation and length of ICU stay. The incidence of pneumonia and ARDS tended to decrease in the group who received supplementation; however, a statistically significant difference was not reached. Several other studies document the effects of an enteral solution enriched with vitamin A, vitamin C, and/or vitamin E in critically ill patients. Overall these studies demonstrate improved clinical outcome variables especially pulmonary function parameters and duration of mechanical ventilation (322;323).

**Poly(ADP-ribose) polymerase (PARP) inhibitors**

PARP is an enzyme that is constitutively active and participates in the DNA repair mechanism. During oxidative stress, when DNA damage is excessive, PARP is overactivated and depletes nicotinamide adenine dinucleotide (NAD+) and consequently ATP, potentially exacerbating ischemic injury. NAD+ is critical for oxidative phosphorylation and without it the cell cannot form ATP and dies by necrosis further exacerbating inflammation in the surrounding tissues. PARP inhibitors block oxidant-mediated NAD+ depletion and aim at preserving cellular ATP stores. PARP inhibitors have been studied in several ischemia models (324;325). PARP knock-out mice were demonstrated to exhibit diminished cerebral infarct sizes when compared to wild-type counterparts (326). In humans, PARP inhibitors have not been fully tested and several clinical trials are currently ongoing. PARP inhibitor INO-1001, has been entered into Phase II clinical trials for various indications such as myocardial infarction, cardiopulmonary bypass and thoracoabdominal aortic aneurysm surgery (327). PARP inhibition, although with potentially wide spread clinical applications, is sill being developed as the potential long-term side effects of PARP inhibitors are yet unknown.

**Mitochondrial permeability transition (MPT) inhibitors**

MPT is a process of opening of permeability transition pores in the inner mitochondrial membrane. This process is important in ischemia reperfusion as both cell apoptosis and necrosis can be initiated by damage to mitochondria resulting in mitochondrial permeability transition (MPT) (328). MPT leads to matrix swelling, outer membrane rupture and release of apoptotic signalling molecules. During ischemia, Ca^{2+} overload, long-chain fatty acid accumulation, and reactive oxygen species all cause translocation of cyclophilin-D from the matrix to the MPT pore allowing flux of solutes from the matrix to the intermembrane space.
Consequently, a number of inhibitors of MTP exist, cyclophilin binding protein cyclosporin A (CsA), Sanglifehrin A, bongkrekic acid and others (330).

In general, however, potent MPT inhibitors such as CsA and sanglifehrin A, have been found to be protective only in animal studies when delivered only during reperfusion, although the extent of protection varies depending on the experimental species (331-333). In conclusion, inhibition of the opening of the mitochondrial permeability transition pores may provide novel targets for ischemia reperfusion protective therapies, however clinical studies have yet to be undertaken.

**Spin traps and peroxynitrite scavengers**

Nitrone spin traps and peroxinitrites are chemically engineered compounds that are able to trap oxygen free radicals and have been used as antioxidants (334). Second-generation nitrone spin traps such as NXY-059 (disodium 4-[(tert-butylimino)-methyl]benzene-1,3-disulfonate N-oxide), and Stilbazulenyl nitrone (STAZN) have been demonstrated to be neuroprotective in models of focal ischemic stroke and traumatic brain injury (335;336-338;339;340). NXY-059 has been shown to maintain Akt activation and inhibit cytochrome c release after ischemia (341). These compounds are currently being investigated in human trials (342) and represent novel potentially therapeutic approaches to the pathological process involving ischemia reperfusion injury and ensuing oxidative stress.

In summary, multiple antioxidant strategies are available today, however most antioxidants have a limited bioavailability, undefined secondary effects and human trials with these agents have yet to show definitive long-term results.
Section IV  Innate Immunity and Toll-Like Receptors: Molecular Mechanisms of Sepsis

- Innate vs. Adaptive Immunity
- LPS Structure
- Recognition of Pathogens Through Toll Like Receptors
- Structure of Toll Like Receptors
- The LPS Receptor Complex
- The TLR-4 Mediated LPS Signalling Cascade:
  - MyD88-dependent pathway
  - MyD88-independent pathway
- Regulation of TLR4 Expression
- Cellular Distribution and Trafficking of the LPS Receptor Complex
- LPS Independent TLR4 Signalling
- TLR4 Independent LPS Signalling
**Innate vs. Adaptive Immunity**

The word “sepsis” is derived from the Greek origin “sepsios” meaning rotten, a condition that is not accepted passively by living organisms. Humans have pursued to understand the mechanisms responsible for the decay of living organisms and especially the human body for centuries, as the answers hold the key to the universal human condition – mortality. One of the key events in understanding the origin of sepsis was the discovery of microbes in 1674 by Antony van Leeuwenhoek. It took several hundred years of rigorous studies to determine that there are a number of systemic responses of the body to defend itself against microbial pathogens and their toxins. The immune response to microbial pathogens can be broadly divided into the adaptive and the innate components that act locally or systemically (343). The very first and therefore critical point of direct contact between the invading pathogen and the host that determines the fate of both living organisms is the innate immunity. Mediated largely by white blood cells: neutrophils, macrophages, dendritic cells and Natural killer cells, innate immunity is not pathogen or antigen specific and it lacks immunologic memory (344). The role of innate immunity is to prevent or attenuate any microbial invasion or to reduce and sequester the pathogen load, however, full clearance of invaders requires a complex interplay between innate and adaptive immunity. Innate immunity can be further subdivided into two parts; the first part includes nonspecific systemic responses such as the activation of the hypothalamic-pituitary-adrenocortical axis, the sympathetic-adrenomedullary axis, acute phase protein synthesis, and thermoregulation. The second part includes responses to highly conserved structures not found in higher eukaryotes called pathogen associated molecular patterns (PAMPs), that are characteristic of “non-self” and therefore potential “danger” signals (345). PAMPs are highly conserved and they are recognized by their corresponding receptors – the pattern-recognition receptors (345). Some well-characterized PAMPs include lipopolysaccharide, lipopeptides, peptidoglycan, bacterial DNA, double stranded RNA, Lipoteichoic acid, mannans in the yeast cell wall, glucans, zymosan, and heat shock proteins. Recently identified Toll like receptors (TLRs) as well as better-characterized mannan-binding lectin (induces complement activation) and macrophage mannose receptor (facilitates phagocytosis) are examples of proteins that take part in a complex system of pattern recognition of conserved microbial structures. Once engaged, these receptors are capable of activating phagocytosis or intracellular signal-transduction pathways that direct immune cells such as neutrophils, macrophages, dendritic cells and in some cases B cells to produce inflammatory mediators necessary for fighting the invading offenders and for immune homeostasis (345).
The adaptive immune system has been refined by evolution starting some 450 million years ago and is mediated by antigen specific, memory T and B lymphocytes. Small populations of cells with over $10^{12}$ specificities constitute a set of memory lymphocytes that once exposed to their antigen expand clonally, a process taking approximately 3-5 days (346). B cells produce antigen specific antibodies while T cells respond via a T cell receptors to antigens presented on antigen presenting cell. For a number of years the innate and adaptive immune responses were thought to act in a sequential manner, the first activating the latter. However, more recent evidence, some put forth in this thesis, suggests that their relationship is more complex (347). Medzhitov, in his most recent review, outlines that TLRs in addition to functioning as pattern recognition receptors, are also responsible for initiation of adaptive immune responses against pathogen-derived antigens primarily through triggering dendritic cell activation and T and B cell activation and differentiation (348). Therefore, the host defence and survival depends on the sophisticated relationship between these two arms of the immune system generating complex intra and inter cellular signalling cascades and corresponding responses that determine the fate of invaders and hosts.

**Lipopopolysaccharide (LPS) Structure**

The prototype model for innate immune responses is the leukocyte activation by Gram-negative bacteria, and specifically bacterial endotoxin: lipopolysaccharide (LPS). Gram negative bacterial infection accounts for up to 60% of cases of sepsis in humans (349) and is central in the pathogenesis of SIRS. LPS is situated in the outer membrane of Gram-negative bacteria. Although a variation exist between LPS components from different bacterial serotypes, the three elements of LPS have a similar function. Lipid A is responsible for the toxicity of LPS. Lipid A consists of a phosphorylated $\beta$1,6-linked D-glucosamine disaccharide that carries up to six or seven acyl residues. Variations in the length, position and number of the fatty acids as well as their phosphorylation determine the biological toxicity of LPS (350). Synthetic *E. coli* lipid A allowed to firmly establish that this moiety is the active and toxic component of LPS (351).

Hexacylated lipid A from *Escherichia coli* with side chains of 12–14 carbons in length has been demonstrated to maximally stimulate Toll like receptor 4 (TLR4) (352). The O-side chain, a complex polysaccharide, allows for classification of bacteria into serotypes, as different species possess O-antigents of differing structures. Antisera raised against one species do not cross-react with other species (353). Although not all Gram-negative bacteria have an O-antigen, they all possess a core hydrophilic oligosaccharide region containing a sugar, 2-keto-3-
deoxyoctonate (KDO) which is essential for bacterial viability. KDO serves as an attachment point to lipid A, the toxic moiety of LPS (353).

LPS is highly stable, toxic, and abundant and its effects on live organisms have been studied now for over a century. It can activate various cells of the immune system to produce proinflammatory molecules such as tumour necrosis factor α (TNF-α), various interleukins (IL-1, IL-6, IL-8, IL-12), interferon α and reactive oxygen species. LPS is a critical molecule in the origin and development of sepsis and therefore understanding the physiological effects of this endotoxin, on a cellular and molecular level, holds a key to understanding the responses of the innate immunity.

Recognition of Pathogens Through Toll-Like Receptors

To exert its biological activity LPS interacts with a specific LPS-binding molecule – TLR4. The interaction takes place on the surface or intracellularly in LPS-responsive cells such as macrophages, neutrophils and endothelial cells. Other proteins are thought to participate in interacting with LPS and forming functional LPS-receptor complexes that are located in specialized signalling microdomains called the lipid rafts (354).

Several key events laid the groundwork for the discovery of the LPS signalling mechanisms. The work has been performed chiefly in mice and insects, specifically in Drosophila. In 1965 Heppner and colleagues observed that a sub-strain of C3H mice, the C3H/HeJ strain, is hypo-responsive to LPS, which was attributed to a spontaneous mutation (355). A second spontaneous mutation was discovered in a C57BL/10ScCr strain of mice and was shown to affect the same locus as the C3H/HeJ mutation. When cells from the C3H/HeJmice were irradiated and then 'reconstituted' with hematopoietic cells from the C3H/HeN non-mutated sub-strain, their sensitivity to LPS was regained. Alternatively, when the C3H/HeN mice were irradiated and “reconstituted” with hematopoietic progenitors from C3H/HeJ mice they lost their LPS sensitivity. These results demonstrated that the lethal effects of LPS are somehow transferred with the hematopoietic cells. These cells were ultimately recognized as macrophages (356). The phenotype was later mapped to a single locus on chromosome 4 by J. Watson and colleagues in 1978 (357). This genetic locus was termed Lps and its mutant allele equivalent responsible for defective LPS responses, was termed Lps<sup>d</sup>. These findings indicate that likely a single protein is responsible for LPS responsiveness.

Initially the protein thought to be responsible for LPS signalling was a plasma membrane LPS binding protein discovered in 1990 by Wright et al., now known as Cd14 (358). However,
mice lacking CD14 were still able to respond to LPS, albeit in a much higher dose. In addition, CD14 is a glycosylphosphatidylinositol-linked membrane protein that lacks a cytoplasmic domain, therefore a different candidate was sought to explain the intracellular signalling of LPS. Subsequently, after many failed Lps cloning attempts, strict positional cloning and sequencing of the lps<sup>d</sup> locus by numerous groups led to the identification of a single authentic gene, encoding the TLR4 by Poltorak <i>et al.</i> in 1998 (359). This seminal work lead to a conclusion that the genetic defect in two LPS hyporesponsive strains of mice is linked to a single Tlr4 gene. In C3H/HeJ the codominant allele of lps<sup>d</sup> is a result of a missense mutation within the TLR4 coding sequence resulting in a Pro 712 to His substitution. C57Bl/10ScCr mouse strain was found to be homozygous for a null mutation of the TLR4. These genetic studies were fundamental in the conclusion that although many proteins may recognize, bind and be required for effective LPS signalling only one protein is absolutely essential to transducer the LPS signal intracellularly, and it was shown to be the TLR4 receptor.

In parallel to the studies performed in vertebrates, analysis of mutant flies that are hyporesponsive to various pathogens contributed to the understanding of the Toll-like receptor family and its relevance to innate immunity pathways. Although insects lack the adaptive immunity response arm, their innate immune system has proven to be closely related to the human one as it is highly conserved through a common ancestor some 800 million years ago. A critical discovery occurred in 1996 when Lemaitre and colleagues established that the <i>Drosophila</i> protein Toll responsible for developmental dorso-ventral axis formation was also involved in fruit fly immunity (360). Mutation in the Toll gene caused fruit flies to succumb to infections from Aspergillus fumigatus due to failure to induce the antifungal peptide Drosomycin. Activation of membrane Toll receptors in <i>Drosophila</i> leads to a production of antimicrobial peptides through a pathway that activates transcription factors Dif and Relish. These transcription factors are homologues of human NF-κB, a transcription factor for a number of proinflammatory mediators. Although Toll receptors serve an immune as well as a developmental function in fruit flies, it does not have such a dual function in mammals.

Mammals were discovered to have a homologous system to the <i>Drosophila</i> Toll signalling now coined Toll-like receptors. An important discovery was made by Medzhitov <i>et al.</i> of the first human homologue of Drosphila Toll – TLR4 (361). Constitutively active mutant of TLR4 exhibited activation of the NFκB pathway and production of a number of proinflammatory cytokines as well as the induction of the adaptive immune responses through activation of B7 family of molecules required for naïve T cell activation. Following the
discovery of TLR4, twelve mammalian types of TLRs have been identified all recognizing different PAMPs on bacteria and viruses or other pathogens (Fig. 1). TLR1 to 9 are conserved between the human and mouse. TLR2 recognizes lipoproteins, peptidoglycan and lipoteichoic acid from Gram-positive bacteria. In addition, it can bind some forms of LPS not recognized by TLR4. Other ligands of TL2 are: lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from Trypanosoma cruzi, a phenol-soluble modulin from Staphylococcus epidermis, zymosan from fungi and glycolipids from Treponema maltophilum. TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2 and recognize triacyl lipopeptides and diacyl lipopeptides respectively (362). TLR4 and TLR5 are other surface receptors that recognize LPS and flagellin respectively. TLRs: 3, 7, 8 and 9, reside inside the cell and recognize bacterial or viral nucleic acids. TLR3 is thought to recognize dsRNA and viruses. TLR7 and 8 are structurally similar and recognize guanosine- or uridine-rich single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus, vesicular stomatitis virus and influenza virus (362). TLR9 recognizes unmethylated CpG DNA also from bacteria or viruses but is rare in mammals. TLR10 is functional in the human, but its ligand is not yet well characterized (363). The ligand for TLR11 has also not been identified, but studies indicate that it may mediate anti-uropathogenic bacterial responses in mice (362). Ligands of TLR12 are not yet well characterized.

Better understanding of the interaction of TLRs with other receptors as well as their cellular signalling responses to threats of infectious and non infectious nature are key to understanding the pathogenesis of many disease processes such as: SIRS, autoimmune reactions or sterile inflammation. TLR4 function under oxidative stress conditions is the fundamental topic of this thesis.

**Structure of Toll-Like Receptors**

Although they recognize different molecular patterns, Toll receptors and different TLRs share structural similarities. They are single transmembrane proteins. The cytoplasmic portion of both Drosophila Toll (dToll) and TLRs contain domains homologous to the interleukin (IL)-1 receptor family. This domain is now called the Toll/IL-1 receptor (TIR) domain. The extracellular regions of Toll receptors contain 21 tandem leucine-rich repeats (LRRs) separated by a non-LRR region. The carboxy-terminal end of the LRR domain contains cysteine-rich domains and the N-terminal end of the LRR contains a 31-amino-acid long N-flanking region (361). The function of the ectodomain C-flanking cysteine-rich region in dToll was determined
to be involved in controlling the signalling activity of dToll, as disrupting the sulfide bonds in this region with a mutation in one of the four conserved cysteine residues (substitution to tyrosine), or deleting this and the LRR region altogether, rendered dToll constitutively active (361). As cysteine residues are a target of many oxidant species and therefore this region is of particular interest in our studies.

To correlate the observations in insects with those in humans, Medzhitov and colleagues demonstrated that expression of a recombinant human TLR that mimics features of the dominant-positive mutants of dToll also resulted in the induction of the IL-1, IL-8 and B7.1 genes. Further, expression of the recombinant human TLR caused an induction of NF-κB similar to dToll confirming that Toll/NF-κB signalling is conserved in *Drosophila* as well as in humans (361). Although the control center for initiation of the signalling activity by TLRs resides in its extracellular portions, the signal itself originates from the cytoplasmic TIR domains. A proline residue in the TIR domain was identified as critical for signalling as point mutations substituting proline residue at position 712 to histidine rendered the mouse strain C3H/HeJ hyporesponsive to LPS (359). This proline residue has been since shown to be conserved among all TLRs, except for TLR3 (364). The TIR domain is conserved not only in TLRs but also in other transmembrane receptors IL-1 receptor, IL-18 receptor and others (365). Of importance to TLR4 signalling, a number of TIR containing adaptor molecules have been identified that participate in TLR4 intracellular signal propagation. They are: Myeloid differentiation factor 88 (MyD88) (366), TIR-domain-containing adaptor protein (TIRAP), also called MyD88 adaptor-like (Mal); TIR domain-containing adaptor inducing interferon-β (TRIF), also called TICAM-1; and TRIF-related adaptor molecule (TRAM), or TICAM-2 (367).

**The LPS Receptor Complex**

In order to propagate LPS signalling TLR4 recognizes LPS in the context of an LPS receptor complex. TLR4 associates with three other proteins: LPS binding protein (LBP) (368), CD14 (358) and MD-2 (369). When LPS is present in the blood stream, it is immediately captured by LBP, and the complex is transferred to CD14, which lacks a transmembrane domain and is located on the surface of macrophages. CD14 is known to bind LPS, and this triggers a physical association between CD14 and TLR4 as well as MD-2 (370). CD14 has been initially suggested as the primary receptor for LPS (358;371) however, its lack of transmembrane or cytoplasmic regions made it a poor candidate for inducing intracellular signalling. CD14 is not absolutely necessary for LPS signalling as cells deficient in CD14 are still able to mount an LPS
mediated response, however mice lacking CD14 are shown to be highly resistant to LPS-induced shock (372). TLR4 has an intracellular TIR domain that can induce downstream signalling, however to do this TLR4 must associate with other proteins. MD-2, a small cysteine-rich glycoprotein has been demonstrated to be critical for LPS signalling via TLR4 (369). Cells expressing TLR4 alone or expressing mutant MD-2 were hyporesponsive to LPS and transfection with MD-2 cDNA reversed this effect (373). MD-2’s seven cysteine residues and a heterogeneous collection of disulfide-linked oligomers (374) are a possible target for oxidation and therefore are of particular relevance to our work. Further, surface expression of TLR4 requires the co-expression of MD2. MD-2 binds to the ectodomain of TLR4 in the endoplasmic reticulum and then transits to the cell surface in an active TLR4/MD-2 complex or is also secreted into the medium as a soluble, active protein (sMD-2) (375). Thus, in macrophages, LPS recognition is mediated by a plasma membrane complex of at least three proteins: TLR4, MD2 and CD14 (354).

Recently, studies by Triantafilou et al. revealed that following LPS stimulation, TLR4 and CD14 are also in physical proximity to other cell surface proteins such as heat shock proteins 70 and 90, the chemokine receptor CXCR4, and growth differentiation factor 5 (GDF5) (376). After stimulation with LPS, biochemical techniques were used to isolate specialized membrane microdomains – the lipid rafts. CD14, hsp70 and 90, were constitutively found in the membrane microdomains, while TLR4, CXCR4 and GDF5 were not constitutively expressed there, but were recruited following LPS stimulation. Similar results were obtained using Fluorescent Resonance Energy Transfer imaging technology (described in the following section). These findings are supported by data from Schromm et. al., who found that LPS cellular activation occurs in the plasma membrane by LPS intercalating into the cytoplasmic membrane of mononuclear cells initiating signalling by steric stress (377). Further, Pfeiffer et al. have recently shown that LPS and ceramide can provoke ligand-specific receptor clustering in lipid rafts (378).

Based on these findings the current model of LPS signalling involves CD14 delivery of LPS bound to LBP to a TLR4-MD-2 complex. Following ligation of CD14 by LPS, different signalling molecules are recruited at the site of the ligation within lipid rafts, where LPS is then briefly released into the lipid bilayer where it finally interacts with a complex of receptors, which involves hsp70, hsp90, CXCR4, GDF5 and TLR4 (354). This interaction promotes signal transduction down an intracellular pathway (379).
The TLR-4 mediated LPS signalling cascade

The interaction of TLR4 with its ligand LPS leads to complex protein associations and downstream signal propagation that leads to the activation of the transcription factor NF-κB which then induces the activation of the inflammatory genes such as TNF alpha, IL-1, IL-6 and IL-8. To date there are two TLR signalling pathways identified: a MyD88-dependent pathway, essential for production of proinflammatory cytokines common to all TLRs and a MyD88-independent pathway partial to TLR3 and TLR4 signalling and responsible for Interferon β production (Figure 2).

**MYD88-dependent pathway**

As elucidated in recent studies by Medzhitov *et al.*, in the classical pathway, MyD88 first associates with the TIR domain of TLRs and in its activated state it recruits TIRAP. TIRAP contains a phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain that targets this adaptor to discrete regions of the plasma membrane (380). The function of TIRAP is to recruit MyD88 to the plasma membrane. Further in TLR4 mediated signalling, MyD88 activates serine-threonine kinases, IL-1 receptor-associated kinase 1 and 4 (IRAK1 and 4) to the TLR complex through interaction of the death domains of both molecules. To date, four members of the IRAK family have been identified: IRAK-1, IRAK-2, IRAK-M, and IRAK-4. IRAK-4 has been demonstrated to play a central role in TLR signalling as it acts upstream of, and phosphorylates IRAK-1 upon stimulation (381). Phosphorylated IRAK 1 associates with tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (379). The IRAK-1/TRAF6 complex dissociates from the receptor and travels to the cell membrane where it associates with TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2 (382). IRAK-1 is degraded whereas the complex of TRAF6, TAK1, TAB1, and TAB2 moves into the cytoplasm to interact with proteins that induce TRAF6-mediated activation of TAK1 (383). Activated TAK1 activates IKK complex, which phosphorylates IκB (an inhibitor of NF-κB) and targets it for ubiquitination and degradation. Finally NF-κB is released and moves to the nucleus to regulate transcription of target genes encoding proinflammatory cytokines (383).

**MyD88-independent pathway**

An observation that LPS stimulation in MyD88 knockout cells was capable of inducing NF-κB activation albeit with delayed kinetics prompted the discovery of the MyD88 independent signalling pathway in mammalian cells. A number of newly discovered molecules mediate the MyD88 independent TLR signalling. TRIF (TICAM-1), and TRAM (TICAM-2) were reported to be some of them. The MyD88-independent pathway begins with TRAM
anchoring to the plasma membrane by myristoylation (367). TRAM is phosphorylated and in this state is capable of inducing downstream TLR4 signalling.

**Regulation of TLR4 Expression**

The regulation of TLR4 gene transcription, trafficking and surface expression are areas of active research and these mechanisms are not yet fully elucidated. Since TLR4 stimulation results in production of potent proinflammatory cytokines and their overexpression can potentially have serious deleterious effects, organisms have evolved mechanisms for modulating their TLR-mediated responses. Evidence from recent studies suggests that the levels of TLR4 mRNA expression in macrophages are regulated at the transcriptional and posttranscriptional levels (384;385). At the transcriptional level, TLR4 is regulated by transcription factors PU.1 (384), as well as AP-1 (386). The TLR4 promoter region has been shown to contain three PU.1-binding sites (387). At the posttranscriptional level, Fan *et al.* demonstrated that TLR4 expression can be regulated by altering mRNA stability as TLR4 mRNA stability was decreased during LPS stimulation and increased under an oxidative stress environment in rodents (385).

Src kinases may be another group of molecules regulating TLR4 expression. Recently, hypoxia was demonstrated to produce a 3- to 4-fold increase in TLR4 mRNA expression in Kupffer cells of proestrus mice, although, the exact mechanism of this phenomenon not yet clear. Hypoxia was found to stimulate Src kinase activity. Treatment of males with Src inhibitor PP1 prevented the increase in IL-6 production following hypoxia. Therefore Src, one of the several members of Src family tyrosine kinases, may be involved in the regulation of TLR4 expression in Kupffer cells following hypoxia (388).

Since LPS transduces signals via TLR4 on the cell surface and overexpression of TLR4 results in increased cytokine secretion, negative regulators of surface expression have evolved. One of these mechanisms is known as endotoxin (or LPS) tolerance (389). With LPS stimulation of macrophages surface expression of the LPS receptor complex diminishes (390;391). Further, expression of IRAK-1 and its phosphorylation by IRAK-4 is also reduced further downmodulating the LPS response (392). Several other mechanisms and molecules have also been shown to negatively regulate TLR 4 expression and thus signalling. SOCS1 is thought to directly down-modulate TLR signalling pathways, although its precise mechanism is ill defined (393). In addition, TIR domain containing proteins, such as SIGIRR (single immunoglobulin IL-1 receptor-related molecule) and T1/ST2, have also been shown to negatively regulate TLR signalling (394).
Based on the findings to date, it is clear that a number of mechanisms are in place to modulate TLR4 expression. However, the complexity of these signalling pathways is vast and the exact effects that oxidant stress has on these interactions remain vastly unknown.

**Cellular distribution and trafficking of the LPS receptor complex**

Understanding regulation of subcellular localization, trafficking, and fate of the TLR4 receptor is important in understanding the LPS response. Nomura *et al.* revealed that stimulation of TLR4 with its ligand LPS results in downregulation of this receptor from macrophage surface, which desensitizes receptor signalling (390). This could be an important negative feedback to prevent an over response of the innate immune system. In contrast, studies by Jiang *et al.* demonstrated that both CD14 and TLR4 are up-regulation following LPS. They suggest that perhaps TLR4 surface expression is upregulated in the first few minutes of LPS stimulation and subsequently declines over a period of hours as it is temporally associated with LPS internalization and changes in TLR4 gene expression (370).

Currently, the model of LPS signalling involves the cell surface assembly of the signalling receptor complex. This complex, upon LPS exposure, has been demonstrated to assemble in cell surface signalling microdomains that are rich in cholesterol and sphingolipids called the lipid rafts. Espevik and colleagues have recently utilized fluorescent TLR fusion proteins to discern the localization of this LPS receptor complex and its fate upon LPS stimulation (395). They have observed that in an epithelial cell line, TLR4 resides in the plasma membrane as well as in a juxtanuclear area that corresponds to the Golgi apparatus (395). In human monocytes, localization of TLR4 to perinuclear compartments was suggested to be evidence of its presence in the Golgi, although no formal localization studies have been performed (396). In RAW 264.7 cells as well as in rat AMs, the intracellular localization of TLR4 appears more diffuse, consistent with its presence in preformed vesicles. Using a Fluorescence Recovery after Photobleaching (FRAP) method Espevik *et al.* describe that both TLR4, CD14 and MD-2 are highly mobile proteins that rapidly and continuously recycle between the Golgi complex and the plasma membrane (396). The CD14- and LPS-containing vesicles and entire lipid rafts with TLR4/MD-2 and CD14 and were observed to recycle between the plasma membrane and an intracellular compartment at a speed of 1–2 µm/s. Although the mechanisms regulating basal TLR4 surface expression are not well understood, alterations in the normal rapid cell surface cycling of this receptor are thought to be responsible.
Both TLR4 and MD-2 were shown to follow the trans-Golgi secretory pathway and reside on the cell surface as mature protein complexes, with only the heavily glycosylated mature forms of TLR4 and MD-2 being expressed on the cell surface. TLR4 was found to be expressed in the ER and the Golgi without mature glycosylation. Recent reports suggest that MD-2 is essential for correct glycosylation and intracellular distribution and surface expression of TLR4 (397). In addition, a recently identified chaperone protein: ER-resident heat shock protein gp96 was reported to regulate the subcellular distribution of TLR4, TLR2, TLR1, and some integrins (398). The TLR4 was shown localized to the Golgi apparatus of intestinal epithelial m-ICc12 cells and lack of free gp96 lead to intracellular accumulation of nonfunctional TLR4 and protein degradation (398). HSP gp96 was postulated to play a role in facilitating formation of gp96–TLR4-dimer–MD-2 complex that allows N-linked glycosylation of both TLR4 and MD-2, and thus transport from the ER toward the Golgi apparatus (374;399). Clearly further definition of the subcellular distribution and its regulation for TLR4 in monocytes and macrophages is necessary to provide insight into the regulatory mechanisms of LPS signalling. The effect of oxidant stress on TLR4 trafficking is largely unknown and defining it may be a critical to explain the augmented LPS signalling following oxidative stress.

**LPS independent TLR4 signalling**

Recent experimental findings suggest the magnitude of TLR4 surface expression plays a critical role in the intensity of the LPS induced signal and in addition, that Toll receptor clustering alone may induce signalling. Transgenic studies from Bihl *et al.* and Kalis *et al.* found that the expression level of TLR4 mRNA and TLR4 surface protein correlates with the height of the host response to LPS (400;401). Medzhitov *et al.* showed that even without LPS, a constitutively active mutant of human Toll that transfected into Jurkat cells can induce NF-κB activation and expression of IL-1 and IL-6 and IL-8 as well as the expression of the co-stimulatory molecule B7.1, which is required for the activation of naive T cells (361). There are other reports of ligand-independent TLR signalling pathways. Recent studies propose that TLRs may be activated by ligand-independent homo or heterodimerization with other molecules. For example, TLR2 activity increases when complexed with TLR1 or TLR6, while TLR4 has been demonstrated to form homodimers (402;403). In a study by Lee and colleagues a protein complementation assay was used to show that intracellular domains of TLR4 interact to form homodimers which can induce ligand-independent NF-κB activation in HEK293 cells (404). UV irradiation as well as osmotic stress have been shown to induce clustering of other surface
receptors including EGF-R, TNF-R and IL-1R, which in the absence of the corresponding ligand has been shown to be sufficient for the initiation of downstream signalling or facilitate subsequent ligand-induced signalling (260;405). Based on the literature reports, TLR4 may also be able to signal solely by receptor multimerization as a mechanism of mediating early NF-κB activation and a proinflammatory response in the absence of infection. Such a possibility is investigated in this thesis where the conditions of oxidative stress and immune cell priming have been shown to induce leukocyte activation even without an infectious stimulus.

**TLR4 independent LPS signalling**

Recent and accumulating evidence suggests that gram-negative bacteria are capable of activating the proinflammatory response through a TLR4 independent mechanism. Bacterial internalization and intracellular LPS have been demonstrated to initiate the inflammatory response that induces NF-κB and C-Jun-N-terminal kinase (JNK) activation and IL-8 production by epithelial cells (406). The mechanism by which intracellular LPS activates this response is still under investigation; however it appears to involve nucleotide-binding oligomerization domain (NOD) family of proteins. NOD1 was shown to be involved in intracellular activation of NF-κB by LPS from *Shigella flexneri* (407). Inohara et al. was able to demonstrate that overexpressed NOD1 is capable of mediating responsiveness to extracellular LPS when co-incubated with cells for 16 h (408). In addition, overexpression of CARD4/NOD1 as well as NOD2 (similar structurally to NOD1) enabled HEK293 cells to respond to preparations of peptidoglycan (409;410). Recognition of peptidoglycan motifs by NOD1 and NOD2 resulted in their oligomerization, which induces the recruitment of Rip2/RICK, a serine/threonine kinase (408). Rip2/RICK shares sequence similarity with Rip, a factor essential for NF-κB activation through the TNF receptor. These findings suggest the existence of an evolutionarily conserved system of intracellular pathogen recognition proteins with a similar signal transduction function as the Toll receptors.
Figure 1. Structure of Toll-like receptors (TLRs) and recognizing molecules.

TLRs are type I transmembrane molecules containing a large leucine-rich repeat (blue and white square) in the extracellular region and a Toll/IL-1 receptor domain (red square) in the intracellular region. Both heterodimers, TLR2 and TLR1 and TLR2 and TLR6, recognize lipopeptides. TLR4/MD-2 recognizes lipopolysaccharide (LPS). TLR5 recognizes flagellin. TLR3 recognizes double-strand RNA (dsRNA). TLR7 and TLR8 recognize single-strand RNA (ssRNA). TLR9 recognizes DNA derived from bacteria and virus.

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Figure 2. Toll-like receptor 4 (TLR4) signalling pathway.

There are two main downstream signalling pathways in TLR4. One is the myeloid differentiation factor 88 (MyD88) dependent pathway to induce inflammation cytokine (right side). The other is the MyD88-independent pathway to induce interferon-β (left side). MyD88 and TIR domain containing adaptor protein (TIRAP) are required for the MyD88-dependent pathway. TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor inducing interferon-γ (TRIF) are required for the MyD88 independent pathway. Mal, MyD88 adaptor-like; LPS, lipopolysaccharide; IRAK, interleukin receptor associated kinase; TRAF6, INF-receptor associated factor 6; TBK1, TANK binding kinase; IKK, IκB kinase; IFNβ, interferon; IRF3; interferon regulatory factor; INF-α, tumour necrosis factor; IL-6, interleukin-6.

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Section V  Plasma Membrane Composition - Implications for Host-Pathogen Interactions

- Plasma Membrane Signalling Domains – the Lipid Rafts
- Lipid Rafts in Endocytic and Secretory Pathways
- Lipid Rafts in Signal Transduction
  - LPS signalling involves the lipid rafts
  - Lipid rafts in ligand independent signalling regulation
- Technology Available to Study Lipid Rafts
- FRET Technology: Detection of Lipid Raft Associated Receptors
- Oxidative stress Effect on Membrane Lipids and Downstream Signalling
Plasma Membrane Signalling Domains – The Lipid Rafts

The traditional Singer & Nicholson’s fluid mosaic model of cell membrane (1972) had been recently extended from a homogenous fluid membrane to a dynamically compartmentalized membrane with lipid diversity within it (411). The new model of the membrane recognizes areas of lipid diversity, high sphingolipid, cholesterol and glycosylphosphatidyl inositol (GPI)-anchored protein content. Lipids were found to be present in three different phases: the gel phase, the liquid ordered phase (lo), and the liquid crystalline or fluid phase (ld). In the gel phase, lipids are immobile with their fatty acid chains fully extended. Above their melting point, lipids are in a fluid phase, packed loosely, and with disordered fatty acid structures. The liquid ordered phase is an intermediate between the gel phase and the ld phase. All these three phases can exist in membranes suggesting presence of phase-separation in membranes at physiological temperatures and suggesting that lipid lateral heterogeneity occurs spontaneously as a function of the lipid composition of the membrane (411).

The existence of protein-stabilized membrane domains such as caveolae and clathrin-coated pits is well documented; however, glycosphingolipid clusters in the plasma membrane have just recently been described and coined as “lipid rafts”. Lipid rafts are detergent-insoluble and are often termed: detergent-resistant membranes (DERMs), detergent-insoluble glycolipid-enriched complexes (DIGs) or glycosphingolipid-enriched membranes (GEMs). Isolation of these lipid clusters have been aided by their properties of Triton X-100 insolubility at 4 °C as well as a light buoyant density on discontinuous sucrose gradients (412). They can be isolated using ultracentrifugation of non-ionic detergent lysates (413). Although lipid rafts constantly interact with each other to perform membrane protein sorting or construction of signalling complexes (414), their size was proposed to be in the order of 50 nm (415).

Several reports were published with contradicting hypotheses regarding the physical composition of lipid rafts and their interaction with other membrane lipids and proteins. Recent findings suggest that synthetic membranes with similar composition to the lipid rafts (glycosphingolipids and cholesterol) possess identical detergent-resistant characteristics of the glycosphingolipid (416). Anderson & Jacobson et al. suggested that raft proteins are organized into "lipid shells" by surrounding themselves with up to 80 molecules of cholesterol and glycosphingolipid (417). The shells are postulated to make the protein resistant to detergent extraction. Subczynski & Kusumi et al. proposed that raft proteins form dynamic entities with a lifetime of less than 1 ms that can combine and cluster during signal transduction (418). Another
report suggested that lipid rafts are manifestations of the thermodynamic properties of the membrane’s lipid bilayer (419). Rafts have been proposed to be a subset of domains corresponding to one particular phase with defined physical and chemical properties and that they are larger in size (<700 nm) (414). Strongest evidence in support of the existence and composition of lipid rafts comes from immunogold electron microscopy of the T cell receptor (TCR) in fixed cells (420). Most recently a consensus on the definition of lipid rafts was reached at the 2006 Keystone Symposium (421): "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, Cholesterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions.” The difficulty in achieving a complete understanding of these elusive structures stands in the limitations of technology available to study them today. As Jacobson et al. points out “It is our contention that the lipid-raft field is at a technical impasse, largely because the tools to study biological membranes as liquids structured in space and time are rudimentary” (422). Nevertheless, substantial numbers of studies have validated the presence of lipid rafts in the cell membranes and their impact on host interactions with the outside world is an intriguing area of intense investigation.

**Lipid Rafts in Endocytic and Secretory Pathways**

Lipid rafts have been suggested to function in sorting of lipids and proteins in the secretory- and endocytic-pathways. Until recently, the mechanism believed to be responsible for internalizing plasma membrane with or without specific proteins was thought to occur via clathrin-coated pits. Today there are a number of clathrin-independent endocytic mechanisms that have been recognized (423), with lipid raft-mediated endocytosis being one of them (424). Three main findings support the lipid raft-mediated endocytosis theory. First, molecules that are known to be internalized independently of clathrin are found in lipid rafts. Secondly, disruption of lipid rafts by cholesterol depletion results in decreased endocytosis of these molecules. Third, caveolae containing caveolin 1 have been shown to be involved in endocytosis. Caveolae have been implicated to be a type of a lipid raft and as they are resistant to detergent extraction (424). Interestingly, it has also been reported that GPI-linked proteins can be endocytosed by a clathrin-independent mechanism into organelles devoid of caveolin 1 (424).

Lipid rafts have also been implicated in vesicular transport mechanisms. Transport of GPI-anchored proteins from endoplasmic reticulum (ER) to Golgi in yeast is selectively retarded when sphingolipid synthesis is inhibited (425). This hints that rafts may be formed in
the ER and that GPI-anchored proteins possibly partition into them for efficient transport (426). A GPI-anchored protein delivered to early endosomes after internalization was found to recycle to the cell surface more slowly than bulk membrane suggesting that association of GPI-anchored proteins with rafts in the endocytic pathway may slow their recycling (427).

The finding that clathrin-independent endocytic pathways exist is compatible with the notion of lipid raft-specific or raft-mediated endocytosis. However, further studies are necessary to define the molecular mechanisms for this process.

**Lipid Rafts in Signal Transduction**

Raft-associated proteins play an important role in signal transduction in T lymphocytes, macrophages, other leukocytes and endothelial cells (354;417;428;429). Lipid rafts help in receptor signalling that involve sub-unit interactions, cross-talk, clustering of signalling molecules, continuation of downstream signalling processes, and isolation from negative regulators (414;419). The function of lipid rafts in macrophage recognition of LPS and signalling under oxidative stress conditions relevant to this thesis are now described in more detail.

**LPS signalling involves the lipid rafts**

The lipid raft membrane domains have been implicated to function in recruitment of cell surface signalling molecules (415) and in membrane trafficking (412;414). CD14, a GPI-linked cell surface glycoprotein and TLR4 are the main receptors for LPS and were recently found to associate with lipid rafts (359;430). Several lines of evidence suggests that clustering of various other molecules in lipid rafts is important in initiating the LPS signalling cascade in macrophages (354;431). Firstly, in addition to TLR4 and CD14, other molecules such as: hsp 70, 90, Chemokine receptor 4 and growth differentiation factor 5, were found to localize within lipid rafts following LPS stimulation (413). Secondly, raft disrupting agents such as nystatin and methyl-β-cyclodextrin inhibited both enrichment of TLR4 in lipid rafts and LPS-stimulated downstream signalling via the MyD88/NF-κB pathway (413). In addition, lipid rafts were shown to mediate intracellular LPS signalling in intestinal epithelial m-ICcl2 cells. Hornef *et al.* demonstrated that LPS-mediated cellular activation requires ligand internalization in a lipid raft dependent pathway and intracellular transport to the Golgi compartment (431). Therefore, the proximity and interaction among the LPS signalling molecules in lipid rafts is important for LPS signalling (359;370).
Proximal LPS signalling events include the association of TLR4 with various molecules such as MD-2, the adaptor MyD88 and activation of IL-1R-associated kinase 1 (IRAK-1). Downstream events involve activation of MAPKs ERK1/2, p38, and JNK eventually leading to activation of the transcription factors NF-κB and AP-1. Olsson and Sundler have recently shown that the co-receptor CD-14 and the MAP kinases ERK-2 and p38 become translocated to lipid rafts after LPS stimulation (432), suggesting a role of the microdomains in LPS downstream signalling. In addition, a molecule TREM-1 found in PMNs was reported to be recruited into lipid rafts to bind to TLR4 and initiate downstream responses by activating Lyn, AKT, ERK1/2, Jak2 and PLCγ pathways that eventually lead to the phosphorylation of STAT5 and RelA (P65), a subunit of the NF-κB family. These findings suggest that lipid rafts are critical structures not only for clustering LPS receptor clusters but also for recruiting molecules involved in downstream LPS signalling.

Besides playing a role in the initiation of LPS signal transduction, lipid rafts may also play a role in recruiting diverse signalling molecules in order to tailor a variety of downstream responses depending on the stimulus provided. In this regard, recent studies have demonstrated that LPS molecules from different bacteria promote different receptor clusters in lipid rafts, thus leading to different downstream responses (433). In addition, the number of TLR4 molecules recruited to the lipid rafts for signalling was shown to determine the magnitude of the biological response (434).

Based on these observations the focus of this thesis is to determine the involvement of lipid rafts in TLR4 signalling under oxidative stress conditions.

**Lipid rafts in ligand independent signalling regulation**

Lipid rafts were demonstrated not only to induce downstream signalling, but also to modulate signalling by excluding certain molecules from signalling complexes (435). An example of this is the family of multichain immune recognition receptors (MIRRs) including the B cell antigen receptors (BCRs), T cell antigen receptors (TCRs), and high-affinity immunoglobulin E (IgE) receptors (FcεR1s) expressed by mast cells and basophils. These molecules possess ligand-binding chains that are integral membrane proteins with small intracellular domains. Signalling is achieved through the association of the ligand binding chains with subunits that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. Cross-linking or oligomerization of the receptors after ligand binding initiates Src kinase to phosphorylate ITAM tyrosines and thereby triggers the signalling cascade (436). The rafts have been shown to concentrate the Src kinases in order for signalling
to occur (437). Current evidence indicates that the MIRRs are excluded from lipid rafts in resting cells and thus sequestered away from the Src kinases essential for the initiation of signalling (438). The affinity of the monomeric MIRRs for rafts is presumably low, but when oligomerized, the oligomer’s affinity for rafts is increased, shifting the equilibrium toward raft association, which initiates contact with the Src kinases (436).

**Technology Available to Study Lipid Rafts**

Ideally, one would want to study the structure and function of the plasma membranes in live cells at a nanoscale level with adequate simultaneous spatial and temporal resolution to detect their dynamic nature. Such technology is currently limited. The traditional techniques involve disruption of lipid rafts with various agents and determining their function (439). Lipid rafts can be disrupted using polyene antibiotics: nystatin or filipin that have been used to disrupt caveolae formation (440). In addition Cyclodextrins can be used such as β-cyclodextrin (βCD) or methyl-β-cyclodextrin (MβCD) that remove cholesterol from cells (441;442). Cyclodextrins are cyclic oligomers of glucose that sequester lipophiles in their hydrophobic core (443).

Most studies of lipid rafts today rely heavily on fluorescence microscopy to achieve the sensitivity necessary to detect these dynamic and small structures in single and living cells. Two established technologies that provide information on interactions between lipids, proteins and their environment are: 1) Fluorescence recovery after photobleaching (FRAP) and 2) Fluorescence Resonance Energy Transfer (FRET). In FRAP, the mobility of a fluorescently tagged molecule is measured by bleaching molecules that move in a focal area of a light beam. Immediately after bleaching, a highly attenuated light beam measures the recovery of the fluorescence in the bleached area due to diffusion of fluorescent molecules from the surrounding unbleached areas. The diffusion coefficient of the fluorescent molecule can be derived from the recovery of fluorescence in the bleached area. This technique has been used to test mobility of receptors in cell membranes (376). FRET, through the use of a fluorescence donor and acceptor molecules, can resolve as low as 10 nanometers distance between molecules, thus determine colocalization more precisely then coimmunofluorescence alone (444). In addition to FRAP and FRET, other fluorescence microscopy techniques are available and have been reviewed in detail by Lagerholm *et al.* (445). Technologies that can be used in conjunction with Fluorescence microscopy include secondary ion mass spectrometry on the 100-nm scale, atomic force microscopy (AFM) and near field scanning microscopy (NSOM) for membrane topography and microcomposition (422). Other scattering techniques have been reported such as neutron and X-
ray scattering. The limitations of all techniques employed include the difficulty in studying live cells and systems, as they require quick-frozen or processed specimens. Hence, these techniques have limited utility and have therefore been used mostly in studying synthetic model-membranes (422).

**FRET technology: Detection of lipid raft associated receptors**

Fluorescence Resonance Energy Transfer (FRET) has been used in experiments described in this thesis. It is a technique for measuring interactions between two proteins in vivo. In FRET, two different fluorescent molecules (fluorophores) either genetically fused or tagged to antibodies specific to the two proteins are used. Fluorophores are chosen such that the emission peak of one fluorophore (donor) overlaps with the excitation peak of the other (acceptor) (446). The light energy is passed at the excitation frequency of the donor fluorophore, which transfers some of this energy to the acceptor fluorophore, which then re-emits the light at its own emission wavelength. The net result is that the donor fluorophore emits less energy than it normally would as it transfers some of the energy to the acceptor fluorophore, while the acceptor fluorophore emits more light energy at its excitation frequency, as it receives additional energy from the donor fluorophore. Controls are used to nullify the background noise or cross-talk (445;446).

The benefit of FRET technology is that it has finest resolution and it can be used in live cells. FRET only occurs when the two fluorophores are within 20-100 Å (0.002-0.01μm) of each other. FRET is a sensitive and reliable tool to detect enrichment of GPI-anchored proteins in membrane microdomains too small to be resolved by light microscopy as long as GPI-anchored proteins in the domain are within <100 Å of one another (446). Various markers for the glycosphingolipid (GSL) components of lipid rafts are used. Most commonly, fluorescently labelled antibodies or toxins specific for proteins or lipids found in the lipid rafts are employed. One of the most commonly utilized markers is cholera toxin B-subunit (CTXB), which specifically binds the monosialoganglioside GM1. CTXB is enriched in biochemical lipid raft fractions and in caveolae (447;448). Other markers used for the identification and labelling of lipid rafts include Fl-PEG cholesterol (449), filipin and dehydroergosterol (DHE) (445).

A major obstacle to implementation of FRET technique in living cells has been the lack of suitable methods for labelling specific intracellular and extracellular proteins with appropriate fluorophores. Jellyfish green fluorescent protein (GFP) has been cloned next to the target gene such that it shares the promoter site of the target gene. This made studying target gene
expression possible in a wide variety of cell types and the principle had been used for developing markers for both gene expression and structural protein localization. Different spectral mutants of GFP have been generated such as blue fluorescent protein (BFP) and used in FRET analysis (446).

In FRET technique, certain disadvantages do exist. Firstly, the attachment of the fluorophore can potentially change the three-dimensional structure of the protein. Probe multivalency is important as multivalent probes can potentially stabilize lipids into rafts through probe cross-linking. Therefore, monovalent Fab fragment for antibody labelling are preferable and used in studies described here (445). Moreover, methodology for conjugation differs from fluorophore to fluorophore. Youn et al. reported that quenching through intra- and intermolecular energy transfers can reduce the fluorophore mean lifetime (450). Secondly, most of the fluorophores have a narrow excitation spectrum and broad emission bands, resulting in spectral overlap when several fluorophores are used. Lastly, due to the short fluorescence lifetime rejection of the particular fluorophore autofluorescence signal is not possible, limiting sensitivity (445;451;452).

**Oxidative stress Effect on Membrane Lipids and Downstream Signalling**

ROS molecules can play a role in signal transduction through modification of cell membrane lipids. These mechanisms are of particular relevance to pathological states such as ischemia reperfusion and sepsis where leukocytes and cytokines appear to generate ROS at or near the plasma membrane. Phospholipid metabolites are potentially important targets for redox induced signalling. Cholesterol and sphingomyelin play an important structural role in membrane lipid rafts as well as a functional role in modulating different receptor clustering and thus downstream signalling (453). It has been recently demonstrated that activation of sphingomyelinases and/or ceramide synthases by LPS, cytokines, growth factors and oxidative stress results in the liberation of ceramide from Sphingomyelin (453;454). Specifically, it was shown that H₂O₂ induces ceramide generation and reduction in sphingomyelin content in human monoblastic leukemia cells and bovine endothelial cells (455). This effect is blocked by D609, an inhibitor of PC-PLC, and by inhibition (or lack of acid sphingomyelinase [ASM]) such as in ASM deficient animals. In clinical disease models, increased levels of cholesterol and ceramide have been found in Alzheimer’s disease (AD) patients. Drugs that suppress ceramide production have been found to protect neurons against death induced by oxidative stress. Interestingly H₂O₂ was also postulated to modulate ceramide levels and induce apoptosis in lung epithelium by
depleting glutathione levels in lung epithelial cells during diseases such as asthma or ARDS (456).

Plasma membrane cholesterol content can also be affected by oxidative stress and lead to initiation of various signalling pathways. Recent evidence suggests that both PI3 kinase and Akt are enriched in lipid rafts and caveolae after H₂O₂ exposure (457). Src kinases were also demonstrated to localize in rafts with H₂O₂ stimulation. According to a report by Yang et al., H₂O₂ can inactivate phosphatases resulting in loss of opposition to actions of Src kinases which potentially can result in Src activation of PI3 and Akt (457). Their data shows that raft disruption causes a loss of Akt from the lipid rafts and attenuates activation of Akt by H₂O₂. Similarly they demonstrate that disruption of rafts by cholesterol depletion also disrupts the signalling machinery necessary to propagate epidermal growth factor (EGF) receptor responses to ERK1/2 after an oxidant challenge (457).

In summary, oxidative stress acts by activating several signalling pathways and plasma membrane lipids play an integral role as signalling mediators for external stimuli including oxidants.
Figure 3  Domain-length scales and the biomembrane as a protein–lipid composite material.

(a) Length scales of domains in biomembranes. Shells, complexes, and nanoclusters range from 1–10 nm, whereas nanodomains such as caveolae can be as large as 100 nm. (b) A schematic representation of the biomembrane as a composite of lipids and proteins. Estimates of lateral protein concentration are about 30,000 per μm² based on rhodopsin in the rod outer segment and transmembrane proteins in the baby hamster kidney (BHK) cell membrane. Lipids were assumed to occupy a surface area of ~0.68 nm² (diameter ~0.93 nm) and an α-helix ~1 nm² (diameter ~1.1 nm). A 30 × 30 nm² section of membrane is depicted with 32 lipids on a side, 35 transmembrane proteins with 15 single-span, 12 tetraspan and eight heptaspan α-helical proteins, having assumed cross-sectional areas in the plane of the membrane of 1 nm², 4.5 nm² and 8 nm², respectively. Taking into account the area excluded by the proteins, the numerical lipid: protein ratio is ~50. For a single-span helix with a diameter of ~1.1 nm, there are about seven lipids in the first boundary layer; for a tetraspan protein with a diameter of ~2.4 nm, there are about 11 lipids in the first boundary layer; for a heptaspan protein (such as rhodopsin) with a diameter of ~3.2 nm, there would be about 14 lipids in the first boundary layer. Such first-boundary layer lipids are shown in white, whereas the second layer is shown in red. All other lipids are shown in yellow. Lipid-binding proteins and adaptors linking transmembrane proteins to membrane proximate cytoskeletal filaments are also depicted as different coloured structures beneath the plane of the membrane, but ectodomains of the membrane proteins are omitted for clarity.

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Section VI  Oxidative stress effect on receptor trafficking

- Exocytosis: An Overview
- Exocytosis of Cell Surface Receptors including TLR4
- Role of Lipid Rafts in Exocytosis
  - Role of SNARE Proteins Localization to Lipid Rafts
  - Cholesterol Depletion Inhibits Exocytosis
- The Role of Lipid Rafts in Regulation of Exocytosis
  - The ‘Spatial Coordination’ Model
  - The ‘Selective Exclusion’ Model
  - The “lipid Geometry” Model
- Oxidative stress Effect on Expression of Cell Surface Receptors
- Src Kinases in Lipid Rafts
- Src Kinase Activation
- Src Kinase Inhibitors
- Oxidant Effect on Src Kinases Regulation of Receptor Trafficking
- The Role of Src Kinases in TLR4 Signalling
Oxidative stress has been shown to alter receptor trafficking, recycling and cell surface molecule expression (458;459). Change in the level of surface receptor expression results from an interplay of exocytosis of the newly synthesized receptors, recycling of receptors and their rate of endocytosis all contributing to cellular sensitivity to various stimuli. Studies by Nomura et al. (390) reveal that stimulation of the TLR4 with its ligand LPS results in downregulation of this receptor from macrophage surface, which desensitizes receptor signalling. On the other hand studies by Jiang et al. demonstrated that both CD14 and TLR4 up-regulation are very early events in LPS stimulation (370). They suggest that TLR4 surface expression is upregulated in the first few minutes of LPS stimulation and subsequently declines over a period of hours. To date, TLR4 recycling and regulation of surface expression especially under oxidative stress conditions has not been well characterized and may represent an important mechanism of regulating the innate immune response to LPS. This thesis focuses on TLR4 receptor surface expression and its alterations under oxidative stress conditions. Specifically TLR4 surface overexpression is addressed and exocytosis as a possible mechanism is explored.

**Exocytosis: An Overview**

Exocytosis is defined as the release of cellular substances contained in vesicles by fusion of the vesicular membrane with the plasma membrane and subsequent release of the contents of the vesicle to the cellular exterior. This process may be of constitutive or regulated nature (460). Constitutive exocytosis is a process by which newly synthesized proteins and lipids are transported from the ER to the cell surface by vesicular traffic. At the cell surface, proteins and lipids become constituents of the plasma membrane; some are secreted into the extracellular space. Specialized cells, such as endocrine cell types have, in addition to the constitutive secretory pathway, one or more regulated secretory pathways. These cells store vesicles, such as dense core granules and synaptic vesicles that are released only in response to a stimulus. In the regulated pathway, the secretory vesicles form only transient fusion pores, and remain structurally distinct from the plasma membrane. These vesicles may be internalized by endocytosis and recycled. Only a regulated amount of cargo is externalized at each fusion event (460). Neurotransmission in neurons, release of hormones by endocrine cells, release of histamine by mast cells and exocytosis of glucose transporter containing vesicles in fat cells are examples of different types of regulated secretory events.

Regulated exocytosis requires co-ordination of numerous cellular and molecular processes, e.g. cytoskeletal rearrangements. A dense network of actin filaments lies both
between individual vesicles and between the vesicle population and the plasma membrane. The cell must be able to rapidly disassemble the microfilament network to permit the vesicles to fuse with one another and with the plasma membrane during exocytosis (461). It has been suggested that the vesicles formed in the cell body travel to cellular extremities via molecular motors (e.g. kinesin, myosin 1), and are prevented from diffusing from these sites by entanglement in a web of microfilaments. Several studies have shown that secretory vesicles are surrounded by a dense web of actin filaments, which also separates the vesicles from the plasma membrane. It has been suggested that an actin filament severing protein scinderin is responsible for removing filaments underlying the plasma membrane (462). While this well may be the case, it is likely that other severing proteins such as the ADF/Cofilins, are responsible for the rapid depolymerization of the same filaments and ones deeper within the cell. In a variety of cell types, exocytosis is facilitated by the disruption of microfilaments (463);(464) and is inhibited by stabilization of microfilaments by various agents (465). Oxidative Stress plays an important role in regulating the cytoskeletal arrangements and therefore its role in receptor exocytosis is explored in this thesis.

**Exocytosis of Cell Surface Receptors Including TLR4**

The number of surface receptors is an important factor in determining the sensitivity of a cell toward hormones and other ligands. Although surface expression can be regulated by altering receptor synthesis, this is usually a slow process. In contrast, receptor redistribution and recycling is a rapid mechanism that can dramatically change receptor number at the cell surface within minutes.

Exocytosis is a mode of cellular secretion of cytokines, chemokines, enzymes, and hormones; antigen presentation; cell signalling; etc. Exocytosis is a multi step process involving vesicle trafficking, -docking, and -fusion. Vesicle trafficking facilitates transportation of certain cell surface proteins and secretory molecules coming from the Golgi apparatus to the cell surface. Both the actin- and the microtubule-based cytoskeletons are implicated in these processes.

Although studies of receptor exocytosis are limited, there are several examples of upregulation of membrane proteins secondary to exocytosis. For example, regulated exocytosis is a common pathway that many cells use to rapidly deliver ligand-gated channels to the plasma membrane (466). NMDA receptors are known to be upregulated by insulin stimulation in a process dependent on SNARE-mediated exocytosis (467). It has also been demonstrated that
SNARE-mediated exocytosis is involved in the augmentation of α7 nACh receptors by genistein. Functional nACh receptor cycle between intracellular and surface pools by exocytic processes (468). SNAREs (soluble N-ethylmaleimide–sensitive factor attachment protein receptors) are a superfamily of small, membrane-anchored proteins, which catalyze vesicle fusion. Other proteins that translocate to the plasmalemma via exocytosis are: renal water channel, aquaporin-2 (469), the epithelial chloride channel (470), and the cystic fibrosis transmembrane conductance regulator (471). Their surface expression is upregulated by exocytosis as a response to the elevation of cAMP.

The role of MHC-class-I-like Fcγ receptor FcRn is to bind to IgG and recycle it back to the plasma membrane, through a constitutive recycling pathway (472). Recent studies suggest that this receptor is exocytosed using both the constitutive and regulated pathway. In human endothelial cells, FcRn constitutive exocytosis occurs most of the time. However, under certain conditions FcRn–IgG complex can be delivered to specific areas of the plasma membrane. Studies show that FcRn carries IgG all the way from the sorting endosome to the plasma membrane. The role of exocytosis in mediating TLR4 surface upregulation is not well characterized in the literature. However similar to the FcRn receptor, in an epithelial cell line TLR4, CD14, and MD-2 were shown to continuously and rapidly recycle between the Golgi complex and the plasma membrane (395). Others have shown that in fibroblasts TLR4 is localized to the cell surface and cytoplasm. TLR4 preferentially migrates to the lamellopodia or filopodia but in the absence of MD2, TLR4 resides in the Golgi (373). The mechanisms whereby this receptor can migrate to the plasma membrane and the role of exocytosis is not well characterized and may well mimic the mechanism of FcRn trafficking.

**Role of Lipid Rafts in Exocytosis**

As described earlier, cell-surface glycosylphosphatidylinositol-anchoring proteins and TLR/MD-2 reside in phosphoglycolipid/cholesterol-enriched membrane microdomains – the lipid rafts. Two distinct lines of experimental evidence suggest that lipid rafts may be involved in the regulation of exocytosis. The first line of evidence is based on the observation that SNARE proteins, molecules involved in exocytosis, are abundant in lipid rafts. The second is based on the observation that disruption of lipid rafts by the depletion of cholesterol from the plasma membrane inhibits a certain type of exocytosis (regulated exocytosis). In this section, evidence supporting the role of lipid rafts in the regulation of exocytosis is discussed. Putative mechanisms by which this regulation is achieved are described.
**SNARE Proteins Localize to Lipid Rafts**

Vesicle docking during exocytosis involves tight association of the vesicle with its target and possibly includes the molecular re-arrangements needed for triggering bilayer fusion. Vesicle fusion is catalyzed by SNAREs (soluble N-ethylmaleimide–sensitive factor attachment protein receptors), which are represented by a superfamily of small, membrane-anchored proteins with 25 members in Saccharomyces cerevisiae and 36 members in humans (473). Some better characterized SNARE proteins are: syntaxin1, synaptosomal-associated protein (SNAP)-25, and vesicle- associated membrane protein (VAMP) (474). SNAREs are located in opposing membranes and assemble into tight complexes to force the membranes into close apposition, initiating the merging of bilayers. Assembly of SNAREs is mediated by a stretch of 60–70 amino acids, termed SNARE motif, which is characteristic for all SNAREs and usually located adjacent to the C-terminal transmembrane domains. In order for exocytosis to occur, Q-SNARE proteins present on the plasma membrane interact with R-SNARE proteins present in the vesicular membrane and form a SNARE complex.

Numerous SNARE proteins have been found to co-localize with lipid rafts in several cell lines such as Madin-Darby canine kidney (MDCK) cells, the rat adrenal pheochromocytoma cell line PC12, human cervical carcinoma cell line HeLa, and the rat basophilic leukemia cell line RBL-2H3 (475-477).

Evidence showing a direct biological role for the association of SNARE proteins with lipid rafts was obtained by developing SNARE point mutants with altered affinities for lipid rafts. A very recent report suggested that cells harbouring mutations, that increased SNARE affinity for lipid rafts, had impaired exocytosis. On the other hand, cells with mutations that conferred decreased affinity for lipid rafts had enhanced exocytosis (478). Surprisingly, this study suggested that SNAREs are negatively associated with exocytosis; nevertheless, it provides direct evidence that lipid rafts are involved in the regulation of exocytosis.

The first report describing the co-localization of SNARE proteins with sphingolipid-cholesterol rafts was by Lafont et al. using polarized MDCK cells (475). Using detergent treatment and gradient density floatation, they observed that the SNARES were present in the fraction containing detergent resistant membranes. Several other reports corroborate these general observations. Using immunofluorescence, Lang et al. found that some SNARE proteins form separate clusters that colocalize with secretory vesicles that are cholesterol dependent (476). Chamberlain et al. found that these same SNARE proteins are present in lipid rafts (479). Later, Pombo et al. reported that although SNARE proteins are expressed in lipid rafts, SNARE...
regulatory proteins such as Munc-18, are not (477). This observation suggests that lipid rafts may regulate exocytosis through sequestering of SNAREs from their regulators. Oxidant stress, by changing the fluidity of lipid rafts, may serve to include or exclude some of these regulators, thus increasing exocytosis of certain receptors.

**Cholesterol Depletion Inhibits Exocytosis**

A second line of evidence suggesting that lipid rafts are involved in the regulation of exocytosis is that in a number of experimental models, disruption of lipid rafts inhibits exocytosis. These reports examined the effect of raft disruption by cholesterol depletion on exocytosis in many different contexts including regulated exocytosis and antigen stimulated degranulation. Chamberlain et al. observed that treatment of neuronal cells with lovastatin and MβCD and disrupting lipid rafts reduced the amount of ATP-stimulated dopamine release. This is normally a neuronal exocytic process (479). Similarly, raft disruption inhibited the release of β-glucuronidase, an indicator of degranulation (480) and inhibited antigen-induced degranulation in basophils (481). Overall, these data corroborate the notion that lipid rafts are involved in the regulation of exocytosis.

**The Role of Lipid Rafts in Regulation of Exocytosis**

The data reviewed above only establishes that lipid rafts are involved in the process of exocytosis. Their precise role in the regulation of this process is unknown. Several models have tried to answer this:

**The ‘Spatial Coordination’ Model**

A putative role for lipid rafts is the spatial coordination of molecular regulators of exocytosis. The selective inclusion or exclusion of key molecules within lipid rafts may be one means of producing an environment conducive to the process of exocytosis. This is consistent with the observation that lipid rafts and non-lipid raft domains of the plasma membrane express different proteins involved in exocytosis (475;476;479). In this model, lipid rafts facilitate exocytosis by permitting the inclusion of SNARE proteins and excluding SNARE regulators. The inclusion of SNARE proteins in lipid rafts could serve as a means of increasing the local concentration of these molecules to a critical threshold required for membrane fusion to proceed. Moreover, SNARE mediated membrane fusion is a dose-dependent process (482).

**The ‘Selective Exclusion’ Model**

Alternatively, lipid rafts may mediate exocytosis by the selective exclusion of SNARE regulators. Given the importance of specificity in membrane fusion, it is unsurprising that
several proteins exist that regulate SNARE complexes. Complexins are cytosolic proteins that compete with alpha-soluble NSF attachment protein (αSNAP) for the binding of SNARE complexes (483) and are thought to influence the SNARE complex assembly-disassembly cycle. SNAPs help N-ethyl-maleimide-sensitive factor (NSF) proteins to bind to SNARE complexes and result in disassembly of the complex. Both complexin and αSNAP are absent in the lipid raft fractions (479). According to this model, the exclusion of these regulators specifically from the lipid raft makes the SNAREs interact well and help in exocytosis.

The ‘Lipid Geometry’ Model

Another model proposes that the lipid composition of rafts may enable them to regulate exocytosis. Indeed, previous studies have shown that the membrane lipid composition and the geometry of the constituent lipids are important in the regulation of exocytosis. In order for membrane fusion to occur, the cis-monolayer of the plasma membrane (the inner monolayer) and the cis-monolayer of the vesicle membrane (the outer monolayer) must merge to produce a stalk formation. This process then proceeds with the indentation of the trans-monolayer of the plasma membrane (the outer monolayer) and the trans-monolayer of the vesicle membrane (the inner monolayer) to produce a hemifusion formation, which then merges to produce a pore. The lipid constituents of biomembranes possess an intrinsic curvature. The curvature of each leaflet of a membrane bilayer contributes to the overall curvature of the membrane. Elastic curvature stress occurs when the curvature of the lipids constituents oppose the curvature of the membrane leaflet. Cone-shaped lipids have an intrinsic negative curvature, while inverted cone shaped lipids have an intrinsic positive curvature. When cone-shaped lipids are added to cis-membrane leaflets the efficiency of membrane fusion increases. Furthermore, when inverted cone-shaped lipids are added to the trans-membrane leaflets, an increase in the efficiency of membrane fusion occurs. The presence of these inverted cone-shaped lipids inside lipid rafts may lower the energy requirement of membrane fusion.

Oxidative stress Effect on Expression of Cell Surface Receptors

Oxidative stress has been shown to alter receptor trafficking, recycling, and cell surface molecule expression. Regulation of these processes involves the plasma membrane function as well as the cytoskeleton. Oxidative stress has been suggested to alter membrane function by lipid peroxidation and by modifying membrane fluidity and increasing intracellular calcium concentration affecting the cytoskeleton (484). In addition, several surface morphological changes and activity of surface receptors have been shown to be affected by cytoskeletal
oxidative damage (459). For example, EGF receptor exposure to H₂O₂ results in an activated receptor uncoupled from normal internalization, leading to prolonged receptor signalling. H₂O₂ was shown to induce tyrosine phosphorylation of the EGF receptor preventing its down-regulation from the cell surface (485). A similar process may be responsible for TLR4 over-expression with H₂O₂ stimulation; however, the exact oxidant effects on TLR4 endocytosis remains unknown and requires further investigation.

Further, oxidative stress may act by altering the stability of newly synthesized protein in the endoplasmic reticulum. It may also induce translocation of new proteins from the endoplasmic reticulum to the Golgi compartment inducing vesicular transport to the plasmalemma, such as is the case with caveolin-1 or glucose transporter GLUT4 (486). In some instances, oxidative stress influences receptor trafficking negatively. Studies suggested that menadione treated cells internalized receptors of transferrin (Tf) and insulin, but then failed to recycle them back to the surface (459). Further, oxidative alterations have been suggested to affect TfR intracellular trafficking from the Golgi apparatus to the plasma membrane of the cell (487).

It is well established that regulated exocytosis is triggered by an increase in intracellular Ca²⁺, removal of external Ca²⁺ and preloading cells with bis(2-aminophenoxy)ethane-NNN’N’-tetraacetate (BAPTA) (488). Ichimura et al. demonstrated that oscillations in calcium levels induced production of reactive oxygen species, which in turn promoted exocytosis of P-selectin (489). Previous studies have also demonstrated that treatment with H₂O₂ can increase cytosolic Ca²⁺ (257;490). In pancreatic acinar cells, reactive oxygen species generated by the hypoxanthine/xanthine oxidase system were able to induce increased exocytosis of amylase (257). It is possible that oxidative stress can induce macrophage priming and increased LPS responsiveness through increasing exocytosis of TLR4 mediated by an increase in Ca²⁺ release from intracellular stores. By this reasoning, one may be able to prevent a priming response in cells where exocytosis is blocked and induce it in cells where Ca²⁺ release is enhanced by a calcium ionophore. Some of the experiments pursued in this thesis are based on this line of reasoning.

**Src Kinases in Lipid Rafts**

Recent studies have demonstrated that Src kinases are present in lipid rafts and are involved in exocytosis of granules and receptors. Further, both LPS and ROS have been shown...
to induce Src kinase activation. The role of Src kinases in TLR4 expression under oxidative stress is therefore explored.

Src family kinases are a group of protein tyrosine kinase enzymes, first discovered as Rous Sarcoma virus proto-oncogenes. They are a group of intracellular enzymes involved in cellular signalling and include Blk, Fyn, Fgr, Hck, Lck, Lyn, Yes, and Yrk. These enzymes are responsible for transferring phosphate groups onto the tyrosine amino-acid residues in proteins. In haematopoietic cells, Src kinases are involved in the transduction of signals from growth factors, integrins, and antigens. Recently, Src kinases were found to reside in lipid rafts, as they were shown to precipitate together with GPI-anchored proteins found in detergent-insoluble compartments (491). Many T cell signalling Src kinases, including Lck, and Fyn, were also demonstrated to localize in the lipid rafts (492;493).

**Src Kinase Activation**

Src kinases contain an N-terminal SH-2 domain, a SH-3 domain, and a kinase domain. In a basal state, Src kinases are in an inactive state, owing to the fact that their C-terminal kinase domain is folded into a closed conformation. This C-terminal tyrosine is bound to the molecule’s SH-2 domain. Certain tyrosine phosphatases such as CD45 can de-phosphorylate Src kinases and cause unfolding of the kinase domain to activate them. Once active, the Src kinases can phosphorylate other proteins and bind to them through their now exposed SH2 domain. Negative regulation of Src kinases occurs by re-phosphorylation of the C-terminal tyrosine residue, rendering the molecule inactive. C-terminal Src kinase (Csk) is the predominant negative regulatory kinase for Src kinases. It floats freely in the cytosol (494).

While Csk is free in the cytosol, it was recently demonstrated to localize in the lipid rafts on the membrane, where it is bound to its negative regulator: Csk-binding protein (Cbp). Cbp binds to Csk when it is phosphorylated at a specific tyrosine residue (495). In their review of Src kinase regulation, Cary and Cooper describe a molecular negative feedback loop mechanism in lipid rafts for Src kinase activation: “A cell- surface receptor binds to its particular extracellular partner and activates a specific Src kinase. The active Src kinase phosphorylates many proteins of varying cellular functions, and possibly Cbp as well. Cbp can now recruit Csk to the membrane, where it can phosphorylate and inactivate Src kinases, turning off the signalling events at the appropriate time.” (494)
**Src Kinase Inhibitors**

Src kinase inhibitors can be an invaluable tool to study the role of Src kinases in many pathological processes, including ischemia/reperfusion injury. Pharmacological Src kinase inhibitors include 4-Amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo(3,4-d)pyrimidine (PP1) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo(3,4-d) pyrimidine (PP2). Both PP1 and PP2 target the ATP-binding cleft of the Src kinase molecule. PP1 and PP2 selectively inhibit Lck, Fyn and Hck (496;497). Paul et al., reported up to 70% reduction in brain infarcts with administration of PP1 to mice that sustained permanent brain ischemia (498). The two compounds, PP1 and PP2, have only minor differences in the profile of action on different Src kinase members (499). The selectivity of PP1 and PP2 is likely to depend on a specific residue corresponding to Thr338 of Src kinases.

As mentioned, another Src kinase inhibitor is the C-terminal Src kinase (Csk). It was discovered in neonatal rat brain, which was capable of inhibiting c-Src by specifically phosphorylating its tyrosine-527 *in vitro* (500;501). Substituting tyrosine-527 with phenylalanine in the mice expressing the activated mutants of Lck, Lyn, Hck and Blk, prevents the phosphorylation dependent control of CSK. Mutants are thereby converted to the constitutively active conformation. Partanen et al., reported that an enzyme called *cyl* is the human ortholog of rat Csk (502). Csk maintains the activity of Src kinases at basal levels in normal cells and loss of Csk function results in a constitutive Src kinase activation.

There are also Src kinase knockout mice and mutant cells that have been developed to study the role of Src kinases in various disease processes; however, they are not utilized in studies described here.

**Oxidative stress Effect on Src Kinases Regulation of Receptor Trafficking**

Src kinases were recently demonstrated to be involved in macrophage priming by oxidants. Oxidative stress, *in vivo* and *in vitro*, was demonstrated to reprogram LPS induced NF-κB signalling from a Src-independent pathway to a Src-dependent one (503). Src kinases have also been reported to be regulated by various forms of oxidative stress which had implications in receptor expression and various downstream signalling actions (235;504). Signalling through Src kinases has also been demonstrated to be important for receptor trafficking: endo- and exocytosis. For example, Khan et al. recently showed that H$_2$O$_2$ activates Src kinases, which regulate endocytosis of epidermal growth factor. In these experiments, Src mediated phosphorylation of caveolin-1 Tyr-14 was shown necessary for caveolar endocytosis.
of EGFR under oxidative stress conditions. Aberrantly phosphorylated caveolin impeded the clathrin-mediated endocytosis and subsequent lysosomal degradation of this receptor resulting in prolonged downstream activation of molecules such as Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) (505).

Src kinase expression is high in cell types that specialize in regulated exocytosis (e.g. neurons, platelets, and osteoclasts). The fact that Src associates with proteins from exocytic vesicles, such as dynamin and synapsin (506), suggests that these enzymes are involved in regulating the exocytic process. Although little is known about Src kinase regulation of receptor exocytosis, the possible role of Src kinases in degranulation had been suggested. PP1 as well as SU6656, another Src-inhibitor, both prevented degranulation of neutrophils in a dose dependent manner (507). These studies suggested that Src kinases, in particular Lyn, are important mediators of chemokine induced neutrophil exocytosis.

These observations make Src kinases possible regulators of TLR4 cell surface expression and studies here focused on Src kinase involvement in TLR4 exocytosis under oxidative stress conditions.

The Role of Src Kinases in TLR4 Signalling

A number of studies implicate Src kinases in LPS-mediated signalling. Protein tyrosine kinases have been shown to be required for the induction of pro-inflammatory cytokines IL-1, IL-6 and TNF-α in response to LPS in murine macrophages (508), in addition to B cell proliferation (509). Src kinases Hck, Lyn, and Fgr, have been implicated in LPS responsiveness of macrophages (491;510;511). Consistent with this notion, protein tyrosine kinase inhibitors of the tyrphostin AG 126 family were found to protect mice against LPS-induced production of TNF-α and nitric oxide by macrophages (512). Although these observations suggest that Src kinases play a role in the macrophage response to LPS, the mechanisms of this process and its effect on LPS receptors is still unknown and will be investigated in this thesis.

The role of Src kinases in TLR4 signalling has been implicated in a study by Henricson et al. LPS was shown to induce autophosphorylation of Lyn kinase in wild type, but not in TLR4-mutant (C3H/HeJ) macrophages (513). Overexpression of a constitutively active Hck enhanced the production of TNFα in response to LPS in macrophages, whereas antisense oligonucleotides to Hck interfered with LPS-induced TNF-α production (514). Furthermore, recent evidence suggest that LPS-induced CD40 activation and the secretion of pro-inflammatory cytokines IL-6 and TNF-α can be down-regulated in Csk knockdown cells (515).
TLR4-mediated activation of IκB-α, Erk and p38 but not of JNK, were also down-regulated in these Csk knockdown cells implicating Csk as a critical regulator of TLR4-mediated signalling (515). In contrast, LPS-induced cytokine production and tumouricidal activity occurred normally in macrophages isolated from triple knockout mice (Lyn−/−, Fgr−/−, and Hck−/−) (516). Based on the observations to date, the exact role of Src family tyrosine kinases in TLR4 signalling pathways is yet to be fully determined, however, Src activation seems to be one of the positive regulators of LPS signalling.

Although hypoxia is not equivalent to oxidative stress it does often, precede it. Recently, hypoxia was demonstrated to produce a 3- to 4-fold increase in TLR4 expression in Kupffer cells of both males and proestrus female mice. When Src levels were examined in the stimulated Kupffer cells, hypoxia diminished the levels of Src kinases in cells from female mice, whereas it stimulated the levels of Src in cells from males. Furthermore, the increase in Src expression was accompanied by an increase in its phosphorylation, suggesting that hypoxia increases Src kinase activity. Treatment of males with Src inhibitor PP1 prevented the increase in IL-6 production following hypoxia. The authors concluded that Src tyrosine kinase are differently regulated following hypoxia in males and females and that the increase in Src protein and phosphorylation likely plays the predominant role in increased IL-6 production in male Kupffer cells.

Based on a number of studies to date, Src kinases are shown be involved in a multitude of pathways of possible importance to ischemic injury. By using Src kinase inhibitors the role of Src kinases in TLR4 receptor surface translocation can be better ascertained.
CHAPTER 2

OBJECTIVE, HYPOTHESIS, OVERVIEW OF THESIS CHAPTERS AND THEIR RELEVANCE TO THE HYPOTHESIS
OBJECTIVE

Oxidative Stress generated during resuscitated hemorrhagic shock is known to alter macrophage responsiveness as a mechanism leading to organ injury. Oxidative Stress contributes to alveolar macrophage priming, which is defined as augmented responsiveness of cells to the same stimulus such as lipopolysaccharide (LPS) resulting in increased generation of proinflammatory cytokines. The objective of this work is to define the cellular and molecular mechanisms responsible for the priming of macrophages and to investigate potential interventions.

HYPOTHESIS

The overriding hypothesis of this work is that the increased responsiveness of macrophages to LPS, following shock resuscitation, occurs through modulating cellular distribution of the LPS receptor – Toll Like Receptor 4 (TLR4). Furthermore, four ensuing hypotheses emerged: 1) that the process of the LPS signalling complex assembly during oxidative stress involves plasma membrane signalling domains – the lipid rafts, 2) that the lipid rafts, under oxidative stress conditions, serve as signalling platforms capable of facilitating early LPS signalling through the TLR4/MyD88/NF-κB pathway, 3) that the assembly of the LPS receptor complex in lipid rafts, under normal and oxidative stress conditions, occurs via a Src kinases dependent process, 4) that macrophage priming by oxidative stress and resultant TLR4 redistribution can be diminished by early exposure to resuscitation strategies that have antioxidant effects. Collectively, we postulate a novel mechanism whereby oxidative stress generated during resuscitated hemorrhagic shock primes the innate immune system for an exaggerated response to inflammatory stimuli and hence for increased tissue and organ injury.
OVERVIEW OF THESIS CHAPTERS 3-6
AND THEIR RELEVANCE TO THE HYPOTHESIS

The next several pages outline the main findings of my research activity. The work is divided into four separate chapters, each addressing different themes of this unified thesis.

**Chapter 3: Reactive Oxygen Species Induce Lipid Raft Recruitment of TLR4 Resulting in Macrophage Priming and Early NF-κB Signalling that Occurs in Resuscitated Hemorrhagic Shock.**

Published as:

**Powers KA, Szaszi K, Khadaroo RG, Tawadros PS, Marshall JC, Kapus A, Rotstein OD.**


In this chapter, the hypothesis that oxidative stress alters cellular distribution of the TLR4 receptor as a mechanism underlying increased macrophage responsiveness to LPS is explored. Macrophages exposed to oxidative stress *in vivo* or *in vitro* were shown to express increased surface levels of Toll-like receptor 4 (TLR4) via exocytosis, an effect inhibited by antioxidants. LPS receptor complex was demonstrated to redistribute to signalling domains lipid rafts in the plasma membrane. Lipid rafts were demonstrated to be essential for the oxidative stress mediated macrophage priming and NF-κB activation downstream.
Chapter 4: Reactive Oxygen Species Induce Src Kinase Dependent Exocytosis of TLR4.

Presented as:

**Powers KA**, Szaszi K, Khadaroo RG, Kapus A, Rotstein OD.


Src kinases are known to be primary targets for oxidant stress. The premise that is addressed in this chapter focuses on the mechanism of oxidant stress-induced exocytosis of TLR4 and the hypothesis that it is a Src kinase dependent process. It is demonstrated that activated Src kinases can induce recruitment of TLR4 onto the cell surface. Src kinase activity was inhibited in two ways 1) pharmacologically with PP2 and 2) via a molecular approach with macrophage cell lines stably transfected and overexpressing Csk a negative regulator of Src kinases. Src kinase inhibition completely abolished the oxidant stress induced TLR4 exocytosis. These studies suggest a novel mechanism whereby oxidant stress might prime the responsiveness of cells to LPS via increasing surface receptor availability in a Src dependent process.
Chapter 5: 25% Albumin, as an Antioxidant, Prevents Lung Injury Following Shock Resuscitation.

Published as:


The findings in this chapter support the hypothesis that macrophage priming by oxidative stress can be diminished by early exposure to resuscitation regimens with antioxidant capacity that may influence cell signalling in response to LPS. A “two-hit” rodent model of shock resuscitation was used to demonstrate that antioxidants, namely 25% albumin (A25) and N-acetylcysteine inhibit LPS-induced macrophage priming, LPS induced augmented NF-κB translocation, and LPS induced upregulation of the chemokine CINC. A25 but not 5% albumin (A5) attenuated lung injury in the rodent shock/resuscitation model by decreasing LPS-induced pulmonary microvascular permeability and PMN sequestration. The protective property of A25 resuscitation was shown to be partly mediated by its effect on the adhesive properties of neutrophils and pulmonary endothelial cells. The data further suggest that the salutary effects of albumin are related to its antioxidant capacity, in part through its multiple thiol groups. Together, these findings suggest the critical role of reactive oxygen species in macrophage priming during shock resuscitation and suggest that targeting oxidants during the resuscitation phase may offer novel protective effects against delayed organ injury in trauma victims.

Published as:

**Powers KA, Zurawska J, Szaszi K, Khadaroo RG, Kapus A, Rotstein OD.**

**Powers KA, Woo J, Khadaroo RG, Papia G, Kapus A, Rotstein OD.**

**Powers KA, He R, Khadaroo RG, Papia G, Kapus A, Rotstein OD.**
Hyperosmolarity Modulates TLR4 Expression and Distribution.

Having demonstrated the critical role of oxidants in macrophage priming and organ injury following shock resuscitation, the role of Hypertonic saline (HTS) resuscitation in diminishing systemic oxidative stress was explored. In a “two-hit” rodent model of shock resuscitation HTS was shown to prevent gut ischemia/reperfusion injury and consequently decreases oxidative stress and distant priming in alveolar macrophages. Specifically HTS was demonstrated to alter the balance between the proinflammatory and the counter-inflammatory response and to modulate macrophage TLR4 gene and protein expression. These HTS effects lead to a less LPS responsive milieu, inhibited immune cell activation and decreased macrophage priming. These findings provide unique insight into regulation of macrophage priming and activation under oxidative stress conditions revealing a novel potential mechanism whereby decreasing LPS receptor availability may be associated with decreased global cell responsiveness.
CHAPTER 3

OXIDATIVE STRESS GENERATED BY HEMORRHAGIC SHOCK RECRUITS TOLL-LIKE RECEPTOR 4 TO THE PLASMA MEMBRANE IN MACROPHAGES

Published as:

SUMMARY

Model

The study design involved the use of a previously developed rodent model of acute lung injury in which resuscitated shock primes for increased lung injury in response to a small dose of intratracheal lipopolysaccharide. Animals were bled to a mean arterial pressure (MAP) of 40mmHg, and maintained in a shock phase for 1h. Animals were then resuscitated by transfusion of the shed blood plus an equal volume of RL with or without N-acetylcysteine (NAC, 0.5g/kg) or Trolox (10 mg/kg) over a period of two hours. Animals were sacrificed by a pentobarbital overdose and alveolar macrophages were recovered by bronchoalveolar lavage at different time points.

Alternatively, the murine macrophage cell line, RAW 264.7, was used and stimulated with 100 μM (or the indicated concentration) of H₂O₂; 0.1 μg/ml LPS (E. coli O111:B4); 0.1 mM xanthine and 3U/ml xanthine oxide; 50 μM menadione. Where indicated, cells were treated with 10 μM of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxyethyl) ester (BAPTA/AM) or 1 μM Jasplakinolide prior to oxidant exposure.

Main results

The present studies show that alveolar macrophages recovered from rodents subjected to hemorrhagic shock/resuscitation (S/R) express increased surface levels of TLR4, an effect inhibited by adding the antioxidant N-acetylcysteine to the resuscitation fluid. Consistent with a role for oxidative stress in this effect, in vitro H₂O₂ treatment of RAW 264.7 macrophages similarly caused an increase in surface TLR4. This increase was prevented by depleting intracellular calcium or disrupting the cytoskeleton, suggesting the involvement of exocytosis in TLR4 surface upregulation. Further, Fluorescent Resonance Energy Transfer between TLR4 and the lipid raft marker GM1, as well as biochemical analysis of the lipid raft components, demonstrated that oxidative stress redistributes TLR4 to lipid rafts in the plasma membrane. Inhibition of the oxidant-induced movement of TLR4 to lipid rafts using methyl-β-cyclodextrin prevented the increased responsiveness of cells to LPS following H₂O₂ treatment.
INTRODUCTION

Acute Respiratory Distress Syndrome following resuscitated hemorrhagic shock in trauma patients is an important contributor to late morbidity and mortality (130;517). It has been suggested that shock/resuscitation (S/R) promotes organ injury by priming cells of the innate immune system for excessive responsiveness to a subsequent pro-inflammatory stimulus (32;33). Several groups, including our own, have modeled this “two-hit” phenomenon both in vivo and in vitro in order to gain insight into the pathophysiological mechanisms underlying the priming events. Our previous work has defined a central role for activated alveolar macrophages (AM) in augmented lung injury following S/R. AM recovered from resuscitated animals exhibited exaggerated LPS responsiveness with excessive generation of proinflammatory molecules including Cytokine-Induced Neutrophil Chemotaxtractant (CINC), the rodent orthologue of IL-8, and TNF-α (35;36). Increased expression of these proteins was due to enhanced gene transcription, a result of earlier and heightened nuclear translocation of the transcription factor NF-κB in response to LPS. Further, oxidative stress during ischemia/reperfusion appeared to be responsible for this priming phenomenon, as inclusion of the antioxidant N-acetylcysteine in the resuscitation fluid prevented the increased responsiveness to LPS. To date, the mechanisms responsible for the ability of oxidative stress to prime for increased LPS responsiveness are not fully elucidated.

One potential mechanism of S/R-induced priming for enhanced inflammatory response is through potentiation of various components of LPS-induced signalling. Several intracellular pathways whereby LPS stimulation leads to dissociation of NF-κB from the cytosolic IκB/NF-κB complex and translocation of NF-κB into the nucleus have been described. Recent studies have identified the Toll-like receptor 4 (TLR4) as the main upstream sensor for LPS both in vitro and in vivo. Optimal activation of the TLR4 signalling pathway by LPS involves the formation of an LPS signalling complex consisting of surface molecules such as CD14 and MD2, as well as intracellular adaptor molecules, including MyD88 and IRAK(362). Recently a number of factors have been identified which contribute to the responsiveness of TLR4. These include receptor dimerization, mobilization to lipid rafts and formation of a TLR4 activation cluster including other proteins such as heat shock proteins 70 and 90, chemokine receptor 4, and growth differentiation factor 5 (433;518). The total number of functional TLR4 molecules expressed on the cell surface may also influence LPS response. Cells lacking TLR4 or expressing a mutant receptor exhibit marked hyporesponsiveness to LPS (359), and
overexpression of surface TLR4 was shown to augment LPS responsiveness (400;401). Together, these observations imply that physiological/pathophysiological alterations in the expression of surface TLR4 in vivo may be one mode of influencing LPS responsiveness. In this regard, Nomura and colleagues reported that induction of LPS tolerance in peritoneal macrophages correlated with the ability of LPS to downregulate surface TLR4 (390). We have previously reported that S/R prevented the LPS-induced reduction in TLR4 protein and mRNA levels in whole lung tissue (385). However, TLR4 levels did not exceed baseline in shock plus LPS treated animals, making it unlikely that total cellular TLR4 per se influenced LPS reactivity. These observations raised the hypothesis that S/R might alter cellular distribution of the TLR4 receptor complex, as a mechanism underlying increased responsiveness to LPS.

In the present studies, we demonstrate that S/R causes translocation of TLR4 in AM from the cytoplasm to the plasma membrane thus elevating TLR4 surface expression. The central role of oxidative stress in this process was shown by the ability of NAC to prevent this translocation in vivo and by the ability of exogenously added H₂O₂ to recapitulate the translocation in RAW 264.7 cells in vitro. We also show that TLR4 is mobilized into lipid rafts and that this is essential for oxidant-induced translocation. Finally, the ability of oxidative stress to induce the formation of a surface TLR4 receptor complex within lipid rafts contributed to the augmented responsiveness to LPS. Together, these findings suggest a novel mechanism whereby oxidative stress generated during S/R, a form of global ischemia/reperfusion, might prime the innate immune system for an exaggerated response to inflammatory stimuli and hence for increased tissue and organ injury.

MATERIALS AND METHODS

Animal Model

Animal protocol was approved by the Animal Care Committee of St. Michael’s Hospital (protocol nr: ACC756). Shock and resuscitation was carried out as described in (385). Briefly, male Sprague-Dawley rats were anaesthetized and the right carotid artery was cannulated for monitoring of mean arterial pressure (MAP), blood sampling, and resuscitation. MAP was reduced to 40 mmHg by blood withdrawal and maintained between 35-45 mmHg by further blood withdrawal or infusion of Ringer’s Lactate (RL, Baxter, Co.) if necessary. Shed blood was collected into 0.1 ml sodium citrate/ml blood. After 60 min, animals were resuscitated with their shed blood and an equivalent amount of RL with or without N-acetylcysteine (NAC, 0.5g/kg, Mucomyst from Shire, Inc.) or Trolox (10 mg/kg) over a period of two hours. Animals
were sacrificed by a pentobarbital (MTC Pharmaceuticals) overdose and alveolar macrophages were recovered by bronchoalveolar lavage with cold phosphate buffered saline (PBS, Gibco™), followed by centrifugation at 300g (10 min).

**Cell Culture and Activation**

The murine macrophage cell line, RAW 264.7 (ATCC), was cultured at 37 °C in a humidified atmosphere of 5% CO₂ in endotoxin-free Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen Co.), with 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco™) and 10% fetal calf serum (FCS, Hyclone Lab, Inc.). Experiments were carried out in Hanks’ balanced salt solution (HBSS, Invitrogen Co.), supplemented with 2% FCS. The following treatments were applied: 100 µM (or the indicated concentration) H₂O₂; 0.1 µg/ml LPS (E. coli O111:B4, Sigma-Aldrich); 0.1 mM xanthine and 3U/ml xanthine oxide; 50 µM menadione. In the functional studies cells were exposed to H₂O₂ (1 h) followed by LPS (15-60 min). Where indicated, cells were treated with 10 µM of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM, Calbiochem) (10 min) or 1 µM Jasplakinolide (Jas, Molecular Probes) (30 min) at 37°C before adding oxidants. Reactions were stopped by placing cells on ice.

**Immunofluorescence Microscopy**

AM or RAW264.7 cells on coverslips were fixed with 4% paraformaldehyde (Canemco-MARivac) for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 20 min, blocked with 5% bovine serum albumin for 1 h and incubated with the indicated antibody. The following primary antibodies were used: anti-TLR4 antibody (H-80) (1:200) (for rat AM), anti-MyD88 (F-19) (1:200) and anti-p65 (1:50) from Santa Cruz Biotechnology, Inc.; and FITC-conjugated anti-TLR4/MD2 (MTS510) (for RAW 264.7 cells) from Stressgen Biotechnologies. The two anti-TLR4 antibodies differ insofar as the H-80 antibody recognizes TLR4, while the MTS510 recognizes the MD-2/TLR4 complex. FITC or Cy™3 – conjugated Fab fragment secondary antibodies were from Jackson ImmunoResearch Laboratories and Alexa 488-labelled anti-goat antibody from Molecular Probes. GM-1 ganglioside, a component of lipid rafts, was detected by incubating live cells with rhodamine (TRITC)-conjugated Cholera Toxin B subunit (CTxB) (1:500, List Biological Laboratories) before fixation. The coverslips were mounted using DAKO medium (Dako Cytomation) and visualized with a Nikon TE200 fluorescence microscope (x100 objective) coupled to an Orca 100 camera (Hamamatsu Photonics, Japan) driven by the Simple PCI software (Compix Inc, Cranberry Township, PA). Where indicated, a Zeiss LSM 510 confocal microscope with a 100× objective was used to acquire serial optical
slices (0.5 μm thick). Images of representative focal planes are shown. Image analysis was done using the Scionimage software. Peripheralization was defined as a ≥80% increase in the ratio of fluorescence intensities at the cell periphery and the cytosol (at 0.5 μm distance from the membrane). The percentage of cells with peripheralized TLR4 under various conditions was calculated by scoring 100 cells. NF-κB translocation was analyzed as described in (41): the percentage of cells with nuclear p65 staining was determined by counting an average of 100 cells for each group. During quantitation, the observer was blinded to the experimental group.

**Flow Cytometry**

Cell surface expression of TLR4 and CD11b was detected by flow cytometry of live cells stained with anti-TLR4 (H-80), anti-TLR4/MD2 (MTS510) or anti-CD11b/CD18 (Mac-1) (Cedarlane Laboratories, Ontario) and the corresponding FITC-labelled secondary antibody, where required. 10,000 cells/ condition were analyzed in a FACScan (Becton-Dickinson, Palo Alto, CA) using FL1 525 mM Band Pass detector at 488 nm excitation wavelength. Results were expressed as mean channel fluorescence (MCF).

**Fluorescence Resonance Energy Transfer (FRET)**

For FRET experiments, live RAW264.7 cells were stained at 4°C with anti-TLR4 antibody (H-80) followed by a FITC-conjugated anti-rabbit Fab fragment and Rhodamine-CTxB. The fluorescence was determined at 37°C using a DeltaRAM illumination system from Photon Technologies, Inc operated by the FELIX software. The excitation wavelength was 480 ± 5 nm and emission was detected at 510 ± 10nm (green emission) and at >590 nm (red emission) for at least 6 cells/coverslip. For each condition, at least 20 cells from 3-4 coverslips were measured.

**Manipulation and measurement of cellular cholesterol**

Cholesterol was depleted and repleted in RAW264.7 cells using a modified procedure of Furuchi et al (519) and Roy et al (520). Briefly, cells were treated with 10 mM methyl-β-cyclodextrin (MβCD) for 30 min at 37°C in HBSS. Cholesterol repletion was then achieved by incubation with 80 μg/ml cholesterol and 0.2% MβCD for 30 min at 37°C. The total cholesterol content was determined using the Amplex red cholesterol assay kit from Molecular Probes.

**Isolation and detection of lipid rafts**

Lipid rafts were isolated as in (521). RAW264.7 cells (2 x 10^7 cells/mL) were lysed in 0.25ml TKM buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂, and 1 mM EDTA) containing 0.5% wt/vol Brij58 (Sigma Aldrich Chemical Co.) and protease inhibitors (Roche Diagnostics) on ice for 30 minutes. Lysates was mixed with an equal volume cold 80% wt/vol
sucrose in TKM, and overlaid with 4.3 mL cold 36% sucrose followed by 0.2 mL 5% sucrose. The gradients were subjected to ultracentrifugation at 200,000xg at 4°C for 18 h. Fractions were collected from the top of the gradient. Equal volume of each fraction was diluted in 10 µL of TBS-T buffer (20 mM Tris, pH 7.4; 150 mM NaCl; 0.05% Tween 20) containing 0.1% Triton X-100 and loaded onto Protran Nitrocellulose Sheet (0.45 µm pore, S&S BioScience) using Dot Blot (BioRad). The membrane was blocked in 5% milk in TBS-T, followed by incubation with CTxB-horseradish peroxidase (HRP) (1:500, List Biological Laboratories), anti-TLR4 (H-80) or anti-MyD88 (F-19) for 1 hr. Where required, HRP conjugated secondary antibody (1:3000 dilution, 1 h) was used. Labelling was visualized using enhanced chemiluminescence (Perkin Elmer).

Western blotting

Proteins were detected using Western blotting as described in (522). Protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Equal loading was verified using Ponceau S staining of the membranes. The phospho-p38 antibody was from Cell Signalling.

Statistical Analysis

Data are presented as mean ± standard error of n determinations as indicated in the Figure Legends. Data were analyzed by one-way analysis of variance; post hoc testing was performed using the Bonferroni modification of the t-test with significance at p<0.05 level. Blots and images shown are representatives of at least 3 separate determinations.

RESULTS

Resuscitated shock induces cell surface TLR4 expression in alveolar macrophages

To determine the effect of resuscitated hemorrhagic shock on TLR4 distribution, we isolated alveolar macrophages from sham and S/R rats. At the end of resuscitation, total cell numbers obtained by bronchoalveolar lavage (BAL) and percent of macrophages (>90%) did not differ from sham animals (36). Phase microscopy shows that AM from sham and S/R animals exhibited similar morphology (Fig 4A). Immunofluorescence staining of permeabilized cells revealed that in sham cells, TLR4 was distributed throughout the cytoplasm. By contrast, in cells from S/R animals, there was peripheralization of TLR4 with diminished cytoplasmic staining. This phenomenon was observed in 58.2 ± 4.1% of cells from S/R animals, compared to less than 20% in sham groups (Fig 4B). The redistribution of TLR4 to the periphery in S/R cells was confirmed by confocal microscopy (Fig 4C, compare top row to middle row). To confirm
specificity of the TLR4 antibodies used in our studies, we performed immunostaining on macrophages from C57BL10/ScCr animals, known to be null for TLR4 expression. No fluorescence was observed in these cells (Fig 39C). Further, on Western blot the H-80 antibody reacted with a protein at the appropriate molecular mass of TLR4 (data not shown). Figure 4E demonstrates that the observed immunofluorescence-staining pattern was indeed due to binding of the TLR4 antibody.

To determine TLR4 surface expression, TLR4 staining of non-permeabilized cells was quantified using flow cytometry. In a representative study (Fig. 5A), there was a marked increase in cell surface TLR4 in cells from S/R animals compared to sham. Over several studies, TLR4 surface level in S/R cells was significantly elevated (Fig. 5B). Together, these findings demonstrate that S/R induces redistribution of TLR4 from the cytoplasmic compartment to the periphery, including onto the plasma membrane. Previous work from several groups suggests that oxidative stress generated during reperfusion of the gastrointestinal tract contributes to distant cellular priming and activation. N-acetyl-cysteine (NAC) supplementation in the resuscitation fluid prevented both the S/R-induced increase of isoprostanes in the blood (a marker of oxidative stress) and priming of AM (36;523). To test the role of oxidants in the S/R-induced TLR4 redistribution, we added NAC to the resuscitation fluid. NAC blocked both the redistribution of TLR4 following S/R (bottom panel of Figs. 4A and 4C, right bar in Fig. 4B) and the increase in TLR4 cell surface expression (Figs. 5A and 5B). To further substantiate the role of oxidative stress, we tested the effect of Trolox, a water-soluble vitamin E analog that is a well-established antioxidant agent. Similar to NAC, Trolox added to the resuscitation fluid prevented the redistribution of TLR4 in AM (4D). In addition, Trolox impaired lung neutrophil sequestration to the same extent as NAC (data not shown). We next asked whether exposure of cells to oxidants could mimic the effect of S/R on TLR4 distribution. Figure 6A demonstrates that one hour treatment of RAW 264.7 cells with 100 μM H₂O₂ induces peripheralization of TLR4, in a pattern reminiscent to that observed in BAL cells following S/R. Treatment with 50 μM H₂O₂ appeared to accentuate a vesicular pattern of TLR4 distribution. By flow cytometry, H₂O₂ was shown to induce a dose-dependent increase in surface expression of TLR4, with modest effect at 50 μM and marked increase at 100-500 μM (Fig. 6B left panels: representative study). Concentrations of H₂O₂ up to 200 μM did not induce cell death as determined by trypan blue exclusion, while 500 μM caused ~50% cell death (not shown). Quantitation of several studies confirmed the dose-dependency (Fig. 6B, right panel). A time course shows that H₂O₂ (100 μM) induces a progressive increase in cell surface TLR4 starting as early as 1-5 min and
peaking at 30-120 min (Fig. 6C). We also tested the effect of alternative agents known to induce oxidative stress. Menadione accumulates in the cells and induces intracellular oxidative stress, while the xanthine/xanthine oxidase system is able to produce extracellular oxidants. Both agents caused a marked increase in TLR4 expression in the cell surface as shown by flow cytometry (Fig. 6D). In aggregate, these findings are consistent with a role for oxidative stress in the increased surface expression of TLR4 following S/R. In subsequent studies, a dose of 100µM H₂O₂ for 1h will be studied. This protocol corresponds to conditions used in our previous studies showing that H₂O₂ primes RAW 264.7 cells for increased responsiveness to LPS (41;524).

**Effect of Resuscitated Hemorrhagic Shock on localization of TLR4 to lipid rafts**

Lipid rafts are considered to be important contributors to membrane recruitment and clustering of signalling molecules (525). Recent studies have reported that clustering of the LPS receptor complex upon LPS stimulation required lipid rafts (354;370;378;413). Therefore, we next evaluated the role of lipid rafts in the oxidative stress-induced TLR4 redistribution.

AM from S/R animals were stained live using rhodamine-CTxB, and then fixed, permeabilized and stained for TLR4. As shown in Figure 7A, AM from sham animals displayed diffuse intracellular TLR4 staining, while GM1 ganglioside, a classical raft marker was mainly localized to the cell surface with minimal colocalization with TLR4. By contrast, TLR4 in cells from S/R animals displayed a peripheral staining (55±2.3%, n=3, Fig. 7A, bottom row), and it colocalized with GM1 as shown by the overlay image. Supporting the critical role of oxidants in the effect, NAC added in the resuscitation fluid prevented TLR4 redistribution and its colocalization with GM1 (7.5 ± 0.5%, n=3, right panel Fig. 7A).

The effect of oxidative stress on localization of TLR4 in lipid rafts was also examined in vitro by exposing RAW 264.7 cells to H₂O₂. Fig. 7B illustrates that H₂O₂ induces colocalization of TLR4 with GM1 in the plasma membrane. We used methyl-β-cyclodextrin (MβCD) to deplete cellular cholesterol and disrupt lipid rafts (526) before adding H₂O₂ to RAW 264.7 cells. This treatment prevented movement of TLR4 to the cell surface (Fig. 7B, right panels). The inhibitory effect of cholesterol depletion was also confirmed by flow cytometry. Live cells treated with MβCD either before or after H₂O₂ were stained with anti-TLR4/MD2. As shown in Fig. 7C, when applied prior to H₂O₂ treatment, MβCD prevented the oxidant-induced TLR4 surface up-regulation. However, when oxidant exposure was performed before raft disruption with MβCD, increased TLR4 levels persisted on the cell surface (Fig. 7C). MβCD may exert its
effect through altering cellular responsiveness to H₂O₂. To examine this possibility, we tested the effect of H₂O₂ on p38 in MβCD treated cells. As shown in Fig. 7D, cholesterol depletion did not prevent H₂O₂-induced phosphorylation of p38. Although we cannot rule out that cholesterol depletion might interfere with some pathways, these data show that MβCD does not exert a global inhibition of oxidant-induced signalling.

To substantiate lipid raft localization of TLR4, we isolated raft fractions using discontinuous sucrose gradient ultracentrifugation of RAW264.7 cells (521). Effective isolation of membrane rafts was confirmed by the presence of GM1 ganglioside in the non-soluble portion - fractions 1 to 3 of the sucrose gradient (Fig. 8, upper panel). H₂O₂ stimulation induced localization of TLR4 to raft fractions (Fig. 8, lower panel). This effect was prevented by pretreatment with MβCD.

Fluorescent Resonance Energy Transfer (FRET) analysis was applied to examine the spatial proximity of TLR4 to GM1 ganglioside in lipid rafts. TLR4 was labelled using anti-TLR4 primary and a FITC-coupled Fab fragment secondary antibody, and GM1-ganglioside was stained with a rhodamine-coupled CTxB. In the first series of experiments, we validated the system and determined how much of the fluorescence was due to other non-FRET related factors in order to correct for them. We considered two potential problems. First, the excitation light used in these experiments (480±5 nm) might also excite rhodamine and thus give a falsely high quantitation of FRET. To test this possibility, we stained cells with rhodamine-cholera toxin only and measured emission at >590 nm after exciting with 480±5 nm light (settings used for FRET). Figure 9A shows that H₂O₂ treatment did not alter the background fluorescence of unstained cells. Emission in the rhodamine stained cells increased by 23±4% compared to unstained cells. However, this increase was identical in the untreated and H₂O₂-treated cells. This verifies that H₂O₂ treatment does not interfere with the fluorescent measurements. To account for the fluorescence resulting from rhodamine excitation by the 480±5 nm wavelength, the emission measured in double stained cells during the FRET experiments was corrected by 23%. Second, we investigated whether part of the fluorescence detected at ≥590 nm could originate from residual FITC emission at the rhodamine emission wavelength. Indeed, control experiments revealed a linear correlation between green fluorescence intensity and the resulting bleed through emission detected at ≥590 nm (not shown). Because H₂O₂ treatment increases TLR4 surface expression, the bleed through can be different in untreated and treated cells. Therefore, we carefully compensated for any bleed through of FITC into the emission wavelength used for FRET. The fluorescence of similarly treated single (FITC only) and double
(FITC and rhodamine) stained cells were determined for each experiment under identical conditions. To obtain the value of real FRET, we first corrected the fluorescence of the double stained cells by 23% (to compensate for the emission of rhodamine excited by the 480±5 nm light, see above). The difference between the corrected fluorescence of the double stained cells and the fluorescence measured in single stained cells (i.e. the bleed through of FITC emission into ≥590nm) is therefore attributed to FRET between FITC labelled anti-TLR4 and rhodamine labelled GM1. Figure 9B illustrates that H2O2 treatment caused ~5-fold increase in FRET emission. This result is consistent with the conclusion that treatment with H2O2 induced molecular proximity of TLR4 with the raft marker GM1 ganglioside. Together, these results provide evidence that oxidative stress induces translocation of TLR4 to plasmalemmal lipid rafts. Further, the integrity of lipid rafts appears to be essential for this process.

**Role of exocytosis in TLR4 translocation to the plasma membrane following oxidative stress**

Exocytosis represents a general mechanism for delivery of molecules from cytoplasmic compartments to the plasma membrane. In order to study the role of exocytosis in TLR4 upregulation after oxidative stress, we applied two strategies to inhibit exocytosis. First, RAW 264.7 cells were exposed to jasplakinolide, a marine sponge toxin which induces excessive actin polymerization (527) and has been shown to physically interfere with exocytosis (528). Second, cytosolic Ca2+, a requisite for exocytosis, was chelated by loading the cells with BAPTA/AM in a Ca2+-free medium (529). To verify that these manipulations indeed abrogated exocytosis in cells, we tested upregulation of CD11b, which is known to be delivered to the cell surface through exocytosis. Figure 10A and B shows that both jasplakinolide and BAPTA prevented the H2O2- and LPS-induced CD11b upregulation in RAW 264.7 cells. Next, we examined the effect of these strategies on oxidative stress-induced redistribution of TLR4. As shown in Figures 10C and 10D, both jasplakinolide and calcium-depletion prevented H2O2- stimulated increase in TLR4 surface level. Together, these findings suggest a role for exocytosis in the oxidant-induced increase in cell surface TLR4.

**Effect of Oxidative stress on the assembly of TLR4/MyD88 signalling molecules**

Formation of a multi-molecular signalling complex containing cell surface TLR4 and adaptor molecules such as MyD88 is an important early step in LPS-induced signalling leading to downstream events such as nuclear translocation of NF-κB (381). Having shown that oxidative stress induces movement of TLR4 to the plasma membrane, we examined whether oxidative stress might cause colocalization of MyD88 with TLR4 in a signalling complex,
possibly in lipid rafts. Figure 11A shows that similar to TLR4, S/R also causes peripheralization of MyD88. Colocalization of Myd88 and TLR4 is evidenced by the fluorescence overlay pictures. Further, inhibition of Myd88 peripheralization by NAC confirmed a role for oxidative stress in the effect (Fig. 11A, right panels). Addition of H2O2 to RAW 264.7 cells also caused translocation of MyD88 to the membrane (Fig. 11B). The specificity of the anti-MyD88 antibody was confirmed using a blocking peptide (Fig. 11C). Next, recruitment of MyD88 into lipid rafts was examined. As shown in Figure 11D, MyD88 was detected in the raft fractions following H2O2 treatment, an effect that was prevented by cholesterol depletion prior to oxidative stress.

**The functional significance of lipid raft integrity in oxidative stress-induced macrophage priming**

The LPS-induced aggregation of a signalling complex within lipid rafts appears to be a prerequisite for LPS-mediated NF-κB translocation in macrophages (413). We have shown, that oxidative stress primed macrophages for earlier and increased responsiveness to subsequent LPS challenge (503). We therefore hypothesized that induction of assembly of the LPS receptor complex by oxidative stress might represent a mechanism for priming of macrophages to subsequent challenge with LPS. We designed experiments to separate the role of TLR4 accumulation in lipid rafts in LPS signalling from the ability of oxidative stress to mobilize TLR4 to lipid rafts as a priming mechanism. In control studies, LPS caused a time dependent increase in NF-κB translocation with minimal translocation observed by 15 minutes, and more than 80% of cells showing translocation by 30 minutes (Fig. 12B). MβCD treatment caused an almost 40% drop in the cellular cholesterol content (Fig. 12A) and prevented LPS-induced NF-κB translocation. Repletion of membrane cholesterol restored LPS responsiveness (Fig. 12A and B). These studies established that cholesterol repletion following antecedent MβCD treatment permitted normal LPS signalling.

Consistent with our prior studies, Figure 12C shows that oxidative stress hastens NF-κB translocation in response to LPS, occurring by 15 minutes (41;503). To test whether lipid raft integrity was required for this H2O2-induced priming, we treated cholesterol-depleted cells with H2O2, where TLR4 movement to lipid rafts was prevented. Next, we restored cholesterol levels to permit normal LPS signalling (as shown in Fig. 12B). As illustrated in Figure 12C, MβCD treatment prior to H2O2 exposure followed by cholesterol repletion prevented the augmented p65 translocation by LPS observed in H2O2 treated cells at 15 min. Further, this was comparable
to the effect of LPS alone added for 15 min. This was not due to toxicity or a non-specific effect of MβCD, since by 30 min, p65 translocation approximated that seen for the combined H₂O₂/LPS treatment. Importantly, cholesterol measurements showed that H₂O₂ treatment did not interfere with cholesterol repletion (Fig. 12A). Moreover, cholesterol depletion does not interfere with H₂O₂-induced signalling (Fig. 7D). Thus, inhibition of TLR4 cell surface translocation by MβCD appeared to prevent the primed macrophage phenotype. These experiments strongly suggest that lipid rafts are not only integral for LPS mediated signalling but also critical for oxidant-induced macrophage priming for LPS responsiveness.

**DISCUSSION**

The clinical scenario of resuscitated hemorrhagic shock is known to render patients susceptible to the development of a systemic inflammatory response and organ dysfunction. While a number of mechanisms undoubtedly contribute to this, the ability of antecedent shock/resuscitation to prime inflammatory cells for increased responsiveness to a second stimulus such as gram negative bacterial LPS has provided a mechanistic framework for the investigation of post-resuscitation organ injury. The present studies focus on the regulation of surface TLR4 expression following ischemia/reperfusion, a pathological process known to sensitize innate immune responses for enhanced LPS signalling both *in vitro* and in the *in vivo* setting (33;36;43). These experiments demonstrate that S/R, through the generation of oxidative stress, causes recruitment of TLR4 to the cell surface and that through the formation of these TLR4-containing receptor complexes, the cell becomes poised for increased responsiveness to LPS stimulation. These studies are the first to show that oxidative stress generated during ischemia/reperfusion alters subcellular TLR4 distribution *in vivo*, and thus they provide insights into the cellular mechanism whereby oxidative stress primes for enhanced LPS responsiveness.

Recent reports have suggested an important role for lipid rafts, detergent-insoluble cell membrane microdomains, in LPS signalling. Specifically, clustering of TLR4/MD2 and other molecules including CD14, heat shock proteins 70 and 90, the chemokine receptor CXCR4 and Growth/differentiation factor 5 within lipid rafts and their confinement in these microdomains were determined as early steps in LPS signalling. Raft disrupting agents such as nystatin and methyl-β-cyclodextrin inhibited both enrichment of TLR4 in lipid rafts and LPS-stimulated downstream signalling via the MyD88/NF-κB pathway (413). In the present studies, we used both biochemical approaches and cell imaging techniques to demonstrate that oxidative stress generated by S/R *in vivo* or by H₂O₂ *in vitro* was able to induce migration of TLR4 into lipid rafts.
rafts. Coupled with the immunofluorescence microscopy and the flow cytometry data demonstrating increased surface TLR4 expression after oxidative stress, these findings suggest movement of TLR4 from the cytoplasmic compartment to lipid rafts in the plasma membrane. Since lipid rafts are known to be present in various intracellular compartments such as the Golgi apparatus or vesicles, it is possible that TLR4 would first move to one or more of these locations en route to the plasma membrane. It also appears that the integrity of the lipid raft microdomains is critical for TLR4 recruitment to the plasma membrane, since disruption of these domains with methyl-β-cyclodextrin prevented the oxidant-induced increase in TLR4 cell surface expression. This observation is also consistent with our finding that oxidant-induced exocytosis was involved in the translocation of TLR4 to the plasmalemma. Recent studies have reported that lipid rafts are involved in regulated exocytosis in various cell types, based on the observation that cholesterol depletion prevents exocytosis (478). The mechanism of this raft-dependent exocytosis in part involves the clustering of SNARE proteins within raft domains. Conclusions regarding whether TLR4-containing vesicles translocate to the plasmalemmal rafts directly or they migrate to the raft fraction after delivery to the plasma membrane will require further investigation.

The data suggest that mobilization of TLR4 into cell surface lipid rafts following oxidative stress plays an important role in the augmented cellular responsiveness to LPS stimulation. When oxidant-induced TLR4 mobilization to lipid rafts was prevented, using the cholesterol-depleting agent methyl-β-cyclodextrin, the increased responsiveness of the cells to LPS was prevented. One alternate explanation is that H₂O₂ might act through the activation of signalling cascades that are dependent on the integrity of lipid rafts, and thus its inability to prime following MβCD treatment may be due to an effect on H₂O₂-induced signalling. Data regarding p38 kinase activation by H₂O₂ with and without MβCD presented in Figure 7D demonstrate that MβCD does not globally inhibit H₂O₂-induced signalling. In addition, while the present studies do not rule out every conceivable pathway, wherein H₂O₂ might have influenced LPS signalling in a raft-dependent manner, it does provide a clear relationship between oxidative stress, increased surface TLR4, involvement of lipid rafts in this TLR4 redistribution and priming for LPS signalling. Recruitment of TLR4 to plasma membrane lipid rafts may have resulted in augmented signalling by a number of distinct mechanisms. First, the number of surface TLR4 molecules has been shown to correlate with LPS responsiveness. Overexpression of TLR4 in transgenic mice amplifies susceptibility to LPS treatment, both in vivo and in vitro (400). Following oxidative stress in the present studies, there was a substantial
increase in surface TLR4 expression, potentially accounting for the enhanced NF-κB translocation after LPS treatment. Second, LPS-induced formation of the receptor complex within lipid rafts appears to be necessary to transduce the LPS signal. We propose that antecedent oxidative stress induces formation of the complex, even before exposure to LPS, such that the receptor complex is poised to respond rapidly when exposed to LPS. While we did not measure the myriad of proteins known to localize in the LPS receptor complex, the adaptor protein MyD88, known to be enlisted to the complex during LPS signalling, was shown to co-localize with both TLR4 and the lipid raft fraction following oxidant exposure. While early studies investigating an effect of oxidants on cellular activation clearly demonstrated that oxidative stress was able to induce NF-κB nuclear translocation (137), enrichment of TLR4 as well as MyD88 in lipid rafts in response to H$_2$O$_2$ treatment in the present studies was not sufficient to induce downstream signalling in the form of increased NF-κB translocation (Fig. 12C), despite the fact that H$_2$O$_2$ was shown to activate other cell signalling pathways (see Fig. 7D, 10A). This finding is likely due to the relatively low dose of H$_2$O$_2$ used in these studies and the early time course of observation for NF-κB. In our prior work, we showed that 100 μM H$_2$O$_2$ was able to induce a modest rise in NF-κB translocation at a delayed time point (1 hour). This dose was chosen, since it had been shown to be a priming dose for LPS stimulation in this system (41;503). Similarly, the oxidant-dependent NF-κB translocation occurring in the lung following S/R is delayed and modest compared to LPS or the combination of shock plus LPS (36). This is consistent with the low dose oxidant priming of alveolar macrophages occurring in this setting and the effects on TLR4 redistribution seen in the present studies. Besides the TLR4 redistribution per se, recent studies evaluating responsiveness of cells to LPS and its analogues have also shown that the protein composition of the LPS receptor complex may vary depending on the stimulus and may influence downstream signalling (433). Oxidative stress may potentially induce an LPS receptor complex that diverts LPS signalling along an alternative pathway, leading to enhanced NF-κB translocation. In this regard, we recently reported that exposure of RAW 264.7 cells to H$_2$O$_2$ reprogrammed cells such that LPS signalling was dependent on activation of Src kinases and involved the PI3-kinase/Akt pathway (41;503). Src kinases are activated by oxidative stress (530) and move into lipid rafts to facilitate signalling (531). Further investigation evaluating the signalling pathways required for the oxidant-induced effects on TLR4 and the components of the oxidant-induced LPS receptor complex will provide insight into these possibilities.
The present studies demonstrate a marked change in TLR4 receptor density and distribution following oxidative stress, induced either by ischemia/reperfusion or by the addition of H$_2$O$_2$. In various other cell systems, oxidative stress may contribute to cell surface receptor density through altering the stability of newly synthesized protein in the endoplasmic reticulum, by inducing translocation of new proteins from the ER to the Golgi compartment and vesicular transport to the plasma membrane. With respect to the last of these mechanisms, the glucose transporter GLUT4 is translocated from cytoplasmic vesicles to the cell surface by oxidative stress (486). Ichimura and colleagues demonstrated that oscillations in calcium levels induced production of reactive oxygen species which in turn promoted exocytosis of P-selectin (489). In pancreatic acinar cells, reactive oxygen species generated by the hypoxanthine/xanthine oxidase system were able to induce increased exocytosis of amylase (257). Consistent with this mechanism, two distinct strategies aimed at preventing exocytosis of CD11b-containing intracellular vesicular compartments in macrophages also precluded oxidant-induced upregulation of TLR4. Specifically, polymerization of the actin cytoskeleton with jasplakinolide and calcium depletion inhibited translocation of TLR4 to the cell surface by H$_2$O$_2$. These and other studies suggest that the effect of oxidative stress on exocytosis may involve alterations in intracellular calcium levels.

The precise intracellular source of TLR4 requires further elucidation. Espevik and colleagues recently showed that in an epithelial cell line entire lipid raft fractions containing TLR4, CD14, and MD-2 continuously and rapidly recycle between the Golgi complex and the plasma membrane (395). In human monocytes, localization of TLR4 to perinuclear compartments was suggested to be evidence of its presence in the Golgi, although no formal localization studies have been performed (396). In RAW 264.7 cells as well as in rat alveolar macrophages, the intracellular localization of TLR4 appears more diffuse, consistent with its presence in preformed vesicles. Further definition of the cytoplasmic localization of TLR4 in cells of monocyte/macrophage lineage should provide insight into the preferred approaches to studying the mechanisms whereby oxidative stress induces an increase in surface TLR4 expression.

Alterations in the redox status of cells has been shown to affect a multitude of signalling pathways (532). With respect to LPS signalling, the present experiments provide evidence that oxidative stress generated during ischemia/reperfusion may exert its effects by regulating the distribution of the most proximal component of the signalling cascade, namely the LPS receptor complex. Future studies defining the mechanism of oxidant-induced TLR4 redistribution may
provide fundamental insights into the regulation of TLR4 in monocytes/macrophages and suggest novel therapeutic approaches in disease states wherein the process of ischemia/reperfusion contributes to the pathogenesis of disease.
FIGURES

Figure 4. Redistribution of TLR4 in alveolar macrophages following resuscitated hemorrhagic shock.
AM were isolated from Sham animals or from animals that underwent hemorrhagic shock followed by resuscitation for 1 hour (S/R). Where indicated, 0.5 g/kg N-acetylcysteine (NAC) or 10 mg/kg Trolox was present during resuscitation (S/R+NAC; S/R+Trolox, respectively). Cells were allowed to adhere to coverslips, fixed, permeabilized and stained with anti-TLR4. In (A and D) typical phase contrast and fluorescence microscopy images are shown. (B). Peripheralization of TLR4 was quantified by Scionimage software as described under Materials and Methods. (C). Pictures in rows show confocal microscopic images of different focal planes of the same cell. Size bars on all images represent 10 μm. (E). Specificity of the TLR4 antibody. Staining was done with (right image) or without (left image) anti-TLR4. Both samples were stained with FITC-labelled secondary antibody.
Figure 5. Resuscitated hemorrhagic shock increases TLR4 surface levels.

AMs were stained live with anti-TLR4 and FITC-conjugated secondary antibody. Fluorescence was analyzed by flow cytometry as described in Materials and Methods. (A). Profiles of fluorescence intensity of TLR4 staining (black line) or controls (secondary antibody only, black solid). (B). Changes in TLR4 surface expression (mean channel fluorescence, MCF) following the indicated treatments. Data are mean ± S.E.M. n= 4 animals per group.*p<0.05 for S/R vs. all other groups.
Figure 6. Oxidative stress induces increased TLR4 surface expression in RAW 264.7 cells. (A). RAW264.7 cells, exposed to H$_2$O$_2$ for 1 h, were stained with anti-TLR4/MD2-FITC antibody. Representative fluorescence microscopy images (n=4). (B) and (C). Dose and time dependence of the H$_2$O$_2$ effect. Cells were treated with the indicated concentration of H$_2$O$_2$ for 1 hour (B) or with 100 μM H$_2$O$_2$ for the indicated time (C). TLR4 staining was analyzed using flow cytometry. In (B) representative profiles of fluorescence intensity of TLR4 staining (grey solid) or unstained controls (black line) are shown (left panels). (D). Raw cells were treated with 50 μM menadione (Mena) or 100 μM H$_2$O$_2$ or 0.1 mM xanthine and 3U/ml xanthine oxidase for 1h. Surface TLR4 was measured as in B. The graphs show mean ± S.E.M. of n=3 (for D) or 4 (for B and C). *p<0.05 for points indicated vs. control.
Figure 7. Oxidative stress induces clustering of TLR4 in plasma membrane lipid rafts.

(A). *TLR4 and GM1 colocalization following oxidative stress.* AMs were plated on coverslips and stained live at 4°C with Rhodamine-CTxB. The cells were then fixed, permeabilized and stained with anti-TLR4. Representative images are shown of TLR4 (green), Rhodamine-CTxB staining (red), or the merged image. (B). RAW264.7 cells were treated with 100 µM H₂O₂ (1 h). Where indicated, cells were depleted from cholesterol by incubating with 10 mM methyl-β-cyclodextrin (MβCD) for 30 minutes prior to H₂O₂. Cells were stained and visualized as in (A). (C). *H₂O₂-induced increase in TLR4 surface level requires lipid rafts.* RAW264.7 cells were treated with 100 µM H₂O₂ for 1 h. Where indicated, cells were depleted from cholesterol prior to or following treatment with H₂O₂. TLR4 expression was analyzed by flow cytometry. Data are mean ± S.E.M. (n=4 per group). *p<0.05 for H₂O₂, H₂O₂ then MβCD vs. control and MβCD then H₂O₂. (D). *Cholesterol depletion does not interfere with H₂O₂-induced signalling.* Cells were treated with 100 µM H₂O₂ for 30 min, then lysed. Equal amount of protein was loaded on SDS-gels and phospho-p38 was detected using Western blotting. Where indicated, cells were cholesterol depleted prior to H₂O₂ addition.
Figure 8. Recruitment of TLR4 into lipid rafts by oxidative stress.

RAW264.7 cells, treated with 100 μM H₂O₂ (1 h) with or without MβCD pre-treatment, were lysed in 1% Triton X-100 and subjected to discontinuous sucrose density gradient centrifugation as described in Materials and Methods. Fractions were analyzed by dot blotting using either using CTxB conjugated to horseradish peroxidase (GM1, upper blot) or anti-TLR4 primary and peroxidase-coupled secondary antibody (lower blot). Fractions 1-3 correspond to lipid rafts. Representative blots of three separate experiments are shown.
Figure 9. Fluorescence Resonance Energy Transfer (FRET) verifies oxidant-induced molecular interaction between TLR4 and GM1.

**A** Controls for FRET. Untreated or H$_2$O$_2$-treated (100 μM, 1 h) RAW 264.7 cells were either left unstained or stained live at 4°C with Rhodamine-CTxB. Fluorescence of individual cells was determined using the excitation wavelength 480±5 nm and emission ≥590 nm as described under Methods. The emission of all groups was normalized to the unstained untreated cells (100%). Data are mean ± S.E.M. of n=18 cells from three independent experiments.

**B** H$_2$O$_2$ induces FRET between Rhodamine-CTxB (GM1) and anti-TLR4-FITC. RAW 264.7 cells were left untreated or treated with 100 μM H$_2$O$_2$ for 1 h, stained live at 4°C with Rhodamine-CTxB and anti-TLR4 and FITC-labelled secondary antibody. For control, identically treated cells were stained only with FITC-labelled secondary antibody. To obtain the value of FRET, non-FRET related factors were corrected for as detailed in Results. Data are normalized to the untreated cells (100%) and are mean ± S.E.M. n=24 cells, n=4.
Figure 10. Role of exocytosis in TLR4 translocation to the plasma membrane following oxidative stress.

Raw 264.7 cells remained untreated (Control) or exposed to DMSO vehicle or 1 μM Jasplakinolide (JAS) (A and C) for 30 min or 10μM of BAPTA/AM for 10 minutes (Band D). Cells were then exposed to 100 μM H2O2 (1 h) or 0.1 µg/ml LPS (30 min) followed by staining live with anti-CD11b-FITC (A and B) or anti-TLR4/MD2-FITC at 4°C (C and D). Fluorescence was analyzed by flow cytometry. Data are mean ± S.E.M. n=4 per group. *p<0.05 for group indicated vs. all other groups without an asterisk.
Figure 11. Oxidative stress induces colocalization of TLR4 and MyD88 in the plasma membrane. 

(A). Oxidant-dependent colocalization of TLR4 and MyD88 after S/R. AMs were stained with anti-TLR4 and anti-MyD88 primary, and the corresponding secondary antibodies. Representative images (n=4 experiments) are shown of TLR4 staining (red) and MyD88 staining (green) or merged images. 

(B). Colocalization of GM1 and MyD88 after H₂O₂ treatment. RAW264.7 cells, treated with 100 µM H₂O₂ (1 h) were stained live at 4°C with Rhodamine-CTxB, fixed, permeabilized and stained with anti-MyD88 antibody as in (A). Representative images (n=3 experiments) of GM1 staining (red) or MyD88 staining (green) or merged image. 

(C). Specificity of the anti-myd88 antibody. RAW264.7 cells were immunostained as in A. In the left image, the primary antibody was added in the presence of a specific blocking peptide (D). RAW 264.7 cells were exposed to 0.1 µg/ml LPS or 100 µM H₂O₂. Where indicated, cells were pretreated with 10 mM MβCD. Lipid raft fractions were isolated and analyzed by dot blotting using MyD88 primary and HRP- coupled secondary antibody. Fractions 1-3 correspond to lipid rafts.
Figure 12. The role of lipid rafts in NF-κB Translocation.

(A.) *H₂O₂ treatment does not affect cholesterol depletion and repletion.* Cells were incubated with 10 mM MβCD for 30 min. Next, indicated samples were treated with 100 μM H₂O₂ for 1 h. Where indicated, cholesterol levels were restored using MβCD+cholesterol. Total cellular cholesterol was measured. Data are normalized to control levels (100%). Data represent mean ± S.E. (n=3 experiments).

(B and C). **Lipid raft integrity is required for oxidant-induced priming of LPS-stimulated NF-κB activation.** (B.) Cholesterol depletion inhibits, cholesterol repletion restores LPS-induced NF-κB nuclear translocation. RAW264.7 cells were exposed to 0.1 μg/ml LPS for 15 or 30 minutes with or without cholesterol depletion. Where indicated, cholesterol levels were restored prior to addition of LPS (MβCD+cholesterol). Cells were stained with anti-p65. NF-κB nuclear translocation was analyzed by fluorescence microscopy as described in Methods. (C.) Cholesterol depletion prior to H₂O₂ prevents LPS- induced early p65 translocation. Control or MβCD treated cells were incubated with H₂O₂ followed by cholesterol repletion and addition of LPS where indicated. NF-κB nuclear translocation was analyzed as in (A). Data are mean ± S.E.M. n=3. *p<0.05 for groups indicated vs. all other groups without an asterisk.
CHAPTER 4
REACTIVE OXYGEN SPECIES INDUCE SRC KINASE DEPENDENT SURFACE
UPREGULATION OF TOLL LIKE RECEPTOR 4 (TLR4) IN RAW 264.7 MACROPHAGES

Disseminated as:

Powers KA, Szaszi K, Khadaroo RG, Kapus A, Rotstein OD.
SUMMARY

Model

The study design involved the use of a previously developed *in vitro* macrophage priming model using RAW 264.7 murine macrophage cell line. Cells were first incubated with various concentrations of hydrogen peroxide, and then exposed to low dose of LPS. In this model, oxidative stress is able to augment macrophage responsiveness to LPS as evidenced by increased surface TLR4 and earlier and greater NF-κB translocation. To test for Src kinase involvement in TLR4 surface upregulation in RAW cells, Src kinases were inhibited by either pharmacological means using PP2 or through a molecular approach, by cell transfection with Csk. Cells were examined by flow cytometry for TLR4 expression as well subjected to biochemical analysis of lipid raft involvement in TLR4 translocation.

Main Results

Inhibition of Src family by PP2 or through cell transfection with Csk was found to prevent oxidative stress induced TLR4 exocytosis suggesting that Src kinases are involved in this process. We have reported that in response to oxidative stress TLR4 moves into the lipid rafts. Lipid rafts are also known to be rich in Src kinases. Lipid raft localization of TLR4 after oxidative stress exposure was abolished by Src inhibition. Therefore, recruitment of TLR4 into the lipid rafts upon oxidative stress is demonstrated to be Src dependent. The findings suggests that alternative therapeutic strategies aimed at Src kinase regulation may be required to modulate cellular activation and organ injury following shock resuscitation.
INTRODUCTION

Macrophages stimulated by oxidants liberated during ischemia/reperfusion have been shown to play an integral role in mediating organ injury following hemorrhagic shock and resuscitation. Both in vitro and in vivo approaches have been developed to investigate the cellular and molecular mechanisms whereby shock resuscitation and oxidative stress augments LPS signalling in macrophages, the so called “two hit hypothesis” models (36;41). In these studies, animals or macrophages alone when subjected to oxidative stress exhibit a marked increase in LPS induced generation of pro-inflammatory molecules such as Cytokine-Induced Neutrophil Chemoattractant (CINC), the rodent orthologue of IL-8, and TNF-α (36;136) compared to controls. This increase was caused by enhanced transcription of these pro-inflammatory molecules and appeared to be a consequence of augmented nuclear translocation of the transcription factor NF-κB in response to LPS. Despite a clear role for oxidative stress in the priming of macrophages for LPS responsiveness, the mechanisms underlying this effect have not been fully elucidated. Two potential alterations in LPS signalling under oxidative stress conditions have been demonstrated to contribute to priming: 1) Recruitment of LPS receptor Toll-like receptor 4 (TLR4) to signalling domains on the plasma membrane of macrophages via an exocytic process (533) and 2) reprogramming of LPS signalling to a Src kinase-dependent pathway involving the PI3-kinase/Akt dependent mechanism (41). These observations lead us to hypothesize that oxidative stress might exert its effects on macrophages by augmenting TLR4 in the lipid rafts through stimulation of Src kinases.

Src family tyrosine kinases Hck, Lyn, and Fgr, have been implicated in the biological response of LPS-activated macrophages (491;510;511). Data from our laboratory and others demonstrate that Src kinases are primary targets for oxidative stress with Hck, Lyn and Fgr undergoing phosphorylation upon stimulation with H$_2$O$_2$. Consistent with this notion, Src kinase inhibition with pharmacological agents, such as PP2, and molecular approaches, such as transfection of cells with the C-terminal Src kinase (Csk), an endogenous inhibitor of Src kinases, blocked not only the LPS-induced biological responses in monocytes but also oxidant mediated priming of macrophages (41;512). Specifically Src kinase inhibition blocked LPS induced NF-κB translocation under oxidative stress condition, but not in the absence of oxidants. The upstream mechanisms responsible for the switch to a Src dependent signalling process under oxidative stress are not well understood. One of the upstream targets may be the surface LPS receptor – TLR4.
TLR4 is an essential receptor for LPS that has been shown to be exocytosed to into plasma membrane lipid rafts during oxidative stress (533). While, several molecular mechanisms have been associated with receptor trafficking regulation, the precise regulation of TLR4 trafficking especially under oxidative stress conditions remains undefined. Several lines of evidence suggest that Src kinases may be involved in regulation of TLR4 exocytosis into lipid rafts. First, Src tyrosine kinases are known to be involved in receptor sorting and cellular secretion (507;534-536). Additionally, Src kinases have been demonstrated to move into cell surface signalling domains – the lipid rafts, to facilitate signalling (531). Lastly, Src kinases have been shown to be important intermediaries of oxidative stress mediated macrophage priming. Exposure of RAW 264.7 cells to H2O2 reprogrammed cells, such that LPS signalling was dependent on activation of Src kinases and involved the PI3-kinase/Akt pathway (41;503). Interestingly, one of the molecules implicated in receptor exocytosis and vesicular trafficking is phosphatidylinositol 3-kinase (PI3K). PI3K is required for the redistribution of the IGF-II, platelet-derived growth factor, and possibly the stem cell factor receptor (537-539). IGF-II recruits its own receptors to the cell surface in a PI3K-dependent manner (538). Collectively, these observations imply that Src kinase activation by oxidative stress may be one of the ways of regulating TLR4 trafficking and surface upregulation during oxidative stress conditions. Therefore, it is conceivable that by inhibiting Src kinase activation during oxidative stress one might diminish TLR4 movement into the lipid rafts on the cell surface. The findings in theses studies help define a novel mechanism whereby oxidative stress might prime the innate immune system for an exaggerated response to inflammatory stimuli and hence for increased tissue and organ injury.

MATERIALS AND METHODS

Solutions and Reagents

Hydrogen peroxide (30%) (H2O2) was purchased from University of Toronto Medstore (Toronto, ON). Lipopolysaccharide (E.coli O111:B4) and crystalline NaCl was obtained from Sigma (St Louis, MO). Endotoxin-free Dulbecco's modified Eagle's medium (DMEM) and Hanks' balanced salt solution (HBSS) were purchased from Invitrogen, phosphate buffered saline (PBS) were from Gibco BRL (Burlington, Ontario), Fetal calf serum was purchased from Hyclone Lab Inc (Logan, UT) and penicillin/ streptomycin from Gibco BRL. Brij58, TKM buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl2, and 1 mM EDTA), protease inhibitors (tablet), TBST buffer. Enhanced Chemiluminescence Kit for Western blotting was purchased
from Amersham Pharmacia Biotech. All chemicals used were of the highest purity available. An inhibitor of the Src family of protein tyrosine kinases, PP2, was purchased from Calbiochem, Inc. (San Diego, CA).

**Cell culture and activation**

The murine macrophage cell line, RAW 264.7 (American Type Culture Collection), was cultured at 37°C in a humidified atmosphere of 5% CO2 in endotoxin-free Dulbecco’s modified Eagle’s medium (Invitrogen) with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 10% fetal calf serum (Hyclone Lab, Inc.). Experiments were performed in Hanks’ balanced salt solution (Invitrogen) supplemented with 2% Fetal calf serum and 2 mM glucose. The following treatments were applied: 100 μM (or the indicated concentration) H2O2 for one hour followed by 0.1 μg/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich) for 5 to 60 min. Reactions were stopped by placing the cells on ice. In inhibition studies, RAW cells were preincubated in the presence of 10 μM PP2 for 10 min at 37 °C. Alternatively, a PP2 treatment time course was performed at 10 to 60 min of treatment at 37 °C.

**Stable Transfection of Cell Line**

Stable transfection of RAW 264.7 cell lines expressing Csk were kindly provided by Dr. Z. Honda (University of Tokyo, Japan) (10). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, P/S, and neomycin antibiotic G418 (Invitrogen).

**Immunofluorescence microscopy**

RAW264.7 cells on coverslips were fixed with 4% paraformaldehyde (Canemco-MARivac) for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 20 min, blocked with 5% bovine serum albumin for 1 h, and incubated with the indicated antibody. The following primary antibodies were used for staining for one hour: anti-TLR4/MD2 (MTS510) – Fluorescein (FITC) conjugated, rat monoclonal antibody (20 μg per 1x10^6 cells) purchased from Stressgen Biotechnologies (Victoria, BC). Antibodies against p-Hck were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Labelling of GM1 ganglioside, a component of lipid rafts was done using rhodamine (BTRITC) conjugated Cholera Toxin B subunit (1:500 dilution) purchased from List Biological Laboratories, Inc. (Campbell, CA). The staining was visualized using a Nikon TE200 fluorescence microscope (x100 objective) coupled to a Orca 100 camera (Hamamatsu Photonics, Hamamatsu, Japan) driven by Simple PCI software (Compix Inc., Imaging Systems, Cranberry Township, PA).
Flow cytometry

Cell surface expression of TLR4 was detected by flow cytometry of live cells stained with anti-TLR4/MD2 (MTS510). 10,000 cells/condition were analyzed in a FACScan (Becton Dickinson) using FL1 525 mM Band Pass detector at an excitation wavelength of 488 nm. Results were expressed as mean channel fluorescence.

Isolation and detection of lipid rafts

Lipid rafts were isolated as described previously (24). RAW264.7 cells (2 × 10^7 cells/ml) were lysed in 0.25 ml TKM buffer (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl2, and 1 mM EDTA) containing 0.5% wt/vol Brij58 (Sigma-Aldrich) and protease inhibitors (Roche Diagnostics) on ice for 30 min. Lysates was mixed with and equal volume cold 80% wt/vol sucrose in TKM and overlaid with 4.3 ml cold 36% sucrose, followed by 0.2 ml 5% sucrose. The gradients were subjected to ultracentrifugation at 200 000 g at 4°C for 18 h. Fractions were collected from the top of the gradient. An equal volume of each fraction was diluted in 10 μl TBS-T buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) containing 0.1% Triton X-100 and loaded onto Protran Nitrocellulose Sheet (0.45-μm pore; S&S BioScience) using Dot Blot (Bio- Rad Laboratories). The membrane was blocked in 5% milk in TBS-T, followed by incubation with CTxB–horseradish peroxidase (1:500; List Biological Laboratories), anti-TLR4/MD2 (MTS510), for 1 h. Where required, horseradish peroxidase–conjugated secondary antibody (1:3,000 dilution for 1 h) was used. Labelling was visualized using enhanced chemiluminescence (PerkinElmer).

Western blotting

Proteins were detected using Western blotting as described previously (43). Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories). Equal loading was verified using Ponceau S staining of the membranes. The phospho-p38 antibody was from Cell Signalling.

Statistical analysis

Data are presented as mean ± standard error of n determinations as indicated in the figure legends. Data were analyzed by one-way analysis of variance, and post hoc testing was performed using the Bonferroni modification of the t test with significance at P < 0.05. Blots and images shown are representatives of at least three separate determinations.
RESULTS
Oxidant-induced TLR4 translocation to the plasma membrane is Src kinase dependent

H$_2$O$_2$ was previously shown to induce a dose-dependent exocytosis of TLR4, with modest effect at 50 μM and marked increase at 100μM. In order to assess the role of Src kinases in oxidant-induced TLR4 exocytosis we used PP2, a potent and selective pharmacological inhibitor of the Src of tyrosine kinases (496). In vitro kinase assays in previous studies have confirmed the ability for PP2 to prevent the activation of Src kinases with a ten minute pretreatment with the inactive analogue of PP2, PP3, showing no inhibitory effect (41).

Src kinase activity was inhibited by preincubating RAW 264.7 cells for 10 minutes with PP2 followed by H$_2$O$_2$ stimulation or low dose LPS. A time course was performed where the TLR4 surface expression was measured by flow cytometry in nonpermeabilised cells over one hour interval. Consistent with what we showed previously, oxidative stress with H$_2$O$_2$ for 60 minutes induced a 54% (n=5, p<0.05) increase in TLR4 surface expression in RAW 264.7 cells compared to untreated controls. This rise was completely prevented in cells pretreated with PP2, where Src activity was inhibited (Fig. 13A). Similarly, PP2 pretreatment inhibited the rise in TLR4 following oxidant plus LPS by 43% (n=5, p<0.05). However, PP2 did not affect the TLR4 decrease following LPS alone. Interestingly, while PP2 caused an increase in basal levels of TLR4, levels returned to untreated control levels at 60 min, suggesting a role of constitutive Src activity in maintenance of TLR4 surface levels. The ability of PP2 to inhibit oxidative stress but not LPS-induced TLR4 surface upregulation suggests that oxidative stress changes the regulation of TLR4 cellular trafficking to a Src dependent process.

Effect of Csk expression on oxidant-induced TLR4 translocation to the plasma membrane

Recent reports have shown that several proteins become tyrosine-phosphorylated in response to LPS stimulation. Among these proteins is the C-terminal Src kinase (Csk), which negatively regulates Src kinases in RAW 264.7 cells. Csk may be a critical regulator of TLR4-mediated signalling by modifying the levels of Src kinases. Daisuke et al. demonstrated that LPS-induced secretion of pro-inflammatory cytokine IL-6 and TNF-α was down regulated in Csk knockdown cells. Furthermore, overall cellular tyrosine phosphorylation and TLR4-mediated activation of IκB-α, Erk and p38 but not of JNK, were also down regulated in Csk knockdown cells. We hypothesized that Src kinase activation by oxidative stress may be one of the ways of regulating TLR4 trafficking that leads to its surface upregulation and consequent increased sensitivity to LPS. Therefore, Csk overexpression in stably Csk transfected Raw 264.7 cells allowed us to assess the role of Src kinases in oxidant mediated TLR4 exocytosis.
Stable Csk transfectants or untransfected Raw 264.7 cells were stimulated in vitro with 
H$_2$O$_2$ or LPS followed by TLR4 fluorescent staining of nonpermeabilized cells. TLR4 
expression was quantified by flow cytometry. A time course was performed that compared 
oxidant induced TLR4 exocytosis in transfectants vs. wild type cells. TLR4 was measured at 0, 
5min, 30min and 60min. The basal expression level of TLR4 was not significantly different 
among RAW 264.7 cells and transfectants (Fig. 14B). In a representative study (Fig. 14A), there 
was a marked rise in surface TLR4 in untransfected Raw 264.7 cell treated with H$_2$O$_2$ consistent 
with previously published results. In contrast, the H$_2$O$_2$ induced TLR4 exocytosis was lost with 
the overexpression of the Src inhibitory Csk suggesting that Src kinases are critical in regulating 
this process (Fig. 14A). Interestingly LPS stimulation alone, in both transfectants and controls, 
induced the same effect of transient TLR4 increase followed by a decline (Fig. 14B). However, 
with Src inhibition, the observed persistent surface elevation of TLR4 in oxidative stress-primed 
macrophages, stimulated by LPS, was completely abolished (Fig. 15). Collectively, these 
findings strengthen the conclusion that exposure of macrophages to oxidative stress causes an 
alteration in regulation of TLR4 surface expression, such that it becomes dependent on 
activation of Src kinases.

**Effect of Src kinase inhibition on oxidant-induced TLR4 translocation into lipid rafts**

Lipid rafts have recently been demonstrated to be important contributors to membrane 
recruitment and clustering of the LPS receptor complex in response to LPS stimulation or to 
oxidative stress (433;540). Lipid rafts are also known to be rich in Src tyrosine kinases which 
are involved in receptor sorting and receptor mediated signalling (507;541). Therefore, we next 
evaluated the role of lipid rafts in the oxidant induced TLR4 redistribution into lipid rafts on the 
cell surface.

Lipid raft fractions were isolated using discontinuous sucrose gradient 
ultracentrifugation of in vitro stimulated RAW264.7 cells (520). The lipid rafts were examined 
first for the presence of the raft marker GM-1 ganglioside in the nonsoluble portion, fractions 1– 
3 of the sucrose gradient to confirm good raft isolation. The three major Src family members 
expressed in the macrophage are Hck, Fgr, and Lyn (542). Recent data from our laboratory have 
demonstrated that Src kinases are primary targets for oxidative stress. H$_2$O$_2$ (100 µM) induced 
phosphotyrosine accumulation in all three of these kinases in Raw 264.7 cells. Specifically, Hck 
kinase activity could be blocked by the addition of 10 uM PP2 (41). The isolated lipid rafts from 
the treated Raw 264.7 cells were therefore assessed for tyrosine phosphorylation of Hck using 
phosphospecific antibody against pHck (Fig. 15). While control cells had no Hck kinase
activity, H₂O₂ stimulation induced pHck localization in lipid rafts. This effect was prevented by PP2 pretreatment.

We next were interested in whether the oxidant induced recruitment of TLR4 into the lipid rafts may also be a Src dependent process and therefore immunoblotted the PP2 pretreated cell raft fractions for TLR4. Src inhibition with PP2 completely inhibited H₂O₂ induced raft localization of TLR4 suggesting that recruitment of TLR4 into the lipid rafts upon oxidative stress is dependent on Src tyrosine phosphorylation.

**DISCUSSION**

Oxidative stress during shock resuscitation has been implicated in macrophage priming for augmented LPS responsiveness. Two mechanisms were proposed for macrophage priming: first through a Src kinase dependent PI3-kinase pathway (41) and secondly through exocytosis of TLR4 into lipid rafts in the plasmalemma (533). The present studies examine the role of the Src kinases in the TLR4 recruitment to the cell surface and define whether such activation is required for LPS stimulated and/or oxidative stress stimulated TLR4 localization in lipid rafts. To study whether Src activation is essential in TLR4 translocation two approaches were used. First, a pharmacological approach with an inhibitor of Src kinases, PP2, and secondly a molecular approach using stably transfected cells overexpressing Csk, a negative regulator of all Src kinases. Results presented demonstrate that Src inhibition with PP2 or Csk overexpression inhibits the oxidant induced TLR4 surface translocation. However, Src inhibition by both approaches does not affect the usual LPS induced TLR4 surface translocation and downmodulation. The ability of PP2 and Csk overexpression to inhibit oxidative stress but not LPS induced TLR4 surface upregulation suggests that oxidative stress changes the regulation of TLR4 translocation from a non-Src dependent to a Src dependent process. Together, these findings are the first to demonstrate that Src kinases are involved in regulating exocytosis of TLR4 under oxidative stress conditions.

Khan and colleagues recently showed that H₂O₂ activates Src kinases, which are thought to regulate trafficking of epidermal growth factor receptor, specifically its endocytosis (505). Little is known, however about the regulation of TLR4 trafficking and especially under oxidative stress conditions. Nakahira et al reported that TLRs show differential patterns of trafficking after different ligand stimulation (543). For example, carbon monoxide significantly suppressed recruitment of TLR4 to the cell surface. In contrast, NADPH oxidase–dependent ROS generation induced by TLR ligand stimulation induced trafficking of TLR to the lipid raft
component of the cell surface. Similarly, our studies indicate that reactive oxygen species induce TLR exocytosis into the lipid rafts. Although the mechanism for TLR4 intracellular sorting is yet unknown, a conceivable mechanism of H₂O₂ induced, TLR4 exocytosis is through oxidant-induced tyrosine phosphorylation of lipid raft components such that downstream signalling initiates TLR4 recruitment. One of the candidates for such tyrosine phosphorylation are the Src kinases which are known to reside in lipid rafts and are involved in mediating downstream signalling (491;492). Tyrosine kinases such as Fyn as well as other Src family tyrosine kinases are also involved in receptor sorting and cellular secretion processes (535;536). Furthermore, recent evidence suggest that LPS-induced CD40 activation and the secretion of pro-inflammatory cytokines IL-6 and TNF-α can be down-regulated in Csk knockdown cells (515). TLR4-mediated activation of IκB-alpha, Erk and p38 but not of JNK, were also down-regulated in Csk knockdown cells implicating Csk as a critical regulator of TLR4-mediated signalling (515). Consistently, our results indicate that Src kinases are critical for the oxidant induced TLR4 surface translocation, important for LPS signalling.

Having illustrated that the Src family kinases participate in oxidant induced TLR4 exocytosis we investigated whether Src kinase activation was essential for TLR4 localization in lipid rafts. Our data indicates that without Src activation oxidative stress can no longer induce raft localization of TLR4. Lipid rafts are known to be involved in clustering TLR4 and other receptors in response to various stimuli including oxidative stress (533;543) and therefore further studies are necessary to elucidate how oxidative stress alters lipid raft composition to facilitate Src kinase activation. One candidate molecule is the lipid ceramide. It is known that ceramide is generated by oxidative stress and evidence exists that it may contribute to lipid raft formation. Ceramide is known to incorporate in lipid rafts and act as a signalling platform for downstream effects. Further, in colonic smooth muscle cells, ceramide-induced PI3-kinase activation was found to be associated with tyrosine phosphorylation of pp60src (544). Based on these considerations, oxidant-induced ceramide production may contribute to TLR4 translocation as well as the altered signalling via a Src-dependent pathway. The possibility of an alternative signalling pathway that depends on the type and number of receptors recruited to the lipid rafts is consistent with the recently reported observation in RAW 264.7 cells exposed to H₂O₂. These cells become reprogrammed, such that LPS signalling becomes dependent on Src kinase activation (41;503). Our studies are first to illustrate that the Src kinase activation which occurs during oxidative stress may be responsible for TLR4 plasma membrane translocation. Interestingly, Src activation was not proven necessary for TLR4 trafficking as Src inhibition did
not affect LPS induced TLR4 plasma membrane migration. These findings define a novel Src kinase dependent priming mechanism for macrophages. Src kinase family inhibition provides a potential alternative therapeutic strategy in prevention of organ injury following shock resuscitation.
Figure 13. The effect of PP2 on the H$_2$O$_2$-induced increased cell surface expression of TLR4 in RAW 264.7 cells.

(A) and (B) Raw 264.7 cells remained untreated (control) or pretreated with PP2 at 10 µM for 10 min at which point 100 µM H$_2$O$_2$ was added for one hour (A). Alternatively cells were treated with PP2 for 10 min then exposed to 100 µM H$_2$O$_2$ for one hour followed by LPS (0.1 µg/ml) for 5-60 min (B). In some cells, a PP2 treatment time course was performed at 10 to 60 min of treatment at 37°C. Treated cells were stained with anti-TLR4/MD2-FITC at 4°C. Fluorescence was analyzed by flow cytometry. Data are mean ± SEM of $n = 5$ per group. *, $P < 0.05$ for groups indicated versus all other groups without an asterisk.
Figure 14. The effect of Csk expression on H₂O₂-induced increased cell surface expression of TLR4 in RAW 264.7 cells.

(A). Raw 264.7 were exposed to 100 μM H₂O₂ for 5-60 min or to LPS 0.1 μg/ml for 5-60 min. 
(B). RAW 264.7 cells stably transfected with Csk were exposed to 100 μM H₂O₂ for 5-60 min or to LPS 0.1 μg/ml for 5-60 min. In both (A) and (B) treated cells were stained with anti-TLR4/MD2-FITC at 4°C. Fluorescence was analyzed by flow cytometry. Data are mean ± SEM of n = 3 per group. *, P < 0.05 for groups indicated versus all other groups without an asterisk.
Figure 15. Effect of Src kinase inhibition on oxidant-induced TLR4 translocation into lipid rafts.

RAW264.7 cells were treated with 100µM H₂O₂ (1 h) with or without a 10 min 10uM pretreatment with PP2. The cells were lysed in 1% Triton X-100 and subjected to discontinuous sucrose density gradient centrifugation as described in Materials and Methods. Samples from the sucrose gradient fractions were loaded onto dot blot and developed either using CTxB subunit conjugated to horseradish peroxidase to detect GM1-containing lipid rafts (fractions 1-3, upper blot), or assessed for tyrosine phosphorylation using immunoblotting with the phosphospecific antibody against Hck or anti-TLR4 primary and peroxidase-coupled secondary antibodies (lower blot). Representative blots of three separate experiments are shown.
CHAPTER 5
25% ALBUMIN, AS AN ANTIOXIDANT, PREVENTS LUNG INJURY FOLLOWING SHOCK RESUSCITATION

Published as:


**SUMMARY**

**Model**

The study design involved the use of a previously developed rodent model of acute lung injury in which resuscitated shock primes for increased lung injury in response to a small dose of intratracheal lipopolysaccharide. Animals were bled to a mean arterial pressure (MAP) of 40mmHg, and maintained in a shock phase for 1h. Animals were then resuscitated by transfusion of the shed blood plus an equal volume of RL or their shed blood plus 3 ml/kg volume of A25 or their shed blood plus 15ml/kg of 5% Albumin (A5) over a period of two hours. MAP was monitored continuously. One hour after resuscitation, 100 µg of LPS in 200 µl of saline was administered intratracheally.

**Main Results**

Resuscitation with A25 significantly reduced transpulmonary protein flux, bronchoalveolar lavage fluid neutrophil counts, and the degree of histopathological injury compared to resuscitation with RL or A5. To delineate the underlying mechanism of this beneficial effect, the production of cytokine-induced neutrophil chemoattractant (CINC) as well as nuclear translocation of its critical transcription factor NF-κB was evaluated. Both CINC mRNA levels and NF-κB translocation were diminished following A25 resuscitation. Further, A25 significantly decreased lipid peroxidation in plasma as measured by 8-Isoprostane levels. N-ethylmaleimide (NEM) modified A25, possessing lesser antioxidant activity, exhibited an attenuated protection from lung injury. In addition, resuscitation with A25 attenuated the increase in PMN CD11b expression in RL resuscitated animals at end resuscitation and at 4h post LPS. While PMN L-selectin levels remained stable in RL treated animals, A25 resuscitation resulted in a significant decrease in surface L-selectin expression at 4h post LPS. ICAM-1 lung endothelial cell mRNA, was increased in RL resuscitated animals whereas reduced with A25 use by 51%. The LPS induced ICAM-1 endothelial cell protein expression was also prevented with A25 resuscitation. Antioxidant property of albumin was shown to play a critical role in altering CD11b expression.
INTRODUCTION

Civilian trauma remains a significant health care burden, ranking high in terms of patient hospital days, years of life lost and mortality (545). Trauma deaths can divided into two major periods; those occurring in the first seven days, mainly a result of exsanguination and major head injury, and those occurring after seven days, most frequently due to the development of multiple organ dysfunction syndrome (MODS) (130). One paradigm relevant to the development of organ dysfunction following shock/resuscitation has been termed the “two-hit hypothesis”. According to this hypothesis, tissue ischemia at the time of acute blood loss leads to hypoxic cellular damage and the subsequent reperfusion phase is felt to contribute to priming of inflammatory cells, and specifically macrophages, for increased responsiveness to subsequent inflammatory stimuli (130). The over-exuberant activation of the immune system contributes to the development of dysfunction in several organs including lung, kidney, gut, liver, and brain, the so called MODS (130). The lungs are among the earliest and most frequently affected organs after hemorrhagic shock, with acute respiratory distress syndrome (ARDS) occurring in up to 50% of these patients (32).

Reactive oxygen species play an important role in the physiological alterations associated with ischemia/reperfusion (43). Xanthine oxidase generated during ischemia can react with the purine substrates hypoxanthine and xanthine during reoxygenation, leading to the production of the reactive oxygen metabolites superoxide and hydrogen peroxide (546). Traditionally, oxidants were considered to exert their effects in inflammation by having direct toxic effects on cells (231). Recently, there has been considerable interest in the potential role of oxidants in the regulation of cell activation, since several cellular signalling pathways are known to be redox sensitive (232-235). Relevant to inflammation, oxidants have been shown to participate in signalling cascades in activated macrophages which culminate in nuclear translocation of NF-κB, a transactivation factor known to contribute to the induction of several proinflammatory genes, including TNF-α and interleukin 8 (46;47;236). An understanding of the role oxidants in inflammation has provided a strong rationale for the use of antioxidants as anti-inflammatory agents in the in vitro and in vivo settings. For example, the addition of antioxidants to cell culture systems has been shown to abrogate LPS-induced TNF release from cells (44;137), while the antioxidant N-acetylcysteine (NAC) has been reported to preclude lung injury in various rodent models (45;547). In critically ill human patients, antioxidant therapy has been suggested to lessen organ injury, although beneficial effects on patient mortality have not been demonstrated (138;139;548).
Our laboratory has previously reported an animal model of lung injury following oxidant stress in the form of shock/resuscitation. In the model, a low dose of LPS induces a profound lung injury when administered following global ischemia/reperfusion (36). Compelling evidence from this and other models of ARDS indicates that activated PMNs accumulate in the interstitium and alveolar space contributing to and then traversing leaky alveolar-capillary membranes where they release proteases, elastases and generate superoxide which contribute to their toxic effect (549;550). These events are initiated and propagated by oxidant stress activated macrophages, primed for an exaggerated response and the generation of proinflammatory molecules such as the cytokines TNF-α, IL-1, the procoagulant molecule tissue factor and chemokines including IL-8 (in rodents Cytokine Induced Neutrophil Chemoattractant, CINC) and MIP-2. Interactions between PMNs and ECs play a critical role in the development of ARDS. PMN recruitment and lung infiltration are the early steps that may lead to organ damage later. Agents released from activated alveolar macrophages have been shown to in turn activate PMNs and pulmonary capillary endothelial cells (ECs), and induce expression of adhesion molecules on their surfaces leading to PMN sequestration in the affected organ and consequent injury (36;551).

There are three families of cell adhesion molecules: the selectins, the integrins and the immunoglobulins, that mediate the PMN-EC interactions (552). L-selectin is involved in the initial rolling and weak attachment of PMNs to the vascular endothelium, as shown by protection against lung injury where L-selectin is neutralized or genetically deleted. Firm adhesion of PMNs to the EC occurs as a result of strong interactions between β2 integrins, expressed on the PMN surface, and their endothelial cell ligands, intercellular adhesion molecules-1 (ICAM-1). In our lung injury model, a significant increase in lung neutrophil sequestration concomitant with increased CD11b on circulating neutrophils and ICAM-1 expression in whole lung tissue was observed following shock resuscitation (173).

Considered together, these findings suggest the possibility that strategies aimed at altering the priming events that occur during shock/resuscitation might exert beneficial effects in terms of preventing organ injury. In this regard, several lines of evidence suggest that the use of various resuscitation strategies including alterations in fluid composition or the type of resuscitation fluid used may influence the proinflammatory response following shock/resuscitation (153;173;553). Our laboratory and others established the beneficial role of hypertonic saline (HTS) resuscitation in preventing lung injury (173;174). The salutary effect of HTS was shown to be due to a decrease in PMN-EC interaction resulting in reduced lung
neutrophil sequestration. *In vitro* studies by Rhee and colleagues established that both HTS and 25% Albumin (A25) diminished PMN activation. In addition, while CD18 expression was increased with crystalloids, artificial colloids and A5, it was not with A25 incubation and diminished with HTS incubation (153). Therefore, the objective of the present studies was to examine whether resuscitation with albumin might exert protective effects on lung injury in a rodent model where shock/resuscitation primes for augmented lung injury in response to LPS treatment. Based on the integral role of PMN-EC interaction in the pathogenesis of post-traumatic ARDS, we further hypothesized that the protective property of A25 resuscitation may be in part mediated by its effect on the adhesive properties of PMNs and pulmonary ECs.

Albumin is a 66-kDa protein and is the most abundant protein in the human plasma exerting 75% to 80% of the normal colloid osmotic pressure (180;181). Its ability to imbibe fluid into the intravascular space makes it an excellent plasma volume expander during shock/resuscitation. One unique aspect of albumin’s biological function is its antioxidant properties exerted primarily through the presence of 17 disulphide bonds as well as single free thiol at the C-34 position (183). Indeed, much of the antioxidant activity of serum can be attributed to the presence of albumin (180;181). Albumin can act as a direct scavenger of reactive oxygen species, a so-called sacrificial antioxidant, via its thiol group (184). In addition, albumin has been shown to bind iron and prevent iron-dependent oxidizing events such as lipid peroxidation (185). In cell culture systems and *in vivo*, albumin has been shown to augment intracellular and tissue reduced glutathione stores, respectively. In cell culture, the increase was sufficient to prevent hydrogen peroxide-induced cytotoxicity and TNF-induced NF-κB translocation (187). These latter properties appear to be independent of the redox status of Cys-34 residue, since its oxidation does not alter the increase in cellular reduced glutathione. Together, these findings suggest a potential role for albumin as a resuscitation fluid with anti-inflammatory actions based on its antioxidant activity.

The studies demonstrate that resuscitation with A25 decreases LPS-induced pulmonary microvascular permeability and PMN sequestration, in concert with reduced NF-κB translocation and impaired upregulation of the chemokine CINC. Our findings support the hypothesis, as up-regulation of CD11b was diminished and a decrease in L-selectin molecules was observed on circulating PMNs in animals resuscitated with A25. The potential for PMN-EC interactions was further diminished by the ability of A25 to inhibit expression of ICAM-1 in the lung tissue. The data further suggest that the salutary effects of albumin are related to its
antioxidant capacity, in part through its thiol group. Together, these suggest a novel protective effect of albumin resuscitation in trauma victims.

MATERIALS AND METHODS

Materials

Albumin 25% and 5% from Bayer Inc. (Etobicoke, Ontario), Lipopolysaccharide (E.coli O111:B4), PMA, citrate, guanidine-isothiocyananate, bovine serum albumin (BSA), DL-Dithiothreitol (DTT), and N-ethylmaleimide (NEM) were obtained from Sigma (St Louis, MO),125I-albumin and 125I-Fibrinogen was from Frost (Kirkland, Quebec), EDTA from BDH Inc (Toronto, Ontario), and G3PDH was from Clontech (Palo Alto, CA), gelatin from Bio-Rad (Hercules, CA) and 8-Isoprostane EIA Kit from Cayman (Ann Arbor, MI). The aesthetic drugs were pentobarbital from MTC Pharmaceuticals (Cambridge, Ontario), ketamine from Rogar/STB (London, Ontario) and xylazine from Bayer Inc (Etobicoke, Ontario). Tri Reagent (phenol and guanidine thiocyanate solution for RNA/DNA isolation) were obtained from Molecular Research Center Inc.( Cincinnati, Ohio), and gelatin from Bio-Rad (Hercules, CA). Mucomyst (N-acetylcysteine NAC) was obtained from Shire Inc. (Oakville, Ontario). All chemicals used were of the highest purity available.

Solutions

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), phosphate buffered saline (PBS) were from Gibco BRL (Burlington, Ontario), RL was from Baxter Co. (Toronto, Ontario) and 10% buffered formalin from Fisher Chemical (Fair Lawn, NJ). PMNs were isolated in DMEM containing 10% fetal calf serum from Hyclone Lab Inc (Logan, UT) and penicillin/ streptomycin from Gibco BRL. Isotonic NaCl solution contained 3 mM KCl, 5mM glucose, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2 and 10 mM Hepes, pH 7.4. Plasbumin®-25% and 5% Albumin were from Bayer Inc. (Toronto, Ontario) and contained 145 mEq/L of Sodium, 0.02 M Sodium caprylate, 0.02M acetyltryptophan, buffered with sodium carbonate.

Antibodies

All antibodies were monoclonal. The following were anti-rat: anti L-selectin (CD62L) IgG1primary, Fluorescein isothiocyanate (FITC)-labelled anti CD11b both from Serotec Ltd. (Oxford, England), mouse monoclonal (IgG2a) anti ICAM-1(G-5) from Santa Cruz Biotechnology Inc. (Santa Cruz, California). The following were anti human: FITC-labelled anti CD11b and Phycoerythrin (PE)-labelled anti L-selectin both from Cedarlane Laboratories Ltd.
Secondary goat anti-mouse IgG:FITC was used from Serotec Ltd. (Oxford, England).

**Animal Model**

Animals were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. Adult male Sprague-Dawley rats weighting 300g to 350g (Charles River, St Constant, Quebec) were anaesthetized with intraperitoneal ketamine (80mg/kg) and xylazine (8mg/kg). A suprapubic catheter was placed in the bladder for urine output measurements throughout the experiment. The right carotid artery was cannulated with a 22 gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling and resuscitation. Hemorrhagic shock was initiated by blood withdrawal leading to a reduction of the MAP to 40 mm Hg within 15 min. This blood pressure was maintained by further blood withdrawal if the MAP > 45 mm Hg, and by infusion of 0.5 ml of RL if the MAP was < 35 mm Hg. Shed blood was collected into 0.1 ml citrate/ml blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood and an equivalent amount of RL or either 3 ml/kg volume of A25 or 15ml/kg of A5 over a period of two hours. Routinely, this resulted in the administration of 10cc of RL, 5cc of A5 or 1cc of A25 depending on the experimental group. The 2:1 ratio of RL to A5 was based on work by Ernest and colleagues who demonstrated that infusion of normal saline increased the extracellular fluid volume by approximately the volume infused, with a 1:3 ratio of plasma volume expansion to extracellular volume expansion (554). The 5% preparation of albumin increased the extracellular fluid volume by double the volume infused, with plasma volume and the interstitial fluid volume expanding by approximately equal amounts. Administration of either A5 or A25 using the above protocols had similar hemodynamic effects in our model as the use of crystalloid RL for resuscitation. After resuscitation, the catheter was removed, the carotid artery ligated, and the cervical incision closed. One hour later, 100 µg of lipopolysaccharide (LPS) in 200 µl of saline was administered intratracheally through a 14 gauge angiocath tracheostomy. Sham animals were instrumented but not bled and saline alone was instilled intratracheally. Animals were sacrificed by pentobarbital overdose at various time points depending on the specific study. Blood samples were collected during the experiment for serum osmolarity, 8-Isoprostane measurements and for serum albumin and antibody labelling. Plasma was separated by centrifugation and osmolarity measured in an automated osmometer Advanced Osmometer 3D3 (Two Technology Way, Norwood, MA). For histological assessment, the whole lungs were fixed in 10% neutral buffered formalin (pH 7.4), later stained...
with hematoxylin/eosin, and examined using an optical microscope or immunostained with ICAM-1 antibody.

**Bronchoalveolar Lavage (BAL)**

BAL cell counts were determined at 4 hours following LPS or saline intratracheal instillation. Immediately after sacrifice, the lungs were perfused via the tracheostomy cannula with cold PBS/0.1 mM EDTA in 10 ml aliquots and gently withdrawn to a total of 40 ml (555). For cell counts and differential, BAL fluid was centrifuged at 300g for 10 min. After discarding the supernatant, the pelleted cells were resuspended in serum free DMEM. Total cell counts were done on a grid hemocytometer and the differential cell counts on a cytospin-prepared slide stained with Wright-Giemsa. 500 cells were counted in cross-section per sample and the number of neutrophils was calculated as follows: number of neutrophils = total cell count X % of neutrophils in BAL fluid sample.

**Transpulmonary protein flux**

For the measurement of the lung transpulmonary albumin flux, the rats were injected with 1 mCi of $^{125}$I-albumin into the tail vein immediately following intratracheal LPS or saline (555). Six hours later, 1 ml of blood collected by cardiac puncture and 1 ml of BAL fluid were aliquoted into tubes for analysis by a gamma spectrometer. The macromolecule leak was corrected to blood cpm and expressed as the transpulmonary albumin leak index as follows:

$$\text{Transpulmonary albumin leak index} = \frac{\text{cpm/ml in BAL fluid}}{\text{blood cpm/ml}}$$

Specific activity of albumin ($\mu$g/counts per minute) was used to calculate the albumin concentration in the BAL fluid. Data were normalized to the albumin specific activity in serum. We also evaluated the leakage of another macromolecule, fibrinogen, to rule out the possibility that administration of large doses of albumin might, in some way, interfere with the permeability determinations for albumin. As for albumin, $^{125}$I-fibrinogen was given via the tail vein immediately after intratracheal LPS and fibrinogen flux was calculated as per albumin with normalization for specific activity of fibrinogen in the blood.

**Neutrophil isolation**

Human neutrophils were isolated from volunteers by dextran sedimentation followed by Ficoll-Paque gradient centrifugation. Red blood cells were removed by NH$_4$Cl lysis. Neutrophils were counted and resuspended in DMEM supplemented with 10% FCS and penicillin/streptomycin at 10$^6$ cells/ml. Neutrophil viability exceeded 97% as assessed by trypan blue exclusion. Human serum albumin was added to achieve various final concentrations.
**Northern Blot Analysis**

Total RNA from whole lung tissue was obtained using the TRI Reagent® (Molecular Research Center Inc.) phenol and guanidine thiocyanate method (556). Briefly, lungs were harvested and homogenized in an appropriate amount of TRI Reagent. RNA was denatured and electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization was carried out using a [³²P]dATP end-labelled 30-base oligonucleotide probe for cytokine induced neutrophil chemoattractant with the sequence: 5'-GCGGCATCACCTTCAAACTCTGTTCTTCT-3’, which is complimentary to nucleotides 134-164 of CINC cDNA provided by Dr. Timothy S. Blackwell (Vanderbilt University Scholl of Medicine, Nashville, TN, (45)). Alternatively, hybridization was carried out using ICAM-1 cDNA. Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens. To control for loading, blots were then stripped and reprobed for G3PDH, a ubiquitously expressed housekeeping gene (557). Expression of mRNA was quantified using a phosphoimager and accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.), and was normalized to the G3PDH signal or to the 18smRNA signal.

**Nuclear protein extraction**

Nuclear protein extracts were prepared from lung tissue or alveolar macrophage by the method of Deryckere and Gannon (558). Aliquots of 200 - 500 mg of frozen tissue ground to powder with a mortar in liquid nitrogen. The thawed powder or 10x10⁷ cells were homogenized in a Dounce tissue homogenizer with 4 ml of solution A (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, and 0.5 mM PMSF). The cells were lysed with five strokes of the pestle. After transfer to a 15-ml tube, debris was pelleted by briefly centrifuging at 2000 rpm for 30 sec. The supernatant was transferred to 50-ml Corex tubes, incubated on ice for 5 min, and centrifuged for 10 min at 5000 rpm. The supernatant that contains cytoplasm was saved for further use. Nuclear pellets were then resuspended in 300 µl of solution B (25% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 5 µg/ml pepstatin, 5 µg/ml leupeptin, and 5 µg/ml aprotinin) and incubated on ice for 20 min. The mixture was transferred to microcentrifuge tubes, and nuclei were pelleted by centrifugation at 14,000 rpm for 1 min. Supernatants containing nuclear proteins were aliquoted in small fractions, frozen in liquid nitrogen and stored at -70°C. Protein quantitation was performed using the BIO-RAD protein assay dye reagent (BIO-RAD, Hercules, CA).
Electrophoretic mobility shift assay (EMSA)

The probe for EMSA was a 30-bp double-stranded construct (5'-CCT GTG CTC CGG GAA TTT CCC TGG CCT GGA-3') corresponding to a sequence (-72 to -42) in the CINC proximal promoter region containing the NF-κB motif (underlined). End labelling was performed by T4 kinase in the presence of [32P]ATP. Labelled oligonucleotides were purified on a Sephadex G-50 M column (Pharmacia Biotech, Inc., Piscataway, NJ).

An aliquot of 5 μg of nuclear protein was incubated with the labelled double-stranded probe (~50,000 cpm) in the presence of 5 μg of non-specific blocker, poly (dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM DTT) at 25°C for 20 min. Specific competition was performed by adding 100 ng of unlabelled double-stranded CINC oligonucleotide, while for non-specific competition, 100 ng of unlabelled double-stranded mutant CINC oligonucleotide (5'-CCT GTG CTC CAA TTT CCC TGG CCT G GA-3') that does not bind NF-κB was added. The mixture was separated by electrophoresis on a 5% polyacrylamide gel in 1 x Tris glycine EDTA buffer (559). Gels were vacuum dried and subjected to autoradiography and phosphoimager analysis.

8-Isoprostane Assay

8-Isoprostane levels were determined using an eicosanoid immunoassay (EIA) with acetylcholinesterase kit (Cayman Chemical, Ann Arbor, MI) according to a modification of the methods of Bligh and Dyer (560) and Morrow et al (561). In general, 500-μl of plasma sample was vortexed with 1 ml of 100% ethanol, allowed to stand at 4°C for 5 min, and then centrifuged at 1,500 g for 10 min. The supernatant containing 8-isoprostanes was then decanted, an equal volume of 15% KOH was added, and samples were incubated at 40°C for 1 h. After 1 h, samples were diluted to 5 ml with double-distilled water and the pH was lowered below 4 with HCl. Samples were then passed through preconditioned SPE-C_{18} reverse-phase cartridges, followed by 5 ml of pure water and then 5 ml of HPLC-grade hexane. 8-isoprostanes were eluted with 5 ml of ethyl acetate containing 1% methanol. The ethyl acetate was then evaporated with nitrogen and 1 ml of EIA resuspension buffer was added, and samples were vortexed for 30s and sonicated for 5s. The developed plate was read by a microplate reader (Diamed, Mississauga, ON, Canada) at 405 nm. The concentration was calculated by computer using Cayman EIA software. Rat plasma samples, standardized for protein concentration, were assayed in duplicate in a blinded fashion (n = 4 per group).
Modification of Albumin’s thiol group

The thiol group was modified in a similar procedure to that described by Cha and Kim (562). A25 was incubated with DL-Dithiothreitol (DTT, 2mM) for 20 min at 30°C, then N-ethylmaleimide (NEM, 10mM) was added and the mixture was incubated for further 2 h. Before A25 was used, excess agent was removed by 48h dialysis. Protein concentration was measured and adjusted to A25 concentration with Phosphate Buffered Saline.

Detection of surface markers on rat PMN in whole blood

For CD11b detection, 100 μl of whole rat blood was incubated with FITC-labelled anti-CD11b antibody at 1/100 dilution at room temperature for 20 minutes. Erythrocytes were lysed with lysing buffer (0.84% NH4Cl), the remaining cells washed twice with DMEM and analyzed on the FACScan as above. For measurement of L-selectin, PMNs were isolated from whole blood and incubated with anti L-selectin at a 1/100 dilution for 20 min at 4°C followed by a FITC-labelled secondary antibody. PMNs were then analysed on the FACScan as above.

Measurement of human PMN surface CD11b and L-selectin

Isolated human PMNs were incubated in isotonic medium with or without 20, 40 and 60 mg/ml Albumin for up to 4 hours. Subsequently, PMNs were resuspended in isotonic medium and incubated with LPS (1μg/ml) for 1 hour and then FITC-labelled anti-L-selectin or PE labelled anti-CD11b antibody at a 1/100 dilution for 20 min at 4°C. PMNs were then analysed on the FACScan (Becton-Dickinson, Palo Alto, CA) using FL1 or FL2 detectors (488 nm excitation wavelengths respectively). Typically, 5,000 cells were analysed per condition and the results expressed as mean channel fluorescence (MCF) percent of control. The preshock value was considered equal to 100%.

ICAM-1 Immunohistochemistry

At four hours after LPS administration, animals were sacrificed and lungs inflated with 10% buffered formalin and sections obtained for immunohistochemical analysis. Tissues were stained with anti-rat ICAM-1 mAb (G-5), followed by a biotinylated goat anti-mouse IgG (Dimension Laboratories, Mississauga, ON). After rinsing, a streptavidin-horseradish peroxidase conjugate was added. The peroxidase reaction was developed by immersion in a freshly prepared solution of 0.02% 3,3’-diaminobenzidine and 0.005% H2O2 in 0.05 M Tris buffer, pH 7.6, followed by hematoxylin counterstaining. Appropriate negative controls were performed using secondary Ab alone.
Statistical Analysis

The data are presented as mean ± standard error of n determinations as indicated in the Figure Legends. Data were analyzed by one-way analysis of variance; post hoc testing was performed using the Bonferroni modification of the t-test. When individual studies are demonstrated, these are representative of at least three independent studies.

RESULTS

Effect on various resuscitation fluids on animal survival, serum osmolarity, albumin levels, and animal hemodynamics

Animals resuscitated with RL, A5 and A25 had comparable survival rates, over 90%, with most deaths occurring due to tracheal cannula obstruction during the 4-6 hours of incubation with endotracheal LPS. By the end of the shock period, there was a significant rise in serum osmolarity. In animals resuscitated with RL, osmolarity returned to baseline levels by 15 minutes post resuscitation. By contrast, resuscitation with A25 resulted in a persistent elevation of serum osmolarity throughout the experimental period. A5 did not differ from RL (Fig. 16A). As shown in Figure 16B, the serum albumin level in animals resuscitated with A25 was significantly increased compared to both A5 and RL and remained so over the entire study period. As noted in the Methods section, animals were resuscitated with shed blood plus 10 ml of RL, 5ml of A5 or 1 ml of A25, depending on their group. In order to ensure adequate resuscitation by the various regimens, we measured mean arterial pressure at end resuscitation and monitored urine output. As illustrated in Figure 17, there was no difference among treatment groups with respect to mean arterial pressure (Panel A) or urine output (Panel B).

Transpulmonary protein flux following various resuscitation strategies

Figure 18A shows transpulmonary albumin flux in each experimental group. As previously reported, intratracheal LPS following antecedent shock/resuscitation with RL caused augmented transpulmonary albumin flux compared to sham, shock or LPS alone, consistent with the concept that shock/resuscitation resulted in priming for LPS-induced lung injury (36). By contrast, animals resuscitated with A25 exhibited reduced albumin flux to levels comparable to LPS alone. Resuscitation with A5 did not differ from the RL group. We considered the possibility that resuscitation with a large dose of albumin might have diluted the labelled albumin and artificially lowered the measurement of albumin flux. This appeared to be unlikely for two reasons. First, albumin flux was normalized for blood albumin counts. Second, plasma albumin levels at the time of flux measurement (t=6 hours after LPS administration) had
returned close to that observed in RL-resuscitated animals (Fig. 16B). Nevertheless, we performed further studies examining transpulmonary flux of labelled fibrinogen to rule out an artefact of the albumin resuscitation. As illustrated in Figure 18B, A25 significantly reduced the transpulmonary fibrinogen flux in comparison to RL. By contrast, administration of equivalent quantity of albumin in the A5 formulation was less effective in preventing the transpulmonary flux, causing a small but insignificant reduction compared to RL resuscitation.

**Effect of 25% Albumin on lung neutrophil sequestration**

Since lung neutrophil sequestration is one of the hallmarks of acute lung injury and plays a central role in its pathogenesis, we examined the effect of albumin on both total cell and neutrophil counts in the BAL (Fig. 19). At end of resuscitation, using any of the resuscitation regimens, there were few neutrophils in the BAL and there was no difference between groups (data not shown). LPS treatment alone in sham animals caused a modest but significant increase in BAL neutrophils. Shock/resuscitation followed by intratracheal LPS caused a further rise in lung neutrophil counts, consistent with our previous report (36). However, A25 totally prevented the augmentation in lung alveolar neutrophil counts seen following shock/LPS using the RL resuscitation regimen, with neutrophil counts shown to be comparable to that observed for LPS alone. Consistent with its failure to prevent an increase in transpulmonary protein flux, A5 resuscitation after shock did not significantly reduce LPS-induced lung neutrophil sequestration compared to RL resuscitation. Lung histopathology revealed a similar protective effect by A25 (Fig. 20). Resuscitation with RL followed by intratracheal LPS caused a marked neutrophilic infiltrate, alveolar hemorrhage, and thickening of the lung interstitium (Fig. 20B). These alterations were reversed when animals were resuscitated with A25 (Fig. 20C), but not with A5 (Fig. 20D).

**Chemokine expression following resuscitation fluids**

Our previous studies with this model showed that Cytokine-Induced Neutrophil Chemoattractant (CINC) was the critical chemokine causing augmented alveolar neutrophil sequestration following shock plus LPS (36). Since A25 lessened neutrophil accumulation in the lungs, we hypothesized that this effect may be due to altered CINC expression. Figure 21A shows a representative study of the levels of CINC mRNA in lungs recovered from animals after various resuscitation strategies. A25 resuscitation, but not A5, significantly reduced levels of CINC mRNA compared to those observed following shock/LPS in animals resuscitated with RL. Determination of G3PDH levels showed comparable loading among lanes. Figure 21B represents an average of 6 independent studies where values are normalized for loading with
G3PDH. The transcription factor NF-κB is known to be important in CINC gene regulation. We therefore studied NF-κB translocation in animals following shock and subjected to different resuscitation strategies (Fig. 22). Two protocols were used to derive samples for these studies. In the first, whole lungs were recovered four hours after intratracheal LPS administration. Treatment of animals with LPS following shock resuscitated with RL markedly increased NF-κB compared to sham, shock, or LPS alone (Fig. 22A). Resuscitation with A25, however, significantly reduced translocation to levels observed with LPS alone. The A5 preparation was again comparable to that observed for RL. In the second protocol, we recovered alveolar macrophages by BAL at the end of resuscitation and cultured them in vitro with or without LPS (0.1 µg/ml) for two hours before subjecting them to analysis. This approach permits study of alveolar macrophages without contamination by other cells that may express CINC. Our previous studies have shown that numbers and percent macrophages did not differ whether recovered from sham or from shock/resuscitated animals. As for whole lung specimens, EMSA in these cells showed augmented NF-κB translocation in shock/RL/LPS compared to other groups (Fig. 22B). Resuscitation with A25, but not A5, prevented this increase. Considered together, these studies suggest that resuscitation with A25 is able to prevent the increased responsiveness to LPS observed after shock/resuscitation by altering the cellular signalling pathways leading to NF-κB translocation and chemokine gene induction.

**Effect of 25% Albumin on LPS-Induced Up-regulation of CD11b and L-selectin shedding**

One of the interactions between PMNs and EC that is important for neutrophil adhesion occurs through the β2 integrin CD11b expressed on PMNs. To evaluate whether A25 may exert its previously shown protective effect on lung injury through modulating CD11b expression, blood PMNs from sham animals and those resuscitated with RL, A5 or A25 were collected at end resuscitation and at four hours after endotracheal LPS administration. Figure 23 demonstrates CD11b expression in vivo. Resuscitation with A25 significantly attenuated the increase in PMN CD11b expression observed in RL and A5 resuscitated animals at end resuscitation and at 4h post LPS (Fig. 23). Surface adhesion molecule L-selectin was also measured on circulating PMNs from treated animals. While PMN L-selectin levels remained relatively stable in RL treated animals at 4hrs, A25 resuscitation resulted in a significant decrease in surface L-selectin expression at end of resuscitation and at 4h post LPS treatment (Fig. 24).
Effect of Albumin on human PMN’s CD11b expression *ex vivo*

Having shown that A25 resuscitation affects adhesion molecule behaviour on PMNs, we speculated that the increase in serum albumin concentration might be responsible for this effect. Figure 25A demonstrates an increase in Albumin plasma concentration reaching a peak at 5min. after resuscitation and gradually going down to just above pre shock levels at 4 hours post resuscitation. In contrast, RL resuscitation resulted in lower levels of serum albumin than those observed in sham animals. A5 resuscitation maintained albumin concentration at 40g/L. To determine whether CD11b expression on PMNs follows a dose response effect to albumin, human PMN CD11b expression was studied *in vitro* using increasing concentrations of albumin. As shown in Figure 25B, Cd11b expression increased with LPS stimulation above control or no LPS levels at 20 and 40g/L concentrations of albumin, however this increase was prevented by antecedent 60g/L albumin incubation.

**Effect of 25% Albumin on lung ICAM-1 expression**

Having shown that adhesion molecules at the level of the PMN are modulated by A25 the endothelial cell ligand to CD11b, ICAM-1, was examined in lung tissue of Sham, RL, A5 and A25 resuscitated animals. At the level of gene expression, ICAM-1 mRNA was evaluated by Northern blot analysis. As seen in Figure 26, there is little constitutive expression of ICAM–1 in sham animals, and shock alone does not induce this molecule’s expression in our model. LPS alone caused a marked up-regulation of ICAM-1, however most robust up-regulation was observed in the RL group treated with endotracheal LPS for four hours (Fig. 26B). ICAM-1 protein expression was then visualised using immunohistochemistry in whole lung tissue (Fig. 27). Consistent with the upregulation at the mRNA level, animals resuscitated with RL and treated with LPS 4h demonstrated a marked increase in ICAM-1 staining along the alveolar capillary membrane as compared to shams. By contrast, A25 resuscitation diminished ICAM-1 protein expression by shock/resuscitation plus LPS, reducing it to near baseline levels.

**Role of albumin as an antioxidant**

Antioxidants have been shown to inhibit NF-κB translocation *in vivo* and *in vitro*. To discern the potential of albumin as an antioxidant in the present model, we measured 8-isoprostanate levels as a measure of oxidant injury. Figure 28 shows a representative study of plasma 8-isoprostanate measured at various times during shock/resuscitation using different fluid resuscitation strategies. Rat plasma oxidant activity rises during the resuscitation phase and peaks at one hour after starting resuscitation. Activity then subsides over the ensuing hour. Resuscitation with A25 suppresses evidence of oxidant activity in the plasma over the entire
period of observation. Its effects over the first one hour were mimicked by use of an RL-based resuscitation regimen containing the antioxidant N-acetylcysteine (NAC). By the second post resuscitation hour, it appeared that the antioxidant effects of NAC had waned. A5 resuscitation demonstrated an intermediate effect between A25 and RL resuscitation. While it suppressed the early (5 min) rise in 8-isoprostane levels, the subsequent two hours demonstrated loss of antioxidant capacity.

To establish a relationship between the antioxidant capacity of A25 and its protective effects *in vivo*, we chemically modified albumin by oxidizing its sulfhydryl groups and its unique free thiol group. This modification is known to significantly reduce the antioxidant activity of the albumin molecule (185). N-ethylmaleimide (NEM) modified A25 was an effective resuscitation fluid (data not shown) and caused a significant reduction in lung neutrophilia in shock/LPS animals compared to animals resuscitated with RL. However, the protective effect was only ~55% of that observed with native A25 (Fig. 29). These data suggest that the antioxidant activity attributable to thiol groups in albumin contributed significantly to the protective effect of A25 resuscitation on lung injury in this model.

**DISCUSSION**

The inclusion of A25 in the resuscitation regimen following hemorrhagic shock exerted a beneficial effect on the development of lung injury in this model as manifested by the prevention of inflammatory changes in the lung and a reduction in transpulmonary protein leak. This is the first experimental study showing that albumin administration is able to exert anti-inflammatory effects *in vivo* and thereby prevent the onset of organ injury. The findings also complement and suggest a potential mechanism for the ability of albumin to reduce infarct volume in a model of focal transient cerebral ischemia in the rat (337). Several lines of evidence suggest that the salutary effects of albumin resuscitation might be related to its antioxidant properties. First, the ability of a given resuscitation fluid to blunt plasma oxidant activity as measured by the generation of 8-isoprostanes during resuscitation correlated with protection from lung injury in this model. A5 nor RL neither exerted antioxidant activity during resuscitation nor provided protection from lung injury following LPS. By contrast, both protective fluid resuscitation regimens i.e. A25 and NAC (36) markedly blunted the rise in plasma 8-isoprostane levels. Second, A25 resuscitation prevented priming of alveolar macrophages for LPS-responsiveness. Several groups, including our own, have demonstrated the ability of oxidant stress to prime for cell responsiveness to a second inflammatory stimulus.
such as LPS (36;44;137). This oxidant-induced priming was, in part, related to the increase in NF-κB translocation and has been shown to be prevented by antioxidants such as NAC and antioxidant vitamin therapy. In the present studies, resuscitation regimens with antioxidant capacity, namely A25, inhibited LPS-induced NF-κB translocation, while A5 and RL did not. Finally, oxidation of the thiol groups in A25 using NEM caused partial reversal of the protective effect of A25. This would suggest that part of the protection is due to the thiol-based antioxidant activity of the molecule. In this regard, studies by Bourdon and Blache suggest that NEM-modification of albumin exerts only ~55% reduction in antioxidant activity (185). The remaining component of the protective effect exerted by A25 may have been through residual antioxidant activity, such as its ability to augment intracellular reduced glutathione levels or prevent iron-catalyzed reactions through iron binding. We favour this possibility due to the correlation between antioxidant activity and degree of protection. Specifically, the residual protection against lung neutrophilia associated with the use of NEM-modified albumin in vivo correlates well with the residual antioxidant activity after NEM-treatment, while native albumin caused near complete inhibition of lung neutrophilia and injury, coincident with complete suppression of ischemia/reperfusion-induced oxidant stress.

The A25 preparation, but not the A5 preparation, was able to exert anti-inflammatory effects as well as protection against lung injury, despite the fact that equal quantities of albumin were administered in each group. Based on the 8-isoprostane data, the most likely explanation for this is the fact that rapid administration of a small volume of high concentration albumin early in the resuscitation phase was a more effective antioxidant than the more prolonged resuscitation with a larger volume of A5. This suggests that a threshold concentration of albumin was required to exert the antioxidant effect or that administration of the entire dose early in the resuscitation phase was critical. An alternate possibility is that the hyperosmolarity associated with use of A25 might have also contributed to the anti-inflammatory effects. Using the present “two-hit” model, our laboratory has previously demonstrated that resuscitation with 7.5% saline solution prevented lung neutrophil sequestration and transpulmonary albumin flux compared to isotonic RL resuscitation (173). In addition, several groups, including our own, have reported the immunomodulatory effects of hyperosmolarity on neutrophil and macrophage activation in vitro (69;563;564). However, most of these effects occurred at osmolarity levels exceeding 350mOsM. A25 was able to achieve serum osmolarity in the range of 330-340 mOsM, a level that may have been insufficient to exert anti-inflammatory effects by virtue of hyperosmolarity alone. Further, resuscitation with hypertonic saline in vivo had little effect on
alveolar macrophage function in terms of their responsiveness to LPS. This contrasts to the marked inhibitory effects of A25 on macrophage activation in the present studies (173). Finally, Rhee and colleagues have suggested the possibility that the apparent protective effects of alternate resuscitation fluids are due more to the proinflammatory effects of lactate resuscitation than the anti-inflammatory effects of other solutions. They recently reported that incubation of neutrophils with either RL or A5 induced greater neutrophil CD18 adhesion molecule expression and oxidative burst than A25 (153). The protective effect of the albumin did not appear to be due to the osmolarity of the incubation solution.

The mechanism of A25’s modulation of the adhesion molecule expression warrants further investigation. Our data are consistent with a conclusion that the major effect of A25 was primarily due to its antioxidant properties. A25, in addition to having osmotic properties, is an antioxidant that may influence not only the extracellular but also the intracellular redox environments. It is capable of modulating redox sensitive MAPK p38 activation and thus preventing LPS induced adhesion molecule surface changes. The intracellular redox state plays a critical role in altering enzyme activity, protein phosphorylation, and most signalling pathways. One of the enzymes sensitive to the ROS is the mitogen-activated protein kinases (MAP kinases), which can be activated by hydrogen peroxide and lead to intracellular signalling that modulates gene expression (565). We have demonstrated previously that Cd11b and L-selectin regulation by LPS is in part mediated by MAPK p38 activation and that hypertonicity prevented this effect through a cell volume dependent mechanism (68;69). It is also possible that the down regulation of PMN CD11b surface numbers with hyperosmolar concentrations of albumin in vivo and ex vivo alone is not entirely responsible for reduced neutrophil sequestration, but rather the activation state of the β2 Integrins dependent on the redox state, may be affected (60). Future studies examining the effect of A25 on the priming and avidity of these integrin molecules need to be undertaken.

In the cell, thiol groups are buffered against oxidation by a highly reduced environment and only accessible thiols, like Cys-34 in albumin, with high thiol-disulphide oxidation potential, are redox sensitive (566). In critically ill patients with septic shock the free thiol content and hence the antioxidant activity of plasma is markedly reduced (567). The free thiol content of infused albumin may be important in scavenging the ROS and preventing the PMN activation that mediates increased adhesive properties and organ failure in those patients. In this regard, we have performed preliminary studies (data not shown), wherein the albumin thiol group was reduced using dithiothreitol (DTT) and subsequently the sulphydryl group was
blocked using N-ethylmaleimide (NEM) to prevent its oxidization, effectively diminishing albumin’s antioxidant activity. This modified albumin, even at 60 g/L, was not able to ameliorate the LPS induced CD11b upregulation in *in vitro* stimulated PMNs, pointing to the importance of the redox state of albumin’s free cysteine group. N-acetyl cysteine (NAC), a potent antioxidant, has been similarly demonstrated to down regulate PMN CD11b expression *in vivo*, in shock resuscitated animals exposed to LPS (304).

Although studies presented point to the major effect of A25 as primarily due to its antioxidant properties, the hyperosmolar properties of albumin should not be discounted in modulating PMN activation and adhesion. A protective effect secondary to hyperosmolarity would be consistent with the reported ability of hypertonic saline to exert protection through hyperosmolarity. PMN-endothelial cell interaction is an early and rate limiting step in transmigration of PMNs into tissues. Several studies, have shown that β2 integrins including CD11b/CD18 are up-regulated and activated during inflammation (68;551). The early contact with the endothelium followed by the slow rolling of neutrophils is mediated by L-selectin (CD62L), which is usually shed from the cell surface once a firmer β2 integrin mediated adhesion takes place. Our laboratory has shown that HTS prevented the LPS-induced up-regulation of CD11b and caused adhesion-independent shedding of L-selectin *in vivo* and *in vitro*. In our “two hit” rodent model of lung injury, similar to HTS, A25 use for resuscitation was previously demonstrated to have a beneficial effect in attenuating the lung injury, as evidenced by reduced pulmonary microvascular permeability and PMN sequestration. In the present studies, we have investigated whether A25 exerts its protective effect through modulating PMN-EC interactions by altering adhesion molecule. Both *in vivo* and *in vitro* data show that A25 albumin significantly reduced LPS induced CD11b upregulation and induced selectin shedding on circulating PMNs.

Since neutrophil activation and cytotoxicity in organ tissues occurs subsequent to their adhesion to endothelium and interaction with extra cellular matrix proteins, it follows that modulating those interactions may be a mechanism of A25’s salutary effect on lung injury. ICAM-1 is constitutively expressed at low levels by endothelial cells but is rapidly up-regulated during inflammation or by direct application of oxidants, resulting in increased leukocyte-endothelial cell adhesion (568;569). Therefore, we examined the effect of different resuscitation fluids on endothelial cell activation by studying ICAM-1 expression in our *in vivo* model. Resuscitation with A25 prevented ICAM-1 up-regulation in shock plus LPS stimulated animals both at the mRNA and protein levels. ICAM-1 is known to be regulated largely at the mRNA
level, with surface expression occurring within 4h after stimulation, which is consistent with our findings (570;571). Like the PMN alterations in adhesion molecules, albumin may conceivably work by either effects of hyperosmolarity or antioxidant capability. The promoter region of ICAM-1 contains a binding motif for NF-κB. There are two steps in NF-κB signalling that can be affected by thiol antioxidants like albumin: first, at the time of NF-κB release from its inhibitor IκB and second at the time of NF-κB binding to DNA. Intracellular thiol oxidation has been previously shown to prevent NFκB-DNA interaction, which may lead to a decrease in gene activation. In a recent report albumin was shown to possess intracellular antioxidant redox signalling effects through preserving the intracellular Glutathione (GSH) levels (187). The increased intracellular GSH levels protected cells from hydrogen peroxide mediated cytotoxicity and decreased TNF-α-mediated NF-κB activation (187). Other thiol antioxidants like alpha-lipoic acid and alpha-lipoate have been previously shown to decrease ICAM-1 expression in activated cell lines through inhibiting NF-κB activation (572). Thus, the antioxidant effect of A25 may play a role in attenuating NF-κB induced ICAM-1 mRNA transcription. Independent of NF-κB activation and DNA-binding, A25 induced intracellular redox changes may alter mRNA stability and this way decrease ICAM-1 expression. NF--κB induced gene expression has also been shown to be sensitive to calcium oscillations (573), and changes in albumin concentrations are known to affect calcium levels, hence ICAM-1 mRNA may be calcium dependent. Both of these possible mechanisms require further investigation in future studies. Studies by Rizoli et al demonstrated an effect of cell shrinkage on LPS induced p38 MAPK activation and hence an inhibition of upregulation of CD11 by LPS. Hyperosmolar albumin may be similarly operative (68).

The decrease in pulmonary endothelium ICAM-1 immunostaining, as observed with A25 resuscitation, can result from decreased protein expression or from loss of membrane bound protein. Circulating ICAM-1 has been previously demonstrated in various inflammatory conditions, and has been shown to bind competitively with CD11b/CD18 complex thus inhibiting PMN adhesion and transmigration. Whether A25 resuscitation induces ICAM-1 shedding and thus abrogates lung injury is yet to be addressed.

Albumin is commonly used in the fluid management of acutely ill patients. Recent meta-analyses evaluating its use for a number of indications have not clearly shown a mortality advantage related to the use of albumin (189;191). In fact, one suggested excess mortality related to albumin use (189). Recent randomized controlled trials have demonstrated that some
benefit may be realized from the ability of albumin infusion to augment plasma oncotic pressure and promote diuresis (548). In one study, combined therapy with A25 and furosemide of patients with ARDS, elevated serum albumin levels, increased PaO2/FiO2 and improved long term hemodynamic stability with a trend towards shorter mechanical ventilation and ICU stay (548). One might speculate that properties other than albumin’s ability to raise plasma oncotic levels may have also contributed to this effect. For example, albumin has been shown to reduce microvascular permeability (574-576) as well as inhibit endothelial cell apoptosis (577;578). Both would tend to favour a net mobilization of fluid from the extravascular space back into the vasculature. Since oxidant stress is one of the factors implicated in both these processes, the antioxidant activity of albumin may have contributed to these effects. Consistent with this potential mechanism, Quinlan and colleagues reported that 20% albumin infusion was able to increase plasma thiol levels in patients with sepsis syndrome (186).

The present study examined the mechanisms by which shock resuscitation with A25, a potent antioxidant, attenuates lung injury in the “two-hit” rodent model of ARDS. Together with the experimental data presented in this chapter, studies to date suggest that the antioxidant capacity of infused albumin may have therapeutic benefit in the prevention and management of lung injury in the critically ill patient. In addition, albumin as a resuscitation fluid may provide immunomodulatory advantage over other resuscitation strategies in preventing PMN-mediated disease process ensuing from ischemia reperfusion injury. Well-designed randomized studies in select patient populations examining endpoints relevant to systemic inflammation and organ dysfunction are required to evaluate this further.
Figure 16. Serum Osmolarity and Calcium Concentrations.

(A) Serum osmolarity following shock/resuscitation and LPS. Following hemorrhagic shock, rats were resuscitated with either RL or A25. Serum osmolarity was measured by a freezing point osmometer. (B) Serum albumin concentration at various time points following shock/resuscitation and LPS. Following hemorrhagic shock, rats were resuscitated with one of RL, A5 or A25 and plasma albumin concentration was determined at indicated time points. Data are expressed as mean ± SEM of five animals per treatment group. Statistical differences were detected using a one way ANOVA and post hoc Bonferoni multiple comparisons testing at each time point. A. A25 vs. RL and A5 *, p< 0.05. B. A25 vs. RL and A5 *, p< 0.05
Figure 17. Mean arterial blood pressure and urine output following shock/resuscitation.

(A) Mean Arterial Blood Pressure following shock/resuscitation with different regimens. Following hemorrhagic shock, rats were resuscitated with one of RL, A5 or A25. MAP was measured throughout the experiment and is shown here at the end of the resuscitation phase. (B) Urine output following shock/resuscitation with different regimens. Following hemorrhagic shock, rats were resuscitated with one of RL, A5 or A25. Urine output was monitored from the beginning of the hypotensive phase. Data are expressed as mean ± SEM of five animals per treatment group. There were no significant differences among animal groups for mean arterial pressure or urine output.
Figure 18. Transpulmonary Protein Flux.

(A) Transpulmonary albumin leak index. (B) Transpulmonary fibrinogen leak index. Following shock/resuscitation and intratracheal LPS, $^{125}$I-albumin or $^{125}$I-fibrinogen was administered intravenously and 4 to 6hrs later BAL fluid gamma counts were measured as described in Materials and Methods. Data are expressed as mean ± SEM of at least three animals per treatment group. (A) RL/LPS vs. all other groups *, $p<0.05$. (B) RL/LPS and A5/LPS vs. all other groups*, $p<0.05$
Figure 19. Bronchoalveolar lavage fluid neutrophil count.

Following shock/resuscitation with different regimens, animals were treated with intratracheal LPS or saline and four hours later, BAL was performed. Cells in the BAL fluid were analysed and counted as described in Materials and Methods. Data are expressed as mean ± SEM of at least five animals per treatment group. RL/LPS and A5/LPS vs. all other groups *, p< 0.05.
Figure 20. Representative photomicrographs of lung histology after resuscitation with various fluid regimens.

(A) Sham (B) Shock with RL resuscitation followed by LPS (C) Shock with A25 resuscitation followed by LPS (D) Shock with A5 resuscitation followed by LPS. A representative picture of three independent studies is shown. Hematoxylin-eosin stain, magnification, X 40.
Figure 21. CINC mRNA expression in whole lung tissue following different resuscitation regimens.

Following shock/resuscitation with different regimens, animals were treated with intratracheal LPS or saline and four hours later lungs were recovered and processed for Northern blot analysis as described in Materials and Methods. (A) Representative Northern blot analysis of CINC mRNA levels with corresponding G3PDH mRNA bands is shown. (B) Densitometric analysis of CINC mRNA, normalized for G3PDH mRNA. Data are expressed as mean ± SEM of at least five animals per treatment group from five Northern blot results. A25 vs. LPS, RL/LPS and A5/LPS. *, p< 0.05.
Figure 22. Shock/LPS-induced NF-κB nuclear translocation in macrophages.

Representative Electrophoretic mobility shift assay (EMSA) autoradiograph, showing shock/LPS-induced NF-κB nuclear translocation in (A) whole lung tissue of animals resuscitated with different regimens and treated for 4 hours with intratracheal LPS, (B) Alveolar macrophages treated ex vivo with LPS (0.1 μg/ml) for 2hr. The probe for EMSA was a [$^{32}$P]ATP end-labelled 30-bp double-strand construct corresponding to a sequence in the CINC-proximal promoter region containing the NF-κB motif. Cold competition (cold; lane7, 9) and non-specific competition (NS; lane 10) are also shown. A representative of three independent experiments is shown.
Figure 23. Effect of Albumin resuscitation on CD11b expression on rat neutrophils.

Animals underwent hemorrhagic shock and were resuscitated with RL, A5 or A25 over two hours. Blood samples were obtained prior to the controlled hemorrhage at the end of the shock phase and at various time points after initiation of resuscitation. Whole blood was incubated with FITC-labelled anti-CD11b antibody, and after erythrocyte lyses, was analysed by flow cytometry. Data are expressed as mean ± SEM of four to ten animals per group. *, $p<0.05$ vs. preshock and vs. A25 at the same time point. The control value for each animal was normalised to 100, and subsequent readings were compared to this.
Figure 24. Effect of Albumin resuscitation on L-selectin expression on rat neutrophils.

Animals underwent hemorrhagic shock and were resuscitated with RL, A5 or A25 over two hours. Blood samples were obtained prior to the controlled hemorrhage at the end of the shock phase and at various time points after initiation of resuscitation. Whole blood was incubated with FITC-labelled anti-L-selectin antibody, and after erythrocyte lyses, was analysed by flow cytometry. Data are expressed as mean ± SEM of four animals per group.

*, p< 0.05 vs. preshock and vs. RL at the same time point. The control value for each animal was normalised to 100, and subsequent readings were compared to this.
Figure 25. The effect of 25% on PMN CD11b expression.

A, Effect of 25% Albumin resuscitation on the serum albumin concentration *in vivo* and B, Effect of Albumin on *in vitro* human PMN CD11b expression. For *in vivo* study animals underwent hemorrhagic shock and were resuscitated with RL or A25 over two hours. Blood samples were obtained prior to the controlled hemorrhage at the end of the shock phase and at various time points after initiation of resuscitation. Data are expressed as mean ± SEM of four to ten animals per group. *, p< 0.05 vs. sham and RL at the same time point. For *in vitro* studies, cells were incubated in various concentrations of albumin for 4h then LPS (1μg/ml) was added for 1h to neutrophils in DMEM media. CD11b expression was assessed by flow cytometry. Data are expressed as mean channel fluorescence ± SEM of three studies per group. *, p< 0.05 vs. LPS treated cells.
Figure 26. Effect of Albumin resuscitation on ICAM-1 expression in rat lungs.

(A) Total lung RNA was obtained at 4 hours after endotracheal LPS administration, and probed for ICAM-1 mRNA, and detected by autoradiography. Equivalent RNA loading was confirmed both by 18S rRNA and ethidium bromide staining of the original gel. A representative Northern blot of three animals per group is displayed. (B) densitometric analysis of ICAM-1 mRNA normalised for 18s rRNA. Data are expressed as mean ± SEM of three animals per group. *, p < 0.05 vs. sham, **, p < 0.05 vs. RL. C, ICAM-1 protein expression. At 4 hours post LPS, animals were sacrificed and lungs were homogenized in 1% Triton, and equal amounts of protein were separated on 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed for ICAM-1. A representative Western blot of three independent studies is shown.
Figure 27. Immunohistochemical staining of ICAM-1 on pulmonary endothelium after shock and resuscitation.

Animals underwent hemorrhagic shock and were resuscitated with RL or A25 over two hours. Six hours after endotracheal LPS or saline administration lungs were fixed by intratracheal instillation of 10% Phosphate buffered saline and stained with anti ICAM-1 antibody as described in Materials and Methods. Representative of one of three animals per group is shown at high power magnification.
Figure 28. Plasma 8-isoprostane levels following different resuscitation regimens.

Blood samples were obtained at different time points following shock/resuscitation with RL, A25, A5 or NAC and analyzed by eicosanoid immunoassay (EIA) with acetylcholinesterase kit as described in Materials and Methods. Data are expressed as mean ± SEM of four to ten animals per treatment group. Statistical differences were detected using a one way ANOVA and post hoc Bonferroni multiple comparisons testing at each time point. RL and A5 vs. A25 and NAC, * p ≤ 0.05 at indicated time points. RL and A5 vs. A25 and not NAC **, p< 0.05 at indicated time points.
Figure 29. Effect of NEM modified albumin on bronchoalveolar lavage fluid neutrophil count.

Following shock/resuscitation with different regimens, animals were treated with intratracheal LPS or saline and four hours later, BAL was performed. NEM modified A25 used for resuscitation is abbreviated A25NEM. Data are expressed as mean ± SEM of at least three animals per treatment group. RL/LPS and NEMA25/LPS vs. all other groups *, p< 0.05.
CHAPTER 6
HYPERTONIC RESUSCITATION OF HEMORRHAGIC SHOCK PREVENTS SYSTEMIC OXIDATIVE STRESS, UPREGULATES THE ANTI-INFLAMMATORY RESPONSE AND MODULATES TLR4 EXPRESSION IN MACROPHAGES

Disseminated as:


SUMMARY

Model

A rodent model of acute lung injury was used in which resuscitated shock primes for increased lung injury in response to a small dose of intratracheal LPS. Animals were bled to a mean arterial pressure (MAP) of 40mmHg, and maintained in a shock phase for 1h. Animals were then resuscitated by transfusion of the shed blood plus an equal volume of RL over one hour or their shed blood plus 4 ml/kg volume of 7.5% NaCl (HTS) over a period of two hours. Routinely, this resulted in the administration of 10cc of RL, 1cc of HTS. MAP was monitored continuously. One hour after resuscitation, 100 µg of LPS in 200 µl of saline and in some animals followed by endotracheal anti interleukin-10 (IL-10) neutralizing antibody were administered intratracheally. Alternatively, alveolar macrophages from bronchoalveolar lavage fluid (BALF) at end resuscitation were incubated ex vivo with LPS (0.1 µg/mL) or harvested alveolar macrophages were preincubated ex vivo with iso (300 mOsm) or hypertonic (500mOsm) and then treated with LPS (0.1 µg/ml).

Main Results

HTS resuscitation prevented leukosequestration in the alveolar space and abrogated the progressive rise in blood 8-iso-prostaglandin production observed with RL resuscitation. Inhibition of oxidative stress with a previously studied antioxidant N-acetylcysteine (NAC) corresponded with the ability of HTS to prevent shock and resuscitation induced edema, villus flattening and mucosal sloughing in the mid-ileum. LPS-induced NF-κB translocation in alveolar macrophages following RL was 42%, compared to 20% after HTS. Similar attenuation was observed with NAC resuscitation (16%). HTS resuscitation also inhibited LPS-induced tumour necrosis factor-alpha (TNF-α) production, while enhancing IL-10 release in response to LPS administered ex vivo and in vivo. Anti IL-10 antibody in vivo partially reversed the lung protective effect of HTS resuscitation. In addition, systemic administration of HTS affected the distribution pattern of TLR4 on alveolar macrophages, preventing cell surface recruitment of TLR4 in response to shock resuscitation. Ex vivo pretreatment with HTS, although transiently augmented TLR4 expression, lead to diminished TLR4 polarization into the leading edge of the cell when LPS was introduced. To further look at effects of HTS, TLR4 mRNA expression was examined. While isotonic saline had no effect on constitutive expression of TLR4 mRNA, HTS induced disappearance of TLR4 message after 2 hours.
INTRODUCTION

More than fifty percent of patients with severe trauma develop a systemic inflammatory response (SIRS) that can lead to multiple organ dysfunction syndrome (MODS). MODS is most often initially manifested as the acute respiratory distress syndrome (ARDS) which carries a mortality of 30-50% (130;579). Global ischemia reperfusion is thought to prime the immune cells, macrophages and neutrophils, for an exaggerated response to inflammatory stimuli, the so-called “two-hit” hypothesis (32;33). To study this clinical problem, a rodent model of ARDS was developed in this laboratory, where animals undergo shock resuscitation and are subsequently exposed to a small inflammatory stimulus in the form of lipopolysaccharide (LPS) (36). In this animal model a small dose of LPS induces an augmented response with increased activation of the proinflammatory transcription factor - nuclear factor kappa B (NF-κB), and consequent increased production of proinflammatory mediators which leads to lung injury similar to ARDS in humans (36). Importantly, the period between the initial shock and the second inflammatory insults provides a window of opportunity for an appropriate intervention designed not only at restoring the necessary hemodynamic parameters but also at aborting priming of macrophages and neutrophils, diminishing the magnitude of the second proinflammatory response leading to organ failure.

Strategies aimed at reducing the oxidant load during the window of opportunity period have been shown beneficial in modulating the inflammatory processes. Antioxidants used in vivo as well as in vitro such as N-acetylcysteine (NAC), vitamin E or pyrrolidinedithiocarbamic acid use have been shown to prevent macrophage activation and proinflammatory responses (43-46). Consistently, recent studies from our group demonstrate that oxidative stress alone is able to prime macrophages in vitro and results in greater and earlier NF-κB translocation in response to small doses of LPS (41). Recently small volume resuscitation with hypertonic saline (HTS) has been proposed and shown beneficial in the resuscitation from hemorrhagic shock. Based on the findings that oxidants are critical contributors to leukocyte priming and activation leading to the developments of SIRS, MODS, and ARDS we hypothesized that HTS resuscitation may exert its beneficial effects via diminishing the systemic oxidative stress that consequently results in reduced distant activation of macrophages.

Numerous clinical studies have evaluated the use of HTS in trauma and have shown that resuscitation with 4ml of 7.5% NaCl per kilogram is safe and effective (160;162;580). Two randomized controlled trials, one by Mattox et al. and the other one by Rizoli et al. demonstrated that the use of HTS for resuscitation resulted in lower rate of ARDS, renal failure
and coagulation abnormalities and a more balanced inflammatory response to hemorrhagic shock, in trauma patients (166;179). The major benefit of HTS is its rapid expansion of intravascular volume for the amount of fluid administered. HTS resuscitation has an instant hemodynamic effect with intravascular movement of water from the intracellular and interstitial compartments. HTS has also been shown to increase myocardial contractility, reduce endothelial and tissue edema as well as improve microcirculation and blood viscosity (581).

In addition to its beneficial hemodynamic properties, HTS has recently been demonstrated to have significant anti-inflammatory effects that favourably modulate cellular events predisposing patients to the development of SIRS and ARDS. In our model, the use of HTS for resuscitation conferred significant advantages over isotonic resuscitation in protecting animals from developing lung injury (173). Compared to animals resuscitated with RL, HTS resuscitation reduced neutrophil infiltration into the lungs, demonstrated by lung histology and neutrophil counts in the BAL fluid, as well as decreased lung protein leak. The lung protective mechanisms of HTS are not well understood. Our studies and others have shown that exposure to hyperosmolarity in vitro or in vivo alters the expression of adhesion molecules (CD11b, L-selectin and ICAM-1) (69) on neutrophils and endothelial cells, it decreases the expression and release of elastases and cytokines in response to LPS stimulation, and affects cytoskeletal reorganization of cells critical for receptor mediated signal transduction (69;582). Moreover some evidence exists that hyperosmolarity exposure may directly limit the neutrophil oxidative burst associated with ischemia reperfusion states as well as decrease NF-κB activation (583;584). Further, in vitro hyperosmotic conditions have been demonstrated, in a dose responsive fashion, to dampen the proinflammatory and augment the counter-inflammatory response by macrophages (178;564). HTS is therefore thought to exert its organ protection following shock resuscitation by modulating the critical balance between pro- and anti-inflammatory reactions of immune cells. Whether HTS has systemic antioxidant properties that contribute to its anti-inflammatory properties remains a hypothesis for the studies described.

One of the main sources of reactive oxygen species (ROS) and proinflammatory cytokines during global ischemia reperfusion is the gut (585). Intestinal mucosal ischemia leads to the production xanthine oxidase which during reoxygenation reacts with the purine substrates hypoxanthine and xanthine forming superoxide and hydrogen peroxide (220;546). Oxidants generated during intestinal ischemia are presumed to enter the systemic circulation and predispose patients to distant inflammation (586;587). Consistent with the lungs being the first organ exposed to the mesenteric lymph carrying proinflammatory mediators from the gut,
strategies aimed at diminishing gut mucosal injury have been shown to be effective in decreasing lung injury (588;589). Specifically, resuscitation with hypertonic solutions has effectively been used to improve intestinal perfusion, reduce intestinal damage and apoptosis in villi and increase gut barrier function following hemorrhagic shock (590-592). Based on these findings, we hypothesized that by preventing gut ischemia/reperfusion injury with HTS resuscitation, systemic oxidative stress might be diminished and result in reduced distant activation of macrophages in the alveolar space, as a central mechanism whereby HTS prevents lung injury.

Further to its systemic antioxidant effects, we investigated the action of HTS directly on macrophages’ activation by LPS. Exposure to hyperosmolar conditions has previously been shown to prevent macrophage activation by LPS in a dose dependent manner in vitro as well as in vivo (69;564). Innate immune recognition of LPS is initiated by its binding to a serum protein - LPS binding protein and transfer to a membrane CD14 molecule which in turn transfers LPS for association with a complex of surface receptors such as TLR4, MD2 and possibly others (354;413). TLR molecules are phylogenetically conserved receptors that recognize different pathogen associated molecular patterns (PAMPS) (593). The TLR4 signalling complex can induce downstream signalling and activation of NF-κB transcription factor. Since LPS activates macrophages through the TLR4 receptor pathway, we further hypothesize that the effect of hyperosmolarity on macrophage activation may be due to modulation of macrophage TLR4 gene and protein expression and distribution.

The data presented demonstrate that HTS prevents gut ischemia/reperfusion injury and consequently decreases oxidative stress and distant priming in alveolar macrophages. Specifically, HTS alters the balance between the proinflammatory and the counter-inflammatory response. In vivo, HTS inhibits TNF-α expression and augments IL-10 production by alveolar macrophage suggesting a potential mechanism for HTS protection from developing a systemic inflammatory response. Moreover, HTS was demonstrated to modulate macrophage TLR4 gene and protein expression leading to a less LPS responsive milieu. Although a clear survival advantage of hypertonic over isotonic resuscitation has not yet been established, the beneficial effects of HTS on the immune system as well as its hemodynamic advantages, ease of transport and rapidity of administration make it an attractive choice as a resuscitation agent.
MATERIALS AND METHODS

Materials

Lipopolysaccharide (E.coli O111:B4) and crystalline NaCl was obtained from Sigma (St Louis, MO). Sodium Citrate was purchased from Sigma Aldrich Chemical Co., Inc., Milwaukee, A 3.8% solution was made using sterile water, stored at 4°C, and filtered before use in resuscitation. Endotoxin-free Dulbecco's modified Eagle's medium and Hanks' balanced salt solution were purchased from Invitrogen, phosphate buffered saline (PBS) were from Gibco BRL (Burlington, Ontario), Ringer's Lactate (RL) was from Baxter Co (Toronto, Ontario). G3PDH was from Clontech (Palo Alto, CA), gelatin from Bio-Rad (Hercules, CA). Macrophages were isolated and cultured in DMEM containing 10% fetal calf serum from Hyclone Lab Inc (Logan, UT) and penicillin/ streptomycin from Gibco BRL. The anaesthetic drugs were pentobarbital from MTC Pharmaceuticals (Cambridge, Ontario), ketamine from Rogar/STB (London, Ontario) and xylazine from Bayer Inc (Etobicoke, Ontario). Isotonic NaCl solution contained 3 mM KCl, 5mM glucose, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2 and 10 mM Hapes, pH 7.4. Anti-IL-10 neutralizing antibody (ab9969) was obtained from Abcam limited, Cambridge, UK). TNF alpha and IL-10 ELISA kits were obtained from R and D Systems (Minneapolis, Minn). All chemicals used were of the highest purity available.

Animal Model

Animals were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. Male Sprague-Dawley rats weighting 300g to 350g (Charles River, St Constant, Quebec) were anaesthetized with intraperitoneal ketamine (80mg/kg) and xylazine (8mg/kg). The right carotid artery was cannulated with a 22-gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling, and resuscitation. Hemorrhagic shock was initiated by blood withdrawal leading to a reduction of the MAP to 40 mm Hg within 15 min. This blood pressure was maintained by further blood withdrawal if the MAP > 45 mm Hg, and by infusion of 0.5 ml of RL if the MAP was < 35 mm Hg. Shed blood was collected into 0.1 ml citrate/ml blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood followed by administration of a volume of RL, which was equivalent to the volume of shed blood. In studies on the effect of antioxidants, the RL infused was supplemented with the antioxidant N-acetyl-cysteine (NAC, 0.5g/kg). Alternatively, animals were resuscitated using a 4 ml/kg volume of 7.5% NaCl (HTS) over a period of two hours. Routinely, resuscitation resulted in the administration of 10cc +/- 3cc of RL, 1cc +/- 0.5cc of HTS. After fluid
administration, the catheter was removed, the carotid artery ligated, and the cervical incision closed. Animals were then allowed to rest to make the total resuscitation time of two hours in order to standardize the time between the end of shock and the administration of intratracheal LPS. After resuscitation, animals were sacrificed by a pentobarbital overdose. Immediately after sacrifice, a bronchoalveolar lavage was performed (see BAL below). Alternatively, 100 µg of LPS in 200 µl of saline was administered intratracheally through a 14-gauge angiocath tracheostomy. Some animals received intratracheal anti IL-10 neutralizing antibody (5 µg in 100µl of saline). Sham animals were instrumented but not bled and saline alone was instilled intratracheally. Animals were then sacrificed by pentobarbital overdose at various time points depending on the specific study and a BAL was performed as described below. For histological assessment a 2 cm piece of the mid-ileum was fixed in 4% neutral buffered formalin (pH 7.4), later stained with hematoxylin/eosin, and examined using an optical microscope.

**Bronchoalveolar Lavage (BAL)**

Immediately after sacrifice, the lungs were perfused via the tracheostomy cannula with cold PBS in 10 ml aliquots and gently withdrawn to a total of 50 ml. For cell counts and differential, BAL fluid was centrifuged at 300g for 10 min. After discarding the supernatant, the pelleted cells were resuspended in serum free DMEM. Total cell counts were done on a grid hemocytometer and the differential cell counts on a cytospin-prepared slide stained with Wright-Giemsa. Five hundred cells were counted in cross-section per sample and the number of neutrophils was calculated when required as follows:

\[
\text{number of neutrophils} = \text{total cell count} \times \text{% of neutrophils in BAL fluid sample.}
\]

**Cell Culture and Activation**

Isolated alveolar macrophages or the murine macrophage cell line, RAW 264.7 (ATCC), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin (P/S), and 10% fetal calf serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Where required, alveolar macrophages were allowed to adhere to flamed glass coverslips at 37 °C in 5% CO₂ in the presence of Hanks's balanced salt solution, 2% fetal calf serum and 2 mM glucose. The cells were exposed to LPS (0.1 µg/ml) treatment ranging from 30 min to 1h. Reactions were stopped by placing the cells on ice. Some cells were exposed LPS (0.1 µg/ml) for various time points. When required, the isotonic medium was made hypertonic by the addition of 200 mM or less of sodium chloride.
8-Isoprostane Assay

8-Isoprostane levels were determined using an eicosanoid immunoassay (EIA) with acetylcholinesterase kit (Cayman Chemical, Ann Arbor, MI). 500-μl of plasma sample was vortexed with 1 ml of 100% ethanol, allowed to stand at 4°C for 5 min, and then centrifuged at 1,500 g for 10 min. The supernatant containing 8-isoprostanes was then decanted, an equal volume of 15% KOH was added, and samples were incubated at 40°C for 1 h. After 1 h, samples were diluted to 5 ml with double-distilled water and the pH was lowered below 4 with HCl. Samples were then passed through preconditioned SPE-C18 reverse-phase cartridges, followed by 5 ml of pure water and then 5 ml of HPLC-grade hexane. 8-isoprostanes were eluted with 5 ml of ethyl acetate containing 1% methanol. The ethyl acetate was then evaporated with nitrogen and 1 ml of EIA resuspension buffer was added, and samples were vortexed for 30 s and sonicated for 5 seconds. The developed plate was read by a microplate reader (Diamed, Mississauga, ON, Canada) at 405 nm. The concentration was calculated by computer using Cayman EIA software. Rat plasma samples, standardized for protein concentration, were assayed in duplicate in a blinded fashion (n = 4 per group).

Immunofluorescence

NF-κB is a heterogeneous collection of dimeric proteins composed of members of the Rel family and resides in the cytoplasm of nonstimulated cells because it is complexed with the inhibitory IκB protein. The major NF-κB components are the p50/NF-κB1 and p65/RelA subunits. Immunofluorescence (IF) microscopy can be used to investigate nuclear translocation of the p65 subunit as a surrogate for NF-κB translocation (41) and nuclear transcription inducing activity. In our assays, alveolar macrophages recovered were allowed to adhere to sterile cover slips and then were fixed for 30 min in PBS supplemented with 2% paraformaldehyde. The coverslips were washed three times with PBS for 10 min each, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then blocked with 5% bovine serum albumin in PBS for 1 hour at room temperature. The samples were stained with goat anti-p65 polyclonal primary antibodies (1:50 dilution in PBS) for 1 h at room temperature, washed three times with PBS for 5 min each, and incubated with fluorescently labelled Alexa 555 donkey anti-goat IgG secondary antibodies (1:400 dilution in PBS) (Molecular Probes Inc., Eugene, OR) for 1 h at room temperature. The coverslips were mounted on glass slides using DAKO solution, from Dakocytomation (Carpinteria, CA). The staining was visualized using a Nikon TE200 fluorescence microscope (x100 objective) coupled to a Orca 100 camera (Hamamatsu Photonics, Hamamatsu, Japan) driven by Simple PCI software (Compix Inc., Imaging Systems, Cranberry Township, PA). A
ratio of translocated to non-translocated cells was counted, with an average of 100 cells counted for each group. Alveolar macrophages or RAW 264.7 cells were allowed to adhere to sterile coverslips under cell culture conditions (see above). After treatment with LPS under isotonic or hypertonic conditions cells fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS, and blocked with 5% bovine serum albumin in PBS for 1 hour at room temperature and then incubated with a primary anti-TLR4 rabbit polyclonal IgG antibody (H-80) (1:200 dilution) from Santa Cruz Biotechnology, Inc., for one hour at room temperature. TLR4 antibody used was first tested for specific labelling on macrophages obtained from wild type and TLR4 deletion mutation animals-10SCR mice. The wild type mice had strong fluorescent staining with our primary and secondary antibodies. In contrast macrophages from 10SCR mice that lack TLR4 displayed only background staining attributable to weak non-specific secondary binding (Fig. 39C). Next coverslips were washed with PBS, and incubated with the corresponding fluorescently labelled secondary antibody: FITC – conjugated affinity purified Fab fragment goat anti-rabbit IgG (H+L) from Jackson ImmunoResearch Laboratories, Inc., for 1 h at room temperature. After extensive washes, the coverslips were mounted on glass slides using DAKO mounting medium, from Dakocytomation (Carpinteria, CA). The staining was visualized using a Nikon TE200 fluorescence microscope (x100 objective) coupled to a Orca 100 camera (Hamamatsu Photonics, Hamamatsu, Japan) driven by Simple PCI software (Compix Inc., Imaging Systems, Cranberry Township, PA). Image analysis of TLR4 peripheralization was done using the Scionimage software. Peripheralization was defined as an 80% or more increase in the ratio of the fluorescence intensity at the cell periphery to the fluorescence intensity at the cytosol at 0.5 micron distance from the membrane. The percentage of cells with peripheralized TLR4 under various conditions was calculated by scoring 100 cells.

**Immunofluorescence Flow Cytometry**

Cell surface expression of TLR4 was detected by Immunofluorescence Flow cytometry in 1 x 10^6 live cells by staining with specific antibodies: anti-TLR4 (H-80) from Santa Cruz Biotechnology, Inc., anti-TL4/MD2 (MTS510) from Stressgen Biotechnologies. Cells were stained with fluorophore conjugated primary antibody at 4°C, washed in staining buffer and analyzed (10,000 cells per condition) in a FACScan (Becton-Dickison, Palo Alto, CA) using FL1 525 mM Band Pass detector at 488 nm excitation wavelength. Alternatively, if primary antibody was not fluorophore conjugated, cells were incubated with fluorophore-conjugated secondary antibody for 1 h on ice before washing and analysis. Results were expressed as mean channel fluorescence (MCF).
Measurement of TNF-α and IL-10

BAL macrophages were isolated 1 hour after end resuscitation, or 4 hours after \textit{in vivo} endotracheal LPS administration and cultured at 37°C. TNF-α and IL-10 proteins in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) 120 minutes after culture with or without \textit{ex vivo} LPS (0.1 µg/ml) administration.

Northern Blot Analysis

Total RNA from treated RAW 264.7 cells was obtained using the TRI Reagent® (Molecular Research Center Inc.) phenol and guanidine thiocyanate method (556). Briefly, cells were harvested into an appropriate amount of TRI Reagent. RNA was denatured and electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization was carried out using a [32P]dCTP-labelled TLR4 cDNA (kindly provided by Dr. S. Frantz, Brigham and Women’s Hospital, Boston, MA; (594). Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens. In RAW 264.7 cells the TLR4 probe usually produces two bands. To control for loading, blots were then stripped and reprobed for G3PDH, a ubiquitously expressed housekeeping gene (557). Expression of mRNA was quantified using a phosphoimager and accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.), and was normalized to the G3PDH signal.

Statistical Analysis

The data are presented as mean ± standard error of n determinations as indicated in the Figure Legends. Data were analyzed by one-way analysis of variance; post hoc testing was performed using the Bonferroni modification of the t-test and was performed only when the between groups ANOVA comparison was significant with \( p = 0.05 \) level. In figures presented, significance is indicated with an asterisk (*) and signifies that the mean difference between indicated treatment groups is significant at the 0.05 Bonferroni corrected level.
RESULTS
Effect of Hypertonic Resuscitation on lung neutrophil accumulation following intratracheal LPS

A survival rate of 95% was observed in the animals resuscitated with HTS or RL+/-NAC with all deaths occurring due to tracheal cannula obstruction during the first 3 hours after LPS administration. Animals that died prior to the end of an experiment were excluded from analysis. Resuscitation was carried out to reach the pre-shock MAP levels (~100mmHg) and urine output of over 0.5cc/kg/hr. Consistent with our previous reports, we observed priming for increased lung injury when LPS was administered after shock/resuscitation with RL. As demonstrated in Figure 30, neither resuscitated shock alone nor LPS alone caused as significant rise in BAL neutrophils. Resuscitation alone, regardless of the strategy used, had little effect on leukosequestration with very few neutrophils in the BAL fluid. LPS treatment alone, in sham animals, caused a modest increase in BAL neutrophils. However, when HTS was used for resuscitation, priming was abolished and further LPS stimulation was not able to cause a significant increase in BAL neutrophils. These data recapitulate previous findings that HTS resuscitation is lung protective.

Role of hypertonic saline resuscitation in intestinal ischemia reperfusion injury

Intestinal mucosal damage following ischemia and reperfusion was assessed by histology samples of the mid ileum 4 to 6 hours following resuscitation using various strategies followed by endotracheal LPS administration. Tissues were fixed in 4% paraformaldehyde and sections were stained with haematoxylin eosin for assessment of tissue damage. Representative micrographs of intestinal mucosa 6 hours following LPS administration are shown in Figure 31. In animals resuscitated with RL there was a number of morphological changes observed: diffuse epithelial edema and necrosis of the villi accompanied by neutrophil infiltration in the submucosa and hemorrhage. When compared to RL resuscitated animals, inhibition of oxidative stress with NAC corresponded with the ability to protect the intestinal mucosa from edema, villus flattening, mucosal sloughing and PMN infiltration. HTS resuscitation had a very similar effect; consistent with the findings in the literature, it had a salutary effect on intestinal ischemia reperfusion injury.

Effect of hypertonic saline resuscitation on plasma oxidant activity

To discern the potential of hypertonic saline resuscitation on its ability to limit plasma oxidant activity, levels of 8-isoprostane - a product of lipid peroxidation were measured in rat plasma after various resuscitation fluid strategies. Figure 32 illustrates plasma 8-isoprostane
concentration measured at various times during shock/resuscitation. Following resuscitation using RL, rat plasma oxidant activity increased during the resuscitation phase and continues to increase even at one hour after the end of resuscitation. Resuscitation with the antioxidant NAC in RL suppressed evidence of oxidant activity in the plasma over the entire period of observation. HTS resuscitation mimics the use of an antioxidant in that it decreased the plasma 8-isoprostane levels starting at 5 min. after the end of the resuscitation period.

Hypertonic Saline Resuscitation Effect on alveolar macrophage NF-κB Translocation

To examine the effect of hypertonic saline resuscitation on LPS signalling, we used immunofluorescence (IF) microscopy to investigate nuclear translocation of p65 as a surrogate for NF-κB translocation (41). Figure 33 shows the effect of in vivo resuscitation with HTS, RL alone, or RL with antioxidant NAC on alveolar macrophage NF-κB translocation following LPS stimulation. IF demonstrates that shock resuscitation with RL induced NF-κB translocation at 30 min of LPS stimulation ex vivo, an effect which was prevented by NAC supplementation. Similar to the effect of NAC, HTS resuscitation abrogated NF-κB translocation at 30 min of LPS stimulation. When translocation was quantitated by counting the percentage of cells with NF-κB in their nuclei, the data revealed that resuscitation with either NAC in RL or HTS is able to limit alveolar macrophage NF-κB translocation back down to LPS alone levels (Fig. 34). This indicates that HTS administered in vivo protects alveolar macrophages from priming and although the mechanism of this protection is yet unknown we can speculate that it may in part be due to its ability to limit oxidant generation and activity in blood.

Effect of Hypertonic Resuscitation on Macrophage TNF-α production

Alveolar macrophages were harvested by BAL at end of resuscitation and incubated at 37°C with or without a small dose of LPS (0.1 µg/ml) for 2 hours. Figure 35 shows the effect of in vivo resuscitation with HTS on the proinflammatory cytokine – TNF-α release by alveolar macrophages. Cells from sham animals produced little detectible TNF-α. Shock/resuscitation, with either RL or HTS, did not induce a significant increase in TNF-α levels. In contrast, TNF-α production rose significantly after LPS stimulation and was further potentiated by antecedent shock RL resuscitation. This is an effect of the ischemia/reperfusion priming for an augmented proinflammatory response. However, HTS resuscitation in vivo inhibited the LPS-induced TNF-α production by over 90%. These data support the hypothesis that the protective effect of HTS resuscitation may be related to impairing alveolar macrophages from mounting a proinflammatory response to subsequent inflammatory stimuli.
Effect of Hypertonic Resuscitation on Macrophage IL-10 production

To determine the effect of HTS resuscitation on the counter-inflammatory response by alveolar macrophages, IL-10 protein production was measured in macrophages recovered by BAL after resuscitation. Figure 36 shows the effect of in vivo HTS exposure on ex vivo IL-10 response to LPS. Alveolar macrophages from sham animals had a baseline IL-10 expression and shock resuscitated with RL decreased IL-10 production below baseline levels. Although stimulation with LPS caused a significant increase in IL-10 release, HTS resuscitation alone without LPS stimulation was able to further induce the counter-inflammatory IL-10 response above LPS levels. This effect was sustained when cells from HTS resuscitated animals were treated with LPS ex vivo, with IL-10 levels higher by over 50% than in the RL resuscitation group.

Subsequent studies were performed to discern whether the HTS induced IL-10 release also occurs in a more clinically relevant model of endotracheal LPS administration. Figure 37 shows that when LPS is administered in vivo there is an increased production of IL-10 protein by alveolar macrophages. In addition, IL-10 is significantly augmented in macrophages from HTS resuscitated animals. Considered together, these data support the concept that the protective effect of HTS observed in our ARDS model may be attributed to an early increase in a counter-inflammatory response by alveolar macrophages with augmented IL-10 production.

The role of IL-10 in HTS mediated protection from lung injury in the ARDS model

Since HTS was shown to augment IL-10 production and decrease TNF-α release in vivo, we hypothesised that blocking IL-10 activity in vivo may impair the protective effects of HTS resuscitation. To test the role of IL-10 released by alveolar macrophages, its biological activity was blocked with the intratracheal administration of anti-IL-10 neutralizing antibody (5µg) following LPS administration. Neutrophil (PMN) sequestration in the lungs was used to measure inflammation in these studies, as it represents a hallmark of ARDS and a measure of lung injury in our model. BAL fluid from different treatment groups was evaluated for differential cell counts. Figure 38 illustrates rat alveolar PMN counts under different treatment regimens. While sham and shock resuscitated animals displayed baseline levels of lung neutrophilia four hours after intratracheal administration of saline vehicle, four hours following LPS stimulation in vivo, there was a significant increase in BAL neutrophils. Consistent with the “two-hit” hypothesis, shock plus LPS in animals resuscitated with RL caused an even greater increase in BAL neutrophils than with LPS alone. As anticipated, HTS resuscitation was protective and decreased the number of neutrophils in the alveolar space significantly.
Interestingly, the administration of IL-10 neutralizing antibody to the HTS resuscitated rat partially abolished its protective effects and restored LPS induced lung neutrophilia to that seen in LPS alone group. These findings suggest a causal role for HTS induced augmented IL-10 as one of the mechanisms rendering this resuscitation strategy protective.

**Hypertonic resuscitation prevents macrophage cell surface TLR4 redistribution induced by shock/ resuscitation and LPS**

TLR4 is an essential component of the LPS surface receptor complex responsible for transmitting downstream signalling to activate the proinflammatory NF-κB transcription factor. Resuscitated hemorrhagic shock alone has been demonstrated to stimulate an increase in macrophage responsiveness to LPS with enhanced NF-κB activation and proinflammatory signalling. To evaluate whether HTS resuscitation may modulate the macrophage TLR4 cell surface expression as one of its anti-inflammatory mechanisms, alveolar macrophages were harvested following each resuscitation protocol. These macrophages were stimulated *ex vivo* with LPS and evaluated by immunofluorescence (IF) microscopy for TLR4 expression using anti-TLR4 (H-80) antibody in permeabilized cells. The antibody was first tested for specificity by staining macrophages from C57BL/10ScCr mice with a TLR4 deletion mutation and non-specific fluorescence was not observed in these cells (Fig. 39C). As depicted in Figure 39A, in untreated alveolar macrophages from sham animals TLR4 was distributed throughout the cytoplasm with some increased staining in perinuclear areas, consistent with the location of the Golgi apparatus. BAL macrophages from RL resuscitated animals harvested one hour after the end of resuscitation, had markedly decreased cytoplasmic TLR4 staining since their TLR4 almost completely peripheralized to the plasmalemma. By contrast, in the HTS-treated animals, TLR4 peripheralization was prevented. TLR4 membrane peripheralization was quantitated as described in Materials and Methods and is shown in Figure 39B. In sham groups, less than 10% of cells had TLR4 peripheralized to the cell membrane, while in Shock RL group cells had 75% surface TL4 staining. HTS resuscitation significantly diminished TLR4 peripheralization to less than 30% level.

Studies by Jiang *et al.* have suggested that stimulation of the TLR4 with its ligand LPS results in initial early upregulation followed by a downregulation of this receptor from the macrophage surface (370). To discern whether systemic hypertonic treatment alters the described macrophage response to LPS, AM from each resuscitation protocol were stimulated *ex vivo* with 5min or 60 min of LPS. As shown in Figure 39, macrophages from sham animals exposed to LPS for 5 min exhibited TLR4 peripheralization as well as an activated appearance.
with numerous phylopodia formation and vacuolization. This effect was not sustained over 60min of LPS stimulation as TLR4 returned to its diffuse intracellular location at that time. In animals resuscitated with RL, macrophages responded to 5 min of LPS stimulation with intense plasmalemmal TLR4 staining, significantly greater then in sham/LPS macrophages, and a return to diffuse TLR4 distribution at 60 min nevertheless above the sham/LPS levels. By contrast, hypertonic resuscitation not only prevented the shock resuscitation induced plasmalemmal redistribution of TLR4 but also prevented the LPS induced TLR4 redistribution and morphological changes at both 5 and 60 minutes of LPS.

To quantitate TLR4 cell surface expression more accurately and confirm that HTS resuscitation is able to prevent localization of TLR4 to the plasma membrane as opposed to submembrane regions, flow cytometry was performed on non-permeabilized cells. Figure 40 demonstrates TLR4 surface expression on alveolar macrophages from animals resuscitated with RL or HTS treated with 5 or 60min of LPS ex vivo. Consistent with our immunofluorescence studies, RL animals showed a significant increase in TLR4 cell surface expression at the end of resuscitation compared to sham animals. This increase was further potentiated in both sham and RL groups with exposure to LPS for 5 min, however in both groups cell surface TLR4 returned to pre-shock levels at 60 min of LPS stimulation. By contrast, in the HTS-resuscitated animals, increased surface TLR4 expression following shock or LPS was completely averted. Considered together, these findings demonstrate that hypertonic resuscitation is able to impair both shock/RL and LPS induced upregulation of TLR4 on the macrophage surface. This represents a potential mechanism by which small volume resuscitation may impair global cell responsiveness to extracellular stimuli and thus exert its anti-inflammatory properties.

**Hyperosmolarity effect on TLR4 expression in vitro**

Having demonstrated in vivo that HTS is able to prevent TLR4 redistribution on alveolar macrophages subsequent studies were performed to discern whether hyperosmolarity alone is capable of inducing this effect at a cellular level. Our laboratory has previously reported that resuscitation with HTS in the animal model also used here results in a transient and rapid increase in serum osmolarity peaking at approximately 500 mOsm at 5 minutes followed by a return to approximately 350 mOsm by one hour (173). To mimic the in vivo environment, alveolar macrophages were treated in vitro with various concentrations of NaCl for 5 minutes and TLR4 surface expression was studied by flow cytometry. Figure 41 demonstrates that in vitro treatment of alveolar macrophages with NaCl at 500 mOsm increased TLR4 surface
expression followed by a subsequent significant decline in surface TLR4 with decreasing salt concentrations.

HTS resuscitation has been demonstrated to have anti-inflammatory properties and demonstrated here to prevent the LPS induced TLR4 surface up-regulation. Paradoxically in vitro hyperosmolar treatment increased TLR4 surface expression on macrophages. We hypothesised that hyperosmolarity may exert its protective effect by inducing a rapid transient increase in macrophage surface TLR4 followed by an equally rapid downmodulation of TLR4 once exposed to LPS. To test this hypothesis, alveolar macrophages were pretreated with hyperosmolarity and then exposed to low dose LPS under isotonic conditions. Figure 42 demonstrates surface TLR4 expression in these experiments. Consistent with our dose response data hyperosmolarity of 500 mOsm induced a significant upregulation of TLR4 as compared to isotonic control, however similar to in vivo findings; hypertonic pretreatment also prevented the early LPS induced upregulation of TLR4, which was sustained over 60 minutes. These data suggest that HTS resuscitation may protect from an exaggerated response to LPS after shock by upregulating macrophage TLR4 expression under high osmolarity such that subsequent LPS exposure can no longer induce further upregulation of this receptor and results in its quick and sustained downmodulation.

**Hypertonic saline effect on regulation of TLR4 mRNA expression in macrophages**

Since hyperosmolarity has been shown to regulate gene expression of many proinflammatory mediators we hypothesized that HTS exerts its modulatory effect on the TLR4 expression not only by altering its immediate distribution but also by contributing to its longer-term regulation of expression. We therefore examined the direct effects of hyperosmolarity on macrophage TLR4 mRNA levels in RAW 264.7 cells. The Northern blot analysis of TLR4 mRNA, controlled for comparable loading with G3PDH shown in Figure 43 illustrates that RAW 264.7 cells under isotonic conditions demonstrate a constitutive expression of TLR4 mRNA expression over 4 hours. However when cells are incubated under hyperosmolar conditions there was a rapid and significant reduction in the TLR4 message. These data suggests that hyperosmolarity not only affects immediate TLR4 redistribution but also induces changes at the TLR4 mRNA expression level.
DISCUSSION

Our previous work using both in vivo and in vitro models has shown that oxidative stress is able to prime macrophages for an augmented response to a subsequent inflammatory stimulus such as a small dose of LPS. ROS alone either generated in vivo during ischemia reperfusion or in vitro in the form of H₂O₂ have been shown to prime the signalling pathway leading to NF-κB nuclear translocation (36;41). In their primed state, even when a small dose of LPS is added, macrophages become activated and demonstrate an earlier and greater NF-κB activation and production of proinflammatory mediators. In the in vivo model, the use of HTS for resuscitation resulted in significantly reduced microvascular leak, lung neutrophilia and the degree of histopathological injury compared to resuscitation with RL (173). However, the mechanisms of HTS protection against the exaggerated response to LPS are not yet fully elucidated especially since the immune responses to LPS and their regulation under oxidative stress are still being defined and are a subject of this thesis. ROS are thought to modulate the innate immune responses to LPS by leukocytes and therefore we hypothesised that HTS resuscitation may exert its protective effects by an antioxidant mechanism, modulating leukocyte cytokine production and LPS receptor expression.

Considerable evidence imputes the gut as a significant source of oxidants and inflammatory mediators contributing to the pathogenesis of SIRS and organ failure (49;595). One of the major in vivo sources of ROS is xanthine oxidase (XO) (596). Coupled with the reduction of NAD+ to NADPH, XO is generated during ischemia from its precursor Xanthine dehydrogenase (XD), a constitutively expressed 150 kDa protein. An important site of this conversion has been shown to be the intestinal microvasculature as XD/XO are especially abundant in the small intestine, where it is expressed predominantly in the villus epithelium (220). During reperfusion, XO preferentially uses molecular oxygen to NAD+ as the electron acceptor which generates superoxide thus contributing to intestinal reperfusion injury (596). Systemic oxidative stress also ensues, presumably both through direct release of oxidants as well as xanthine oxidase into the systemic circulation (221). Together, these may account for injury to distant tissue beds and priming of inflammatory cells for increased responsiveness to a second “hit”. Indeed, strategies aimed at preventing oxidant generation have been proposed as potential therapeutic strategies. For example, allopurinol, an inhibitor of xanthine oxidase, has been shown to prevent lung injury in a hemorrhagic shock model in mice (597). Relevant to the present studies, hypertonic saline resuscitation has been shown to improve intestinal blood flow and consequently decrease the extent of intestinal villus injury (598). In addition, HTS has been
demonstrated to possess edema-reducing and direct vasodilatory properties in the gut and also to prevent capillary narrowing induced by hemorrhagic shock (599;600). The data presented here confirm that resuscitation with HTS (4 ml/kg) may diminish microcirculatory dysfunction in the gut and consequently prevent the generation of oxidative stress in the systemic circulation. Consistent with the findings in this study, Ciesla D.J. and colleagues demonstrated that early HTS treatment (before priming and after priming but before LPS activation) prevents PMN respiratory burst and protease release and thus prevents PMN-mediated tissue injury (583). Similarly in the data presented here, HTS resuscitation protects the gut from the priming effects of shock/resuscitation and is able to reduce plasma oxidant activity to the same level as that observed with the use of the antioxidant NAC. Consistent with an antioxidant effect of HTS resuscitation, NF-κB translocation induced by LPS in HTS-resuscitated animals was reduced to that observed in cells recovered from sham animals. Together, these findings suggest that the gut protective effects of HTS may lead to decreased macrophage activation in the lung possibly via reduced PMN priming and decreased generation of gut-derived oxidants ensuing in decreased systemic oxidant load.

These findings suggest that the gut protective effect of HTS might also reduce distant oxidative stress and hence exert beneficial effects on the priming of leukocytes and their response to LPS. One of the major effector cells in the innate immunity is the macrophage. Activated macrophages play an important role in the pathogenesis of ARDS, releasing proinflammatory cytokines such as IL-8 and TNF-α that act in a paracrine fashion to recruit and activate other immune cells such as neutrophils that further contribute to the proinflammatory state. Specifically in our model, hemorrhagic shock primed alveolar macrophages for increased activation in response to LPS, compared to hemorrhagic shock or LPS alone. Based on these observations, we hypothesised that HTS resuscitation may exert its protective effects by modulating macrophage function during ischemia/reperfusion states. Using our rodent model of ARDS, we demonstrated that HTS resuscitation induces a counter-inflammatory activity in alveolar macrophages with increase IL-10 and decreased TNF-α production that may account for some of HTS anti-inflammatory properties.

A balance between the pro- and anti-inflammatory immune mediators such as TNF-α and IL-10 respectively is critical in influencing the progress or resolution of an inflammatory process. IL-10 is a powerful counter-inflammatory cytokine that is released into the circulation following shock resuscitation (601;602) that is known to inhibit activation of the transcription factor NF-κB leading to a decreased transcription of a number of proinflammatory cytokines:
TNF-α IL-1 and IL-6 as well as adhesion molecules such as ICAM-1 (603). Clinical studies have shown that ARDS patients with decreased plasma IL-10 levels in BAL fluid have a worse prognosis (126). Exogenous IL-10 administration has been shown to protect against lethal endotoxemia and bacteremia in animals (604). HTS may therefore exert its protective role by inducing IL-10 production by alveolar macrophages. Our results have demonstrated that HTS resuscitation augments IL-10 levels and can bring TNF-α production to a halt. The mechanism of augmented IL-10 production under hyperosmotic conditions remains largely unknown. IL-10 production is mostly regulated at the transcriptional level (605). Recent investigations by Alam and colleagues demonstrated by cDNA array technique that the use of HTS for resuscitation alters the genetic profile of different organs (606). It is possible that the increase in IL-10 expression, observed under HTS conditions in our model, is regulated at the gene level as well. This possibility however, requires further investigation.

Our data show that macrophages exposed to HTS resuscitation in vivo can not only mount a strong counter-inflammatory response with IL-10 when stimulated with ex vivo LPS but also retain this effect when a dose of endotracheal LPS is administered. Further, the macrophage through exposure to in vivo HTS is reprogrammed for a lesser proinflammatory response with TNF-α. Our finding on in vivo HTS use support recent reports on its in vitro beneficial effect by Cushieri et al. demonstrating that exposure to hyperosmolar conditions prevents macrophage activation (564). In Cushieri’s studies hypertonic preconditioning resulted in a dose dependent attenuation of LPS induced TNF-α production, extracellular signal-related kinase (ERK ½) phosphorylation and stress fiber polymerization. Similarly, our group previously demonstrated that murine peritoneal macrophages exposed to HTS had an increased counter-inflammatory response with increased IL-10 production and an attenuated TNF-α production (178). Whether alveolar macrophages in vivo are directly exposed to a hypertonic environment or whether they respond to mediators released into the circulation under hyperosmolar conditions needs further investigation. However, our experiments using a neutralizing anti IL-10 antibody in vivo support a causal role of HTS induced augmented IL-10 production by alveolar macrophages in conferring protection against lung injury in our model of hemorrhagic shock and ARDS. Consistent with our previously published results, neutrophil sequestration in the lung was demonstrated to be largely diminished in rats resuscitated with HTS as compared to RL when LPS was given intratracheal. However, blocking endogenous IL-10 with a neutralizing antibody resulted in increased neutrophilia and lung injury. Corroborating
our findings, other investigators found that the use of neutralizing antibodies to IL-10 increase inflammatory cytokines TNF-α and IL-1β in vivo (607).

Other potential mechanisms whereby HTS resuscitation might exert protective effects warrant consideration. HTS itself may act as an oxidant scavenger similar to hyperosmolar albumin, which was shown capable of exerting an antioxidant effect (Chapter 3). In the present studies, HTS was administered in the absence of colloid. Therefore, since HTS has no such known antioxidant properties, this mechanism seems unlikely. In addition, HTS has been postulated to moderate the release of gut-derived proinflammatory factors into the mesenteric lymph and thereby exert its lung protection. This notion has been substantiated by the work of several groups who demonstrated that lymph from hypertonic saline resuscitated animals prevents neutrophil activation, their vascular margination and the release of neutrophil superoxide anion (174;563;608). Thus, local effects of hypertonic resuscitation on the intestinal mucosa and the generation of bioactive mesenteric lymph may either directly lessen oxidative stress or have direct effects by reducing inflammatory cells oxidant generation (609).

Several groups have reported that hypertonicity in vitro or HTS resuscitation in vivo is able to prevent neutrophil respiratory burst, decrease expression of adhesion molecules CD11b and ICAM-1 and inhibit an increase in L-selectin shedding (49;69;173). Similarly, HTS may directly prevent cytokine production by inhibiting LPS signalling at the macrophage level. The results presented here support the possibility that hypertonic saline resuscitation prevents alveolar macrophage activation and intracellular signalling leading to NF-κB nuclear translocation. Following hypertonic saline resuscitation, NF-κB is virtually absent from the nucleus following 30 minutes of LPS stimulation ex vivo. The addition of antioxidant NAC in isotonic medium to the resuscitation fluid recapitulates observations using HTS. Our current findings of HTS salutary effect in vivo on alveolar macrophage signalling contrast those of a previous report by Oreopoulos et al. (178) where, despite the ability of HTS to inhibit TNF-α protein and gene expression after LPS treatment, it had no effect on NF-κB translocation at either 5 or 60 minutes after LPS exposure. The reason for this difference is not evident. However, they may serve to illustrate an important difference in the pathophysiological reaction of primary alveolar macrophages derived from in vivo treated animals versus thioglycolate elicited peritoneal macrophages exposed to hypertonicity in vitro.

The cellular mechanism of the attenuated response by macrophages is largely unknown and we hypothesized that hyperosmolarity may modulate the expression of the LPS receptor – TLR4. Our major and novel findings suggest that HTS may exert its protection in global
ischemia/ reperfusion states by impairing the proinflammatory response at the macrophage LPS receptor level. Our data demonstrates that HTS resuscitation prevents shock resuscitation induced TLR4 redistribution and migration to the leading edge of the cell in vivo. Further, HTS resuscitation decreases LPS induced early rise in surface TLR4 expression in vivo and in vitro. This occurs by inducing a rapid and transient increase in macrophage surface TLR4 that prevents any further LPS induced increase presumably necessary for enhanced downstream signalling. The long-term downmodulation of TLR4 receptor may be due to decreased transcription of TLR4 mRNA independent of TLR4 message stability. Since signalling via the TLR4 receptor complex is a critical first step in NF-κB activation, deciphering the HTS effect on the TLR4 expression provides further insight into potential pharmacologic targets for suppressing the exaggerated inflammatory process in ischemia reperfusion states.

Evidence exists that both CD14 and TLR4 can initially be up-regulated on macrophages and other cells in response to LPS stimulation, however the receptors are internalized and their surface numbers decrease with longer LPS exposure (370;610). It appears that HTS may inhibit the conventional LPS signalling route. This inhibition is accomplished by diminishing the early, LPS induced, TLR4 upregulation possibly necessary for signalling. However, Since hyperosmolarity alone is not known to induce NF-κB activation, and our previously published results indicate that HTS protects macrophages from priming it appears that it is neither HTS induced TLR4 surface upregulation nor subsequent TLR4 internalization that are key steps for augmented downstream signalling. Consistent with this idea are observations by Espivik and colleagues who demonstrated that LPS signalling is not dependent upon LPS trafficking to the Golgi as disrupting Golgi-associated TLR4 expression by brefeldin A preserves LPS signalling (396). Interestingly, cross-linking of TLR4 at the macrophage surface was sufficient to activate NF-κB (396). Based on our findings and the reports of others presented here, we postulate that hypertonic saline resuscitation interferes with events necessary for augmented downstream NF-κB signalling when TLR4 is still at the macrophage surface.

In rat, the plasma osmolality is 300 mOsmol at baseline and routinely exceeds 500 mosmol immediately following HTS resuscitation and is followed by a gradual decline to 350 mosmol over the next 4 hours (173). The mechanism whereby transient hyperosmolarity in vivo exerts its anti-inflammatory effect on macrophages and diminishes their downstream NF-κB activation is complex. Our results indicate that hyperosmolarity may act at the cell surface level by transiently increasing the macrophage surface TLR4 numbers followed by their rapid and sustained downregulation when LPS is introduced. Increasing the number of receptors on the
cell surface often leads to increased downstream signalling, however in the case of TLR4 not only the increased number but association with other surface proteins (MD-2, CD14, Hsp70, Hsp90, CXCR4 and GDF5) as well as cross linking of these receptors in membrane microdomains called the lipid rafts are critical for signal transmission (540). Blocking the cell surface assembly and crosslinking of proteins involved in LPS signalling may be one of the mechanisms of hyperosmolar protection from macrophage priming. A role for this mechanism is indirectly supported by an observation that hypertonicity is known to perturb the formation of cell surface membrane domains. For example, clathrin lattices found in lipid rafts are disrupted by hyperosmolarity by preventing the interaction between clathrin and adaptors proteins (611). In addition, by disrupting the lipid structure of cell membranes and inhibiting endocytosis, hyperosmolarity was demonstrated to accumulate receptors such as GLUT4 and CXCR4 on cell surfaces (612;613). By disrupting the delicate balance between endo and exocytosis of TLR4, hyperosmolarity induces a transient accumulation of receptors at the cell surface. However, the cross linking of receptors within lipid rafts required for downstream NF-κB activation may be disrupted and therefore in the presence of LPS, under isotonic conditions, TLR4 internalization is not sufficient for downstream signalling and hence proinflammatory cascades are halted. Although the exact effect of hyperosmolarity on TLR4 intracellular transport and its assembly in the lipid rafts needs further investigation our observations together with these reports point to a potential novel mechanism of hyperosmolar antiinflammatory effect, namely prevention of the functional assembly of the LPS receptor complex at the cell membrane.

Our study does not preclude the possibility that hypertonicity may inhibit not only TLR4 surface up-regulation but also some distal step(s) required for the activation of the NF-κB transcription factor. Having demonstrated immediate effects of hyperosmolarity on the TLR4 distribution, longer-term changes were studied at the level of gene transcription. The observed reduction in the TLR4 mRNA levels following hyperosmotic treatment could result from decrease in the mRNA stability and/or decreased rate of gene transcription. Further experiments are needed to delineate these processes. TLR4 mRNA level has been recently shown by Roger and colleagues to be regulated by transcription factors at several positive (two Ets and one AP-1 sites) and negative (a GATA-like and an octamer sites) regulatory elements within 350 bp upstream of the TLR4 transcriptional start site (386). Further the transcription factor PU.1 bound to the proximal Ets site and a member of the ESE sub-family of Ets transcription factors bound to the distal Ets site, was also found indispensable for Tlr4 gene transcription (386). Although the effect of hyperosmolarity on the function of these transcription sites and binding
of the corresponding transcription factors is largely unknown, it is conceivable that by modulating this pathway hypertonic resuscitation may alter the regulation of TLR4 expression. Oxidative stress in ischemia/reperfusion is known to activate stress-activated protein kinases and was shown to promote AP-1-dependent reporter activity in rat myocytes (614). Since HTS resuscitation has been demonstrated to diminish oxidative stress, it may also be involved in attenuating the oxidative stress induced effect at the level of positive TLR4 regulators such as AP-1.

In summary, our evidence suggests that hypertonic saline resuscitation is able to:

1) prevent gut ischemia/reperfusion injury and consequently diminish systemic oxidative stress
2) with HTS resuscitation the balance of pro- and counter-inflammatory cytokines was shifted in favour of an anti-inflammatory response by macrophages with a causal role demonstrated for HTS - induced increase in IL-10 production
3) exposure to hypertonicity in vivo diminishes TLR4 migration to the macrophage surface concomitant with a suppressed TLR4 gene expression. The observed HTS protective effect is not only local, but also attenuates distant alveolar macrophage priming, thereby reducing LPS-induced NF-κB nuclear translocation in these cells and resultant lung injury. These findings suggest a novel mechanism for the in vivo protective effect of HTS resuscitation on lung injury following shock/resuscitation and provide preliminary work toward delineating the complex effects of HTS on the leukocyte.
Figure 30. The effect of Hypertonic Saline resuscitation on neutrophil sequestration in the lung as a measure of acute lung injury following Shock, resuscitation, and lipopolysacharide (LPS).

After hemorrhagic shock and resuscitation with RL or 7.5% NaCl (HTS), animals were treated with intratracheal LPS or saline. Four hours later BAL was performed and alveolar macrophages were analyzed and counted as described in Materials and Methods. Data are expressed as mean +/- SEM of 4 animals per group. *p<.005 for shock/RL/LPS vs all other groups.
Figure 31. Representative photomicrographs of small intestine histology after resuscitation with various fluid regimens.

(A) Sham (B) Shock with RL resuscitation followed by LPS (C) Shock with 7.5% NaCl (HTS) resuscitation followed by LPS (D) Shock with RL + NAC resuscitation followed by LPS. A representative picture of 3 independent studies is shown. Hematoxylin-eosin stain, magnification, X 40.
Figure 32. Plasma 8-isoprostane levels following different resuscitation regimens.

Blood samples were obtained at different time points following shock/resuscitation with RL or 7.5% NaCl (HTS) and analyzed by eicosanoid immunoassay (EIA) with acetylcholinesterase kit as described in Materials and Methods. Data are expressed as mean ± SEM of four to ten animals per group. \( p \leq 0.05 \) vs. shock/HTS at same time point.
Figure 33. The effect of Hypertonic Saline or antioxidant NAC resuscitation on LPS induced NF-κB translocation in alveolar macrophages.

After hemorrhagic shock and resuscitation with Ringer’s Lactate (RL) or 7.5% NaCl (HTS) or RL with NAC. One hour later BAL was performed and alveolar macrophages were treated with LPS (0.1 µg/ml) or saline for 30 min, then fixed and stained with a specific anti p65 NF-κB antibody and analyzed by immunofluorescence microscopy. Representative cells following immunofluorescence staining using an anti p65 subunit of NF-κB antibody as outlined in Materials and Methods. (A) Sham (B). Shock with RL resuscitation followed by LPS for 30 min (C) Shock with 7.5% NaCl (HTS) resuscitation followed by LPS for 30 min. (D) Shock with RL + NAC resuscitation followed by LPS for 30 min. A representative picture of 3 independent studies is shown.
Figure 34. The effect of Hypertonic Saline or antioxidant NAC resuscitation on LPS induced translocation of NF-κB into the nucleus in alveolar macrophages.

After hemorrhagic shock and resuscitation with Ringer’s Lactate (RL) or 7.5% NaCl (HTS) or RL with NAC. One hour later BAL was performed and alveolar macrophages were treated with LPS (0.1 µg/ml) or saline for 30 min, then fixed and stained with a specific anti p65 NF-κB antibody and analyzed by immunofluorescence microscopy. Cells with NF-κB translocated into the nucleus were counted as described in Materials and Methods. Data are expressed as mean +/- SEM of 3 animals per group. *p<.005 for shock/RL/LPS vs all other groups.
Figure 35. The effect of Hypertonic Saline Resuscitation on TNF-α production by rat alveolar macrophages with and without LPS stimulation *ex vivo*. Following hemorrhagic shock and resuscitation with Ringer’s Lactate or 7.5% NaCl (HTS), BAL was performed and alveolar macrophages were incubated *ex vivo* with or without LPS (0.1 µg/ml) for 2 hours at 37°C. TNF-α was measured in the culture supernatants by ELISA at the end of LPS incubation period. Data are expressed as mean ± SEM of four animals per group *p< 0.005 for Shock/RL/LPS vs. all other groups and *p< 0.05 for Sham/LPS vs. Sham.
Following hemorrhagic shock and resuscitation with Ringer’s Lactate or 7.5% NaCl (HTS), BAL was performed. Alveolar macrophages were incubated *ex vivo* with or without LPS (0.1 µg/ml) for 2 hours at 37°C. IL-10 was measured in the culture supernatants by ELISA at the end of LPS incubation period. Data are expressed as mean ± SEM of seven animals per group *p* < 0.002 for Shock/HTS and Shock/HTS/LPS vs. all other groups.

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**Figure 36. The effect of Hypertonic Saline Resuscitation on IL-10 production by rat alveolar macrophages with and without LPS stimulation *ex vivo***
Figure 37. The effect of Hypertonic Saline Resuscitation on IL-10 production by rat alveolar macrophages with LPS stimulation in vivo.

Following hemorrhagic shock and resuscitation with Ringer’s Lactate or 7.5% NaCl (HTS), animals were treated with intratracheal LPS or saline and four hours later, BAL was performed. BAL alveolar macrophages were incubated for 2 hours at 37°C in the absence of further stimuli was measured in the culture supernatants by ELISA at the end of the IL-10 incubation period. Data are expressed as mean ± SEM of three animals per group *p< 0.005 for Shock/HTS/LPS vs. all other groups.
Figure 38. The effect of endotracheal anti IL-10 neutralizing antibody on neutrophil sequestration in the lungs as a measure of acute lung injury following shock, resuscitation and LPS.

Following hemorrhagic shock and resuscitation with Ringer’s Lactate or 7.5% NaCl (HTS), animals were treated with intratracheal LPS or saline followed by an intratracheal anti IL-10 neutralizing antibody. Four hours later BAL was performed and alveolar macrophages were analysed and counted as described in Materials and Methods. Data are expressed as mean ± SEM of three animals per group *p< 0.005 for Shock/HTS/LPS vs. all other groups and **p< 0.005 for Shock/HTS/LPS/antiIL10 vs. sham, Shock/RL, Shock/HTS and Shock/HTS/LPS.
Figure 39. The effect of HTS resuscitation on TLR4 distribution in alveolar macrophages ex vivo.

Alveolar macrophages were isolated from sham animals or from animals that underwent hemorrhagic shock followed by resuscitation with RL or HTS as indicated. Cells were allowed to adhere to coverslips and were treated ex vivo with LPS (0.1 µg/ml) for 5min or 60 min. They were then fixed, permeabilized, and stained with anti-TLR4 antibody and analyzed by immunofluorescence microscopy. (A) Typical fluorescence microscopy images are shown. (B) Peripheralization of TLR4 was quantified by Scionimage software as described in Materials and methods. Data are expressed as mean +/− SEM of three animals per group. *p<.005 for shock/RL/LPS vs all other groups, ** p<.005 for shock/RL/LPS vs sham. (C) Specificity of the TLR4 antibody was tested by staining wild type (left image) or 10SCR (right image) macrophages with anti-TLR4 antibody. Both samples were stained with FITC-labelled secondary antibody.
Figure 40. The effect of HTS resuscitation on TLR4 surface levels in alveolar macrophages *ex vivo*. Alveolar macrophages were isolated from sham animals or from animals that underwent hemorrhagic shock followed by resuscitation with RL or HTS as indicated. Cells were allowed to adhere to coverslips and were treated *ex vivo* with LPS (0.1 µg/ml) for 5min and 60 min. AMs were stained live with anti-TLR4 and FITC-conjugated secondary antibody at 4°C. Fluorescence was analyzed by flow cytometry as described in Materials and methods. Mean channel fluorescence is reported [MCF] after the indicated treatments. Data are mean ± SEM. n = 3 animals per group. *, P < 0.05 for a group versus sham and ** p<.005 for shock/RL/LPS vs shock/HTS/LPS.
Figure 41. The effect of Osmolarity *ex vivo* on alveolar macrophage TLR4 surface expression.

Alveolar macrophages were isolated from sham animals. Cells were allowed to adhere to coverslips and were treated *ex vivo* with various concentrations of NaCl for 5 minutes and TLR4 surface expression was studied by flow cytometry. AMs were stained live with anti-TLR4 and FITC-conjugated secondary antibody at 4°C. Fluorescence was analyzed by flow cytometry as described in Materials and methods. Mean channel fluorescence is reported [MCF] after the indicated treatments. Data are mean ± SEM. *n* = 3 animals per group. *, *P* < 0.05 vs. 300 mOsm.
Figure 42. The effect of Osmolarity and LPS ex vivo on alveolar macrophage TLR4 surface expression.

Alveolar macrophages were isolated from sham animals. Cells were allowed to adhere to coverslips and were treated ex vivo with various concentrations of NaCl and then exposed to LPS (0.1 µg/ml) for 5 and 60 min under isotonic conditions. TLR4 surface expression was studied by flow cytometry. AMs were stained live with anti-TLR4 and FITC-conjugated secondary antibody at 4°C. Fluorescence was analyzed by flow cytometry as described in Materials and methods. Mean channel fluorescence is reported [MCF] after the indicated treatments. Data are mean ± SEM. n = 3 animals per group. *, P < 0.05 vs. 500 mOsm.
Figure 43. Hypertonic saline effect on TLR4 mRNA expression in RAW 264.7 macrophages.

RAW 264.7 cells were exposed to various concentrations of NaCl for one hour. Total RNA was extracted from the cells, and Northern blot analysis was performed. (A) A representative result of three independent studies is shown. (B) The densitometric analysis for TLR4 mRNA after normalization with GAPDH. Results are expressed as mean ± SEM (n = 3; *, p < 0.01 vs HTSgroup).
Trauma remains a leading cause of morbidity and mortality. In addition to loss of life, there is considerable long-term disability resulting in an enormous economic and social expense. Late mortality in trauma patients is largely due to a Systemic Inflammatory Response Syndrome with its associated organ dysfunction such as The Adult Respiratory Distress Syndrome (ARDS). Animal and human studies suggest that resuscitated hemorrhagic shock in trauma renders patients more susceptible to the development of SIRS, MODS, and ARDS. This concept of enhanced response to a stimulus that is from a previous exposure of the cell or organism to a different agonist has been used as a definition of “priming” (131). Leukocytes have been demonstrated to be primed for an increased responsiveness to a subsequent inflammatory stimulus following resuscitated shock. Specifically macrophages and neutrophils have been shown to play a pivotal role in the propagation of SIRS. In addition, it is well demonstrated by many experimental studies that reactive oxygen species play an integral role in the pathophysiological alterations associated with hemorrhagic shock that lead to leukocyte priming. However, the precise mechanism of ROS effect on macrophage priming and an exaggerated proinflammatory response to lipopolysaccharide is ill defined in the literature and subject of investigation.

The findings of this thesis help establish a novel cellular mechanism responsible for the priming of macrophages in resuscitated hemorrhagic shock and offer a better understanding of the immunomodulatory mechanisms possessed by select resuscitation agents. This thesis presents the first evidence establishing a direct link between oxidative stress during resuscitated hemorrhagic shock and translocation of the lipopolysaccharide receptor TLR4 to the plasmalemma of macrophages. This present work makes the first demonstration that oxidative stress induces lipid raft recruitment of TLR4 by way of exocytosis. Additionally, Src kinases, residing in lipid rafts sensitive to oxidative stress, are demonstrated to be involved in regulating exocytosis of the TLR4. Furthermore, raft recruitment of TLR4 induces early NF-κB signalling and suggests a novel mechanism for Oxidant-Mediated Macrophage Priming. Lastly, our findings demonstrate that macrophage priming by oxidative stress can be diminished by early exposure to resuscitation regimens with antioxidant capacity that may influence LPS cell signalling via TLR4. Together these findings raise the possibility that specifically targeted strategies at modulating raft recruitment of TLR4 in humans may represent a potential therapeutic target for oxidant mediated disease processes such as SIRS, MODS, and ARDS.

Based on our findings to date our proposed mechanism of oxidant-mediated priming is demonstrated in a schematic (Fig. 44) where macrophages once exposed to oxidative stress are
primed by activation of Src kinases in lipid rafts of the plasma membrane. Src kinase activation induces a rapid translocation of TLR4 into the plasmalemma and into lipid rafts by way of exocytosis. In this ready to signal position, when LPS is introduced, earlier and stronger signalling occurs via the LPS receptor complex assembled in lipid raft fractions resulting in a potentiated downstream response through activation of the transcription factor NF-κB.

Figure 44. Proposed mechanism of oxidant mediated macrophage priming.

Macrophages once exposed to oxidative stress are primed by activation of Src kinases in lipid rafts of the plasma membrane. Src kinase activation induces a rapid translocation of TLR4 into the plasmalemma and into lipid rafts by way of exocytosis. In this ready to signal position, when LPS is introduced, earlier and stronger signalling occurs via the LPS receptor complex assembled in lipid raft fractions resulting in a potentiated downstream response through activation of the transcription factor NF-κB.

Increased responsiveness to LPS following shock resuscitation occurs through modulating cellular distribution of the LPS receptor – Toll Like Receptor 4 (TLR4).

Reactive oxygen species (ROS) play an important role in the physiological alterations associated with HS (43). Xanthine oxidase generated during ischemia can react with the purine substrates hypoxanthine and xanthine during reoxygenation, leading to the production of the ROS superoxide and hydrogen peroxide (H₂O₂) (546). Traditionally, oxidants were considered to exert their effects in inflammation by having direct toxic effects on cells (231). Recently, there has been considerable interest in the potential role of oxidants in the regulation of cell activation, since several cellular signalling pathways are known to be redox sensitive (232-235).
Relevant to inflammation, oxidants have been shown to participate in signalling cascades which culminate in nuclear translocation of NF-κB and the induction of several proinflammatory genes (46;47;236). Although the cellular signalling cascades initiated by ROS have not yet been fully elucidated, oxidative stress generated by ischemia/reperfusion is known to prime inflammatory cells for increased responsiveness to subsequent stimuli. One of such stimuli is LPS. The mechanism(s) underlying this effect remain poorly elucidated. Diminishing oxidative stress either directly by antioxidants or indirectly by decreasing gut ischemia/reperfusion injury was shown in this thesis to abrogate macrophage priming and excessive LPS signalling via the NF-κB pathway. These observations raised the hypothesis that oxidative stress might alter cellular distribution or function of the LPS receptor, TLR4 receptor complex, as a mechanism underlying increased macrophage responsiveness to LPS. This thesis demonstrates that macrophages exposed to oxidative stress in vivo or in vitro express increased surface levels of Toll-like receptor 4 (TLR4) through an exocytic process. This effect is inhibited by antioxidants.

One of the valid criticisms of the studies presented is that the mechanisms affecting the endocytosis of the TLR4 receptor are not addressed. Oxidative stress has been shown to alter receptor trafficking, recycling and cell surface molecule expression (458;459). Increased surface expression of receptors could result not only from increased exocytosis of the newly synthesized receptors but also from the accelerated rate of recycling or inhibition of endocytosis. TLR4 recycling has not been well studied and may represent an important mechanism of regulating the innate immune response. Oxidative stress has been shown to be involved in decreasing receptor-mediated endocytosis (615). For example, EGF receptor exposure to H₂O₂ results in an activated receptor uncoupled from normal internalization, leading to prolonged receptor signalling (485). Considering that stimulation of the TLR4 with LPS results in surface downregulation of this receptor it is conceivable that exposure of macrophages to oxidative stress prevents TLR4 endocytosis and allows for augmented signalling through continuous LPS engagement at the cell membrane without internalization to reduce signal potency. Therefore, in addition to increased exocytosis from secretory vesicles, one of the alternative mechanisms not investigated here is decreased TLR4 internalization from the cell surface.

UV irradiation as well as osmotic stress have been shown to induce the multimerization of several surface receptors including EGF-R, TNF-R and IL-1R (260;405). Receptor oligomerization in the absence of the corresponding ligand was shown to be sufficient for the recruitment of downstream signalling molecules, and is believed to facilitate the efficiency of the ligand-induced signalling. Recent experiments using Fluorescence Recovery After
Photobleaching (FRAP), a method to detect lateral mobility of receptors in the membrane, demonstrated that LPS must bind to CD14 and then is transferred to an immobile receptor or cluster of receptors (376). Clustering of CD14 and TLR4 in response to LPS has been demonstrated by Fluorescence Resonance Energy Transfer (FRET) (370;413). In addition FRET studies revealed that LPS associates with hsp70, hsp90, CXCR4 and GDF5 in the lipid rafts and that way can induce TNF-α production (354). Lipid rafts are detergent-insoluble and glycolipid-enriched cell membrane areas that have been implicated to function in recruitment of cell surface signalling molecules (415) and in membrane trafficking (412;414). The results of this thesis support the proposal that TLR4 distribution on the cell membrane plays a key role in mediating the innate immune response under oxidative stress conditions. The LPS receptor complex is demonstrated to redistribute to signalling domains lipid rafts in the plasma membrane upon *in vivo* or *in vitro* exposure to oxidants. These findings support the hypothesis that hemorrhagic shock exerts its priming effect on macrophages by modulating alveolar macrophage LPS receptor expression. Together, these studies suggest a novel mechanism whereby oxidative stress might prime the responsiveness of cells of the innate immune system.

The generation of oxidative stress, during shock/resuscitation, is an important contributor to priming for an exaggerated immune response to a delayed inflammatory stimulus such as endotoxin (LPS), the so-called “two-hit hypothesis”. Oxidative stress is able to activate Src kinases. Src family of tyrosine kinases were recently demonstrated to be involved in macrophage priming by oxidants and oxidative stress was demonstrated to reprogram LPS induced NF-κB signalling such that it changes from a Src-independent pathway to one that is Src-dependent (503). Src kinases have also been reported to be regulated by various forms of oxidative stress which had implications in receptor expression and various downstream signalling actions (235;504). These observations make Src kinases possible regulators of LPS receptor expression and studies in these thesis explored Src kinase involvement in lipid raft recruitment of TLR4 under oxidative stress conditions. Inhibition of the Src kinases by pharmacological inhibition using PP2, or through a molecular approach of cell transfection with Csk, was previously found to prevent the augmented LPS-induced NF-κB translocation caused by oxidants. These findings lead us to further hypothesize that Src kinase stimulation might contribute to oxidative stress induced TLR4 translocation. The *in vitro* model of macrophage priming employed in this work demonstrates that Src inhibition by both the pharmacological as well as a molecular approach abolishes oxidative stress induced TLR4 exocytosis. These studies demonstrate macrophage priming during oxidative stress to be a Src dependent process.
Early exposure to antioxidant resuscitation strategies modulates macrophage priming and TLR4 signalling during oxidative stress conditions.

In critically ill patients, antioxidant therapy has been suggested to lessen organ injury, although beneficial effects on patient mortality have not been demonstrated (138;139;548). Although an understanding of the role of oxidants in inflammation has provided a strong rationale for the use of antioxidants such as NAC and A25 as anti-inflammatory agents in the in vitro and in vivo settings, further elucidation of their mechanism of protective action is required and is addressed in this thesis.

The findings of this thesis support the hypothesis that macrophage priming by oxidative stress can be diminished by early exposure to resuscitation regimens with antioxidant capacity that may influence cell signalling in response to LPS. A “two-hit” rodent model of shock resuscitation was used to demonstrate that antioxidants, namely 25% albumin and N-acetylcysteine inhibit LPS-induced macrophage priming. A25 but not A5 attenuated lung injury in the rodent shock/resuscitation model by decreasing LPS-induced pulmonary microvascular permeability and PMN sequestration. The protective property of A25 resuscitation was shown to be in part mediated by its effect on the adhesive properties of PMNs and pulmonary endothelium. The salutary effects of A25 resuscitation was confirmed as related to its antioxidant properties. First, the ability of A25 to blunt plasma oxidant activity, as measured by 8-isoprostane levels during hemorrhagic shock, correlated with its lung protective effect. Second, A25 like NAC prevented priming of alveolar macrophages as both LPS induced augmented NF-κB translocation and LPS induced upregulation of the chemokine CINC were diminished. Finally, oxidation of the thiol groups in A25 albumin using N-ethylmaleimide caused partial reversal of the protective effect of A25, which suggests that part of the protective effect is due to the thiol-based antioxidant activity of the molecule. Together, these findings suggest critical role of reactive oxygen species in macrophage priming during shock resuscitation and suggest that targeting oxidants during the resuscitation phase may offer novel protective effects against delayed organ injury in trauma victims.

Further, the findings in this thesis support the hypothesis that Hypertonic saline resuscitation of hemorrhagic shock exerts its anti-inflammatory effect by modulating alveolar macrophage function and LPS receptor expression through a systemic antioxidant effect. HTS previously demonstrated in experimental studies to inhibit immune cell activation in response to LPS in vitro and to reduce lung damage when used for resuscitation of hemorrhagic shock in vivo. Specifically, it was found that HTS resuscitation prevented gut ischemia/reperfusion
injury, thus decreasing oxidative stress and distant priming in alveolar macrophages. In addition, HTS resuscitation altered the balance between the proinflammatory and the counter-inflammatory immune response favouring the latter. Since the TLR4 is known to mediate LPS responsiveness, the effect of hyperosmolarity on macrophage TLR4 gene and protein expression was also investigated and they found to be decreased under hyperosmolar conditions. These findings further support the critical role of oxidants in macrophage priming leading to organ injury following shock resuscitation and delineate possible novel pathophysiological processes that may govern this response. The evidence from these studies also suggests that TLR4 cell surface expression can be influenced by oxidative stress and interventions that prevent it.

**Limitations and Clinical Significance**

This study has several limitations that are directly related to the models that are being used here to study such complex conditions as SIRS MODS and ARDS. Specifically, our studies make use of an endotoxin infusion model of SIRS that may not completely reflect the complexity of the highly regulated inflammatory response to bacterial invasion in humans. Although, an endotoxin bolus introduced into the lung can isolate certain biological responses from particular organs studied, information from this model may not correlate with a complex form of human disease. The animals in our model although displaying various physiologic parameters representative of SIRS MODS and ARDS may have very different immunological derangements then SIRS patients who are resuscitated in an ICU and treated with appropriate antibiotics. Further, the model of a controlled hemorrhage although accurate in the degree of shock induced, again may not translate directly to a clinically relevant condition. Although the severity of hemorrhage may parallel what trauma patients’ experience, other important parameters such as the duration of the shock or the circumstances under which it is sustained may not imitate the human systems directly and provoke immune responses that cannot be generally translated to a trauma patient. Further, confounding variables in our experiments are the use of anaesthetics and heparin used for animal comfort and for catheter maintenance throughout the procedure respectively. These affect cardiovascular and metabolic function as well as may affect microbial translocation from the gut (616). High doses of heparin (1000-500 units/kg body weight) have also been described affect experimental results in animal models of hemorrhagic shock (616).

Despite an abundance of data in animal models, translating preclinical therapeutic strategies into clinically efficacious ones has been a challenge. In a recent review of 101
preclinical animal studies it was found that only for one-quarter of them was a randomized trial ever undertaken in the clinical realm, and fewer than 10% resulted in an approved new therapy (617). Some of these failures can be attributed to interspecies differences others purely to the limitations of the model itself. Notwithstanding their limitations, the purpose of using animal and cellular models in this thesis is to isolate specific pathological processes under controlled conditions with the aim to modify them as a basis for future therapies in human diseases. As Dr. John Marshall points out in his recent editorial, “Perhaps the greatest value of preclinical models arises not from their capacity to demonstrate promise for a novel therapeutic approach, but from the fact that similar models may show considerable variability in the response to the same or a similar intervention. A careful and explicit attempt to understand the sources of that variability may provide insight into the circumstances where the greatest therapeutic benefit might be anticipated, and conversely, where harm may result in subsequent clinical trials.” (618). Without ignoring the complex nature of human syndromes of shock and sepsis the conclusions of this thesis may have broader implications in targeting the TLR4 mediated responses under oxidative stress conditions that may improve long – term outcomes of trauma patients.
CHAPTER 8
FUTURE DIRECTIONS
This work supports the notion that global ischemia-reperfusion resulting from hemorrhagic shock and subsequent resuscitation predisposes to organ injury by priming for an exaggerated immune response to a delayed inflammatory stimulus. Generation of oxidative stress during shock/resuscitation is a critical factor contributor to priming the immune system and specifically macrophages for an exaggerated cellular response to a delayed inflammatory stimulus such as endotoxin (LPS), the so-called “two-hit hypothesis”. By employing the in vivo model of lung injury, it was demonstrated that shock/resuscitation induced oxidative stress is capable of augmenting LPS-induced lung injury and can be inhibited by fluids with antioxidant effects. The fluids evaluated included: A25 and HTS shown capable of diminishing the generation of oxidants. Interestingly, studies using in vivo and in vitro models of the “two hit hypothesis” revealed a novel finding that oxidative stress causes Src kinase dependent translocation of the LPS receptor Toll-like receptor 4 (TLR4) into lipid rafts in the plasma membrane, where it contributes to heightened responsiveness to LPS.

Future studies aim at delineating cell surface and downstream mechanisms of oxidative stress mediated macrophage priming. Specifically, studies will focus on a more in depth understanding of TLR4 association with other proteins in the context of lipid rafts that may be critical in oxidative stress mediated downstream signalling. Future work will therefore be focused in the following areas:

**Effect of HS on clustering of TLR4 and CD14**

Recent studies have demonstrated the importance of lipid rafts for clustering the LPS receptor complex upon LPS stimulation (354;370;378;413). LPS triggers a physical association of CD14 with TLR4. Using FRET, one can demonstrate whether oxidative stress clusters TLR4 and CD14. We hypothesize that priming of Mφ in HS is mediated by lipid raft recruitment of TLR4 and CD14 without LPS stimulation. Rafts may enhance the receptor’s availability for the LPS ligand once present or alternatively by clustering-mediated recruitment of the downstream signalling molecules MyD88 and IRAK4. Macrophages exposed to oxidative stress in vivo and/or in vitro will be recovered and lipid rafts will be isolated and analyzed by Western blotting for presence of TLR4 and CD14. For direct visualization, differently labelled anti CD14 and anti TLR4 antibodies will be used to stain cells from in vitro or in vivo experiments. Cells will be fixed, immunostained and analyzed using coimmunostaining and FRET for CD14 and TLR4 proximity.
Effect of HS on the cell surface mobility of TLR4 and CD14

UV irradiation as well as osmotic stress have been shown to induce the multimerization of several surface receptors including EGF-R, TNF-R and IL-1R (260;405). Receptor oligomerization in the absence of the corresponding ligand was shown to be sufficient for the recruitment of downstream signalling molecules, and is believed to facilitate the efficiency of the ligand-induced signalling. Recent experiments using FRAP demonstrated that LPS must bind to CD14 and then is transferred to an immobile receptor or cluster of receptors (376). Clustering of CD14 and TLR4 in response to LPS has been demonstrated by FRET (370;413). In addition FRET studies revealed that LPS associates with hsp70, hsp90, CXCR4 and GDF5 in signalling lipid rafts and that way can induce TNF-α production(354).

FRAP measures the mobility of a fluorescent molecule by bleaching molecules that move in a focal area of light beam. Immediately after bleaching, a highly attenuated light beam measures the recovery of the fluorescence in the bleached area due to diffusion of fluorescent molecules from the surrounding areas. The diffusion coefficient can be derived from the recovery of fluorescence. This technique has been used to test mobility of receptors in cell membranes. We hypothesize that oxidative stress primes macrophages for increased signalling through increasing TLR4 receptor lateral mobility within the membrane that results in clustering of this receptor with CD14 and/or other proteins within lipid rafts. To study this differently labelled anti CD14 and anti TLR4 antibodies will be used to stain macrophages from in vivo and in vitro treatments. Cells will be stained live and analyzed by FRAP at different time points. These studies will provide information as to whether receptor mobility is important for macrophage priming under conditions of oxidative stress.

Effect of oxidative stress on IRAK docking to TLR4 and subsequent signalling

Lipid rafts have been shown to sequester many signalling molecules such as Src kinases. Myd88 has recently been shown to localize in lipid raft fractions upon LPS stimulation. We hypothesize that oxidative stress induced NF-κB activation is initiated by recruitment of MyD88 and IRAK into proximity of TLR4 in the lipid rafts. This will be examined using immunofluorescence microscopy and coimmunopercipitation. Cells treated in vivo or in vitro will be coimmunostained with TLR4, MyD88, IRAK, and CTXB (for raft GM-1). For coIP experiments, RAW 264.7 cells will be transfected with Flag-tagged versions of TLR4 and gD epitope tagged CD14 and treated in vitro. Antibodies will be used to IP one of the partners and then blot with the other thus determining the association of TLR4 or CD14 with IRAK or
Myd88. If so, raft expression of MyD88 and IRAK will be tested by Western blotting. The effect of raft disruption on Mφ priming will also be tested using Electrophoretic Mobility Shift Assays (EMSAs) to measure NF-κB translocation, expecting decreased priming in MCD and nystatin treated cells.

Effect of oxidative stress on signalling in MyD88 and IRAK deficient cells

Another effective way of testing the involvement of the TLR4/MyD88/IRAK/NF-κB signalling pathway in oxidative stress mediated priming is through using knockout animals or dominant negative mutant transfected cells. To test the signalling cascade, cells will be cotransfected with NF-κB reporter plasmids and/or dominant negative mutants of MyD88 and IRAK. Alternatively, macrophages will be recovered from MyD88 or IRAK deficient mice. NF-κB activation will be measured in these cells following in vitro treatment. It is expected that if MyD88 and IRAK are necessary for macrophage priming, decreased NF-κB translocation will be observed in transfected cells. These studies will elucidate the role of MyD88 and IRAK in HS induced Mφ priming.

The role of ceramide production in oxidant induced macrophage priming

Concurrent work from the same laboratory demonstrated that oxidative stress reprograms LPS signalling such that it involves Src kinase activation and leads to signalling via activated phosphatidylinositol 3 kinase (PI3 kinase). Interestingly, while Src kinase inhibition was able to prevent the LPS-induced NF-κB translocation in oxidant-treated macrophages, this strategy had no effect on NF-κB translocation caused by LPS in the absence of oxidants. These findings suggested that oxidative stress might divert LPS signalling along an alternative signalling pathway. Ceramide is known to be generated by oxidative stress and existing evidence suggests that it may contribute to lipid raft formation. Interestingly, in colonic smooth muscle cells, ceramide-induced PI3-kinase activation was found to be associated with tyrosine phosphorylation of pp60src and to be inhibited by a Src kinase inhibitor (544). Based on these observations, one future hypothesis is that oxidant-induced ceramide production in macrophages is central to the TLR4 translocation into lipid rafts as well as the altered signalling via a Src-dependent pathway. We will use both pharmacological approaches using inhibitors of ceramide generation as well as genetic approaches using macrophages from acid sphingolmyelinase deficient mice to test this hypothesis. Role of ceramide in recruitment of different signalling molecules such as IRAK and Src kinases following oxidant treatment will be studied.
results should provide new knowledge regarding LPS signalling and its modulation by oxidative stress.

RELEVANCE

Targeting upstream components of the LPS signalling pathway may represent alternate anti-inflammatory strategies. Elucidation of these processes will suggest novel therapeutic approaches to the management of the critically ill trauma patients.


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