Effect of Remote Ischemic Conditioning on Lipopolysaccharide-induced Pulmonary Inflammation

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
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Abstract

Remote ischemic preconditioning (RIPC), an intervention whereby an extremity undergoes brief repeated cycles of ischemia/reperfusion (I/R), has been shown to exert protective effects on distant organ I/R injury. Its potential benefit in organ injury induced by other inflammatory stimuli has not been determined. To test whether RIPC would exert protective effects on LPS-induced pulmonary inflammation, mice were subjected to hindlimb RIPC using a tourniquet prior to intratracheal LPS. RIPC significantly reduced leukocyte recruitment and protein leakage in the lungs at 4 hours after LPS. Lung ICAM-1 mRNA expression was also reduced, whereas TNF-α and IL-1β were upregulated, although these changes were statistically insignificant. However, the protective effect of RIPC was lost by 24 hours after LPS. Moreover, delayed conditioning and dual conditioning before and after LPS administration did not prevent lung inflammation. In conclusion, RIPC provides early protection against LPS-induced pulmonary inflammation in mice, but the effect is not sustained.
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Contributions

So Jung Choi performed the majority of the experiments, data analyses, and writing with the following exceptions. Dr. Ori Rotstein offered guidance throughout all aspects of the thesis experiments, analyses, and writing. Dr. Wolfgang Kuebler and Dr. John Semple provided advice and suggestions for future studies as members of the progress advisory committee. Chung Ho Leung provided advice for every experiment, provided training on all surgical techniques, performed a subset of RT-qPCR experiments, assigned lung injury scores to H&E stained tissue sections, and assisted with statistical and data analyses. Dr. Menachem Ailenberg provided assistance with laboratory techniques (e.g. cell counting, assessment of nucleic acid sample purity, PCR, RT-qPCR). Dr. Caterina Di Ciano-Oliveira provided training on bioimaging and MPO assay. Hajera Amatullah provided the HO-1 and GAPDH primers, and gave suggestions for the discussion. Dr. John Marshall lab provided the reagents for MPO assay. Pamela Plant provided training on RT-qPCR and assisted with data analysis. Xiaofeng Lu provided training on histology. Danielle Gifford and the Research Vivarium staff assisted with surgical preparation. Michael Kim provided training on cytopsin and Hemacolor® staining. Alexandra H. Marshall edited the written thesis.
# Table of Contents

ACKNOWLEDGEMENTS ........................................................................................................ III

CONTRIBUTIONS ............................................................................................................... IV

TABLE OF CONTENTS ........................................................................................................ V

LIST OF TABLES ................................................................................................................ VIII

LIST OF FIGURES .............................................................................................................. IX

LIST OF ABBREVIATIONS ................................................................................................ XI

CHAPTER 1 INTRODUCTION ............................................................................................. 1

1 ACUTE LUNG INJURY AND ACUTE RESPIRATORY DISTRESS SYNDROME ............... 1

1.1 Definition and prevalence .......................................................................................... 1

1.2 Diagnosis and outcomes ............................................................................................ 1

1.3 Etiology ....................................................................................................................... 2

1.4 Pathogenesis .............................................................................................................. 2

1.4.1 Disruption of alveolar-capillary barrier ................................................................. 3

1.4.2 Proinflammatory cytokines .................................................................................... 3

1.4.3 Neutrophil-dependent injury .................................................................................. 4

1.5 Current treatments ..................................................................................................... 6

2 ACUTE LUNG INJURY AND ACUTE RESPIRATORY DISTRESS SYNDROME ........ 8

2.1 Acid aspiration model ............................................................................................... 8

2.2 Bleomycin model ....................................................................................................... 9

2.3 LPS model ................................................................................................................ 10

2.3.1 LPS signaling ......................................................................................................... 10

2.3.2 Role of macrophages in LPS-induced inflammation .............................................. 11

2.3.3 Leukocyte transmigration cascade ...................................................................... 12

2.3.4 High- and low-dose LPS model .......................................................................... 17

2.3.5 Advantages and disadvantages of LPS model ..................................................... 17

3 ISCHEMIC PRECONDITIONING (IPC) ...................................................................... 18

4 REMOTE ISCHEMIC CONDITIONING (RIC) .............................................................. 19

4.1 Molecular basis of ischemic conditioning ............................................................... 19

4.1.1 Signaling components of ischemic conditioning ................................................. 19

4.1.1.1 Reactive oxygen species (ROS) ................................................................. 20

4.1.1.2 Nitric oxide (NO) ......................................................................................... 21

4.1.1.3 Protein kinase C (PKC) ............................................................................. 23

4.1.1.4 ATP-sensitive K⁺ (KATP) channel .................................................................. 24

4.1.1.5 Neural and humoral transmission .................................................................. 25

4.1.1.6 Bradykinin ..................................................................................................... 27

4.1.1.7 Heme oxygenase-1 (HO-1) .......................................................................... 29

4.1.1.8 Antioxidant defense ...................................................................................... 31

4.1.1.9 Opioid receptors ............................................................................................ 33

4.1.1.10 MicroRNA-144 .......................................................................................... 35

4.1.1.11 Intercellular adhesion molecule-1 (ICAM-1) ............................................. 36

4.1.2 Signal transduction of ischemic conditioning .................................................... 37

4.1.2.1 RISK pathway ............................................................................................. 37

4.1.2.2 SAFE pathway ............................................................................................. 39

4.2 Effect of RIC on various inflammatory conditions ................................................ 41
4.2.1 Effect of RIC on hemorrhagic shock and resuscitation (S/R) .............................................. 41
4.2.2 Effect of RIC on systemic inflammatory response ......................................................... 41
4.3 Ischemic post-conditioning .................................................................................................. 42
4.4 Combined conditioning protocols ..................................................................................... 43
  4.4.1 Animal studies ................................................................................................................. 44
  4.4.2 Clinical trials .................................................................................................................. 45
4.5 Protection of other organs by RIC ..................................................................................... 47
  4.5.1 Acute kidney injury (AKI) ............................................................................................. 47
  4.5.2 Acute lung injury (ALI) .................................................................................................. 47
  4.5.3 Acute liver ischemia/reperfusion (I/R) injury ................................................................. 48
  4.5.4 Cerebral ischemia ........................................................................................................... 48
4.6 Clinical trials .......................................................................................................................... 48

CHAPTER 2 OBJECTIVES AND HYPOTHESES ........................................................................... 51
1 OBJECTIVES ............................................................................................................................ 51
2 HYPOTHESES ........................................................................................................................ 51
  2.1 Hypothesis I: RIPC attenuates LPS-induced pulmonary inflammation. .................................. 51
  2.2 Hypothesis II: RIPC provides sustained protection against LPS-induced pulmonary inflammation .......................................................... 52
  2.3 Hypothesis III: RIPostC prevents LPS-induced pulmonary inflammation. ......................... 52
  2.4 Hypothesis IV: Dual conditioning exerts protection against LPS-induced pulmonary inflammation at 24 hours after challenge. ........................................................................ 52

CHAPTER 3 MATERIALS AND METHODS ............................................................................... 53
1 REAGENTS .................................................................................................................................. 53
2 ANIMAL MODEL OF PULMONARY INFLAMMATION ............................................................... 53
  2.1 Instrument preparation ........................................................................................................ 53
  2.2 Surgical procedure ............................................................................................................. 54
    2.2.1 Remote ischemic pre-conditioning (RIPC) .................................................................... 54
    2.2.2 Remote ischemic post-conditioning (RIPostC) ............................................................. 55
    2.2.3 Remote Ischemic Conditioning (RIC): pre- and post-conditioning ............................... 56
  2.3 Tissue harvest .................................................................................................................... 57
  2.4 Bronchoalveolar lavage (BAL) ........................................................................................... 57
3 ASSESSMENT OF PULMONARY INFLAMMATION .................................................................. 58
  3.1 Analysis of BALF ................................................................................................................ 58
    3.1.1 Cell count ...................................................................................................................... 58
    3.1.2 Protein analysis ............................................................................................................ 58
  3.2 Myeloperoxidase (MPO) activity assay .............................................................................. 58
    3.2.1 Buffers ........................................................................................................................ 58
    3.2.2 Sample preparation ....................................................................................................... 59
    3.2.3 Assay .......................................................................................................................... 59
    3.2.4 Analysis ........................................................................................................................ 59
  3.3 Histology .............................................................................................................................. 59
    3.3.1 Tissue preparation and staining .................................................................................... 59
    3.3.2 Lung injury score ......................................................................................................... 60
  3.4 Real-time quantitative polymerase chain reaction (RT-qPCR) .............................................. 60
    3.4.1 RNA preparation and reverse transcription .................................................................... 60
    3.4.2 qPCR and analysis ........................................................................................................ 60
List of Tables

Table 1. Primer sequences for qPCR experiments................................................................. 61
List of Figures

Figure 1. Schematic of the remote ischemic preconditioning (RIPC) protocol of LPS-induced pulmonary inflammation. ................................................................. 55

Figure 2. Schematic of the remote ischemic post-conditioning (RIPostC) protocol of LPS-induced pulmonary inflammation. ....................................................... 56

Figure 3. Schematic of the remote ischemic (dual)-conditioning (RIC) protocol of LPS-induced pulmonary inflammation. ................................................................. 57

Figure 4. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) total cell count at 4 hours (n=8-10 per group). .................................................. 62

Figure 5. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) neutrophil count at 4 hours (n=8-10 per group). .................................................. 63

Figure 6. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) macrophage count at 4 hours (n=8-10 per group). .................................................. 64

Figure 7. Representative images of bronchoalveolar lavage fluid (BALF) cytospin at 4 hours following LPS injection. Black arrows indicate macrophages and red arrows indicate neutrophils (40X magnification). ....................................................................................... 65

Figure 8. Representative images of hematoxylin and eosin (H&E) stained sections of lung tissue at 40X magnification, harvested at 4 hours following PBS vehicle/LPS exposure. Red circles indicate polymorphonuclear leukocytes. ....................................................................................... 66

Figure 9. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) total protein content at 4 hours following LPS exposure. ........................................ 67

Figure 10. Effect of remote ischemic preconditioning (RIPC) on lung heme oxygenase-1 (HO-1) mRNA expression at 4 hours following LPS exposure (n=3-6). ........................................ 68

Figure 11. Effect of remote ischemic preconditioning (RIPC) on lung intercellular adhesion molecule 1 (ICAM-1) mRNA expression at 4 hours following LPS administration (n=3-6). .... 69

Figure 12. Effect of RIPC on lung TNF-α mRNA expression at 4 hours following LPS administration (n=3-7). ....................................................................................... 70
Figure 13. Effect of remote ischemic preconditioning (RIPC) on lung IL-1β mRNA expression at 4 hours following LPS administration (n=3-7). ................................................................. 71

Figure 14. Hematoxylin and eosin (H&E) stained lung histology imaged at 20X magnification, 24 hours following PBS vehicle/LPS exposure. ................................................................. 72

Figure 15. Lung injury score determined at 24 hours following PBS/LPS instillation (n=3-4). 73

Figure 16. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) total cell count at 24 hours (n=3 per group). ................................................................. 74

Figure 17. Effect of remote ischemic preconditioning (RIPC) on myeloperoxidase (MPO) activity in the lung at 24 hours following LPS injection (n=3-4). ................................................................. 75

Figure 18. Bronchoalveolar lavage fluid (BALF) total protein concentration measured at 24 hours following LPS exposure (n=3-4 per group). ................................................................. 76

Figure 19. Effect of post-conditioning on bronchoalveolar lavage fluid (BALF) total cell count at 4 hours following LPS injection (n=3-5). ................................................................. 77

Figure 20. Effect of post-conditioning on bronchoalveolar lavage fluid (BALF) neutrophil count at 4 hours following LPS injection (n=3-5). ................................................................. 78

Figure 21. Effect of post-conditioning on bronchoalveolar lavage fluid (BALF) total protein concentration at 4 hours following LPS injection (n=3-5). ................................................................. 79

Figure 22. Effect of pre- and post-conditioning on bronchoalveolar lavage (BALF) total cell count at 24 hours following LPS injection (n=3-5). ................................................................. 80

Figure 23. Effect of pre- and post-conditioning on bronchoalveolar lavage fluid (BALF) neutrophil count at 24 hours following LPS injection (n=3-5). ................................................................. 81

Figure 24. Effect of pre- and post-conditioning on bronchoalveolar lavage fluid (BALF) total protein concentration at 24 hours following LPS injection (n=3-5). ................................................................. 82
List of Abbreviations

5-HD, 5-hydroxydecanoate
8-SPT, 8-(p-sulfophenyl)theophylline
AB, assay buffer
ACE, angiotensin-converting enzyme
ACh, acetylcholine
ACK, ammonium-chloride-potassium lysis buffer
AFT, activating transcription factor 2
Ago, Argonaute-2
AKI, acute kidney injury
Akt, protein kinase B
ALI, acute lung injury
ALT, alanine aminotransferase
AP-1, activator protein 1
A/R, anoxia and reperfusion
ARDS, acute respiratory distress syndrome
ARE, antioxidant response element
ARF, acute renal failure
AST, aspartate aminotransferase
BAD, Bcl-2-associated death promoter
BAL, bronchoalveolar lavage
BALF, BAL fluid
Bax, Bcl2-associated X protein
BMDMs, bone marrow-derived macrophages
BSA, bovine serum albumin
cAMP, cyclic adenosine monophosphate
CAO, coronary artery occlusion
CAT, catalase
CCA, common carotid arteries
C/EBPδ, CCAAT/enhancer-binding protein delta
CO, carbon monoxide
Ct, cycle threshold
CXCL, chemokine (C-X-C) motif ligand
DAG, diacylglycerol
DFP, diisopropylfluorophosphate
DNase, deoxyribonuclease
DOG, 1,2-dioctanoyl-sn- glycerol
ECM, extracellular matrix
ELISA, enzyme-linked immunosorbent assay
eNOS, endothelial nitric oxide synthase
ERK, extracellular-signal-regulated kinase
ERM, Ezrin, radixin, and moesin
ESL-1, E-selectin ligand
FACS, fluorescence-activated cell sorting
FMD, flow-mediated dilation
GATA-1, GATA-binding factor 1
GM-CSF, granulocyte-macrophage colony-stimulating factor
GPCR, G-protein-coupled receptors
GPx, glutathione peroxidase
GSH, glutathione
GSK3β, glycogen synthase kinase 3 beta
HCl, hydrochloric acid
H&E, hematoxylin and eosin
HIF-1α, hypoxia-inducible factor 1-alpha
HO-1, heme oxygenase-1
HUVECs, human umbilical vein endothelial cells
ICAM-1, intercellular adhesion molecule-1
IL-1, interleukin-1
IκB, inhibitor of κ light chain gene enhancer in B cells
IKK, IκB kinase
iNOS, inducible nitric oxide synthase
IP_3, inositol 1,4,5-triphosphate
IPC, ischemic preconditioning
I/R, ischemia and reperfusion
IRAK, IL-1 receptor-associated kinase
JAK, Janus kinase
JAMs, junctional adhesion molecules
KATP channel, ATP-sensitive K⁺ channel
LAD artery, left anterior descending artery
LB, lysis buffer
LBP, lipopolysaccharide-binding protein
LDH, lactate dehydrogenase
L-NAME, NG-nitro-L-arginine methyl ester
LPS, lipopolysaccharide
LSP-1, lymphocyte-specific protein 1
MAC1, macrophage antigen 1
MACCE, major adverse cardiac and cerebrovascular events
MAO, mesenteric artery occlusion
MAPK, p38 mitogen-activated protein kinase
MBF, microcirculatory blood flow
MCA, middle cerebral artery
MCP-1, monocyte chemoattractant protein-1
MD-2, lymphocyte antigen-2
MDA, malondialdehyde
MEK, mitogen-activated protein kinase kinase
MIP-2, macrophage inflammatory protein 2
miR, microRNA
MMPs, matrix metalloproteinases
MnSOD, manganese superoxide dismutase
MP, microparticle
MPG, N-2-mercaptopropionylglycine
MPO, myeloperoxidase
mPTP, mitochondrial permeability transition pore
MyD88, myeloid differentiation primary response gene 88
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS, neuronal nitric oxide synthase
NO, nitric oxide
Nor-BNI, nor-binaltorphimine
NOS, nitric oxide synthases
Nrf2, nuclear factor erythroid 2-related factor 2
NTD, naltrindole
PaO₂, partial pressure of arterial oxygen
PAR-1, proteinase activated receptor-1
PBMCs, peripheral blood mononuclear cells
PCI, percutaneous coronary intervention
PECAM-1, platelet/endothelial cell adhesion molecule 1
PI3K, phosphoinositide 3-kinase
PIP₂, phospholipid phosphatidylinositol 4,5-bisphosphate
PKC, protein kinase C
POC, ischemic post-conditioning
PPARα, peroxisome proliferator-activated receptor alpha
PSGL-1, P-selectin glycoprotein ligand 1
RARα, retinoic acid receptor alpha
RIC, remote ischemic conditioning
RIP-1, receptor-interacting protein 1
RIPC, remote ischemic preconditioning
RIPerC, remote ischemic per-conditioning
RIPostC, remote ischemic post-conditioning
RISK, reperfusion injury salvage kinase
RNase, ribonuclease
ROS, reactive oxygen species
RT-qPCR, real-time quantitative polymerase chain reaction
SAFE, survivor activating factor enhancement
SIRPα, signal-regulatory protein alpha
SOD, superoxide dismutase
S/R, hemorrhagic shock and resuscitation
SRAM, sterile α and HEAT-Armadillo motifs-containing protein
STAT3, signal transducer and activator of transcription 3
TAK1, transforming growth factor-β-activated kinase 1
TANK, TRAF family member-associated NFκB activator
TBK1, TANK binding kinase 1
TIR, Toll/interleukin-1 receptor
TIRAP, TIR domain-containing adaptor protein
TLR4, Toll-like receptor 4
TNF-α, tumour necrosis factor alpha
Tollip, Toll-interacting protein
TRAF6, TNF receptor-associated factor 6
TRAM, TRIF-related adaptor molecule
TRIF, TIR domain-containing adaptor inducing IFN-β
UBC13, ubiquitin-conjugating enzyme 13
UEV1A, ubiquitin-conjugating enzyme E2 variant 1 isoform A
VCAM-1, vascular cell adhesion protein 1
VE-cadherin, vascular endothelial cadherin
VEGF, vascular endothelial growth factor
VVOs, vesiculo-vacuolar organelles
WB, working buffer
WM, wortmannin
W/D ratio, wet-to-dry ratio
Chapter 1 Introduction

1 Acute lung injury and acute respiratory distress syndrome

1.1 Definition and prevalence

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) were first described in 1967 (Ashbaugh, Bigelow et al. 1967) and are characterized by a rapid onset of severe hypoxemia and pulmonary edema. Worldwide estimates of ALI range from 1.5 to 75 cases per 100 000 people per year (Arroliga, Ghamra et al. 2002). Rubenfeld and colleagues estimated that in the United States there are 190 600 cases/year of ALI, of which there are 74 500 deaths and 3.6 million days of hospitalization; therefore, ALI has a tremendous impact on public health (Rubenfeld, Caldwell et al. 2005).

1.2 Diagnosis and outcomes

According to the 2012 Berlin Definition of ARDS, ARDS is divided into three different categories with respect to the degree of hypoxemia: mild (PaO$_2$/FiO$_2$ oxygenation ratio: greater than 200 mmHg, but less than or equal to 300 mmHg), moderate (100 mmHg < PaO$_2$/FiO$_2$ ≤ 200 mmHg), and severe (PaO$_2$/FiO$_2$ ≤ 100 mmHg). Its onset must be within 1 week of a known cause or new or aggravating respiratory symptoms. ARDS is characterized by pulmonary edema of non-cardiac origin, manifested as bilateral opacities on the chest radiograph or CT scan. More severe form of ARDS would have more extensive opacities (Ranieri, Rubenfeld et al. 2012).

Mortality in patients with ALI depends on various factors, such as age, presence of other organ dysfunctions, and shock. Although ALI is characterized by hypoxemia, the extent of gas exchange dysfunction is a poor indicator of outcome, unless it is extremely severe (Luhr, Antonsen et al. 1999). The most recent randomized trials have shown that the rate of 28-day mortality is approximately 25-30% (McIntyre, Pulido et al. 2000). If not resolved after the acute phase, ALI/ARDS may progress to fibrosis with prolonged hypoxemia, increased alveolar dead space, and further loss of pulmonary compliance (Ware and Matthay 2000). In most patients that survive, pulmonary function is fairly restored within 6 to 12 months. However, those individuals still may experience mild pulmonary restriction, obstruction, and impaired gas exchange with
exercise (Ware and Matthay 2000). Patients who were subjected to prolonged mechanical
ventilation due to severe disease are at higher risk for long-lasting impairment of pulmonary
function (Ware and Matthay 2000).

1.3 Etiology

The onset of ALI often follows a triggering event and may be affected by genetic
polymorphisms. Direct injury includes pneumonia, gastric aspiration, drowning, alveolar
hemorrhage, and inhalation of toxic gas and smoke. Indirect injury may involve severe sepsis,
transfusions, shock, and salicylate or narcotic overdose. The probability of developing ALI
depends on the predisposing disorder; certain conditions, such as severe sepsis, are more likely to
lead to development of ALI than others (Arroliga, Ghamra et al. 2002). ALI affects patients of
all ages. Genetic polymorphisms may also play a role in the predisposition of ALI. Marshall and
colleagues have examined allele frequencies for common polymorphisms in angiotensin
converting enzyme (ACE) with respect to ARDS (Marshall, Webb et al. 2002). Activation of the
local renin-angiotensin system in the lung as well as the pulmonary circulation may affect the
progression of ALI/ARDS through a number of ways, as it increases vasoconstriction, vascular
permeability, and reduces lung epithelial cell survival (Marshall 2003). A polymorphism exists
within the human ACE gene, where the deletion of a 287-base pair fragment leads to higher ACE
activity in the tissue and plasma (Rigat, Hubert et al. 1990). The authors reported that a higher
frequency of the deletion alleles was seen in ARDS patients compared to ICU-admitted, non-
ARDS patients and healthy volunteers. Moreover, the genotype was associated with a higher
mortality rate in ARDS patients (Marshall, Webb et al. 2002).

1.4 Pathogenesis

During the acute phase of ALI/ARDS, alveolar macrophages secrete proinflammatory molecules,
such as interleukin-1 (IL-1), IL-6, IL-8, and tumour necrosis factor-alpha (TNF-α), into the
surroundings, which induces neutrophil chemotaxis and activation. IL-1 can also promote
production of extracellular matrix by fibroblasts. Neutrophils cause injury to the epithelium and
endothelium during massive transmigration and through release of oxidants, proteases, and
proinflammatory cytokines. Protein-rich hyaline membranes form on the denuded basement
membrane. Epithelial and endothelial injury increases permeability. An influx of protein-rich
fluid into the alveolar compartment due to compromised cell junctions leads to surfactant
inactivation and depletion, further aggravating the inflammatory state (Arroliga, Ghamra et al. 2002).

1.4.1 Disruption of alveolar-capillary barrier

The alveolar-capillary barrier is formed by the alveolar epithelium and the microvascular endothelium. Under normal conditions, the alveolar epithelium is comprised of two types of cells. Flat type I epithelial cells contribute to 90% of the alveolar surface area. Cuboidal type II cells are responsible for surfactant production, ion transport, and proliferation and differentiation into type I cells following injury (Modelska, Pittet et al. 1999). ALI is an acute inflammatory disorder that leads to disruption of the lung endothelial and epithelial barriers (Modelska, Pittet et al. 1999). As a result, patients suffer from pulmonary edema caused by the influx of protein-rich fluid into the alveolar compartment (Ware and Matthay 2000). The degree of alveolar epithelial injury is a crucial factor in the development and recovery of ALI. Type II cell injury can lead to disruption of epithelial fluid transport, which interferes with the removal of edema fluid from the alveolar space (Modelska, Pittet et al. 1999). Moreover, in severe conditions, inefficient epithelial repair may lead to fibrosis (Bitterman 1992). Other cellular characteristics of ALI involve a massive influx of neutrophils into the lung interstitium and alveolar space, as well as the release of proinflammatory cytokines that act to aggravate the inflammatory state (Arroliga, Ghamra et al. 2002).

Similarly, injury to the microvascular endothelium gives rise to increased capillary permeability. Increased permeability leads to leakage of protein-rich fluid into the lung interstitium, which eventually crosses the epithelial barrier into the alveolar compartment (Pugin, Verghese et al. 1999). Endothelial injury is associated with increased release of von Willebrand factor (vWF), a marker of endothelial activation and injury (Ware, Eisner et al. 2004), and elevated soluble intercellular adhesion molecule-1 (sICAM-1) expression (Flori, Ware et al. 2003).

1.4.2 Proinflammatory cytokines

Proinflammatory cytokines amplify the inflammatory response in ALI and ARDS. Such cytokines are produced locally by immune cells, lung epithelial cells, or fibroblasts. The balance between pro- and anti-inflammatory agents is essential. Numerous endogenous inhibitors of
proinflammatory cytokines have been identified in the context of ALI; for example, IL-1 receptor antagonist, soluble TNF receptor, autoantibodies targeting IL-8, and anti-inflammatory cytokines (e.g. IL-10 and -11) all counteract the inflammatory response (Pittet, Mackersie et al. 1997). The imbalance between the two opposing groups of cytokines may contribute to the development of ALI/ARDS. Non-pulmonary factors may also play a role in the regulation of cytokine production. For instance, the BALF of ARDS patients contains high levels of macrophage inhibitory factor (MIF), which is released from the anterior pituitary (Donnelly, Haslett et al. 1997). MIF enhances production of proinflammatory cytokines IL-8 and TNF-α and abrogates glucocorticoid-mediated inhibition of cytokine production (Ware and Matthay 2000).

1.4.3 Neutrophil-dependent injury

Transepithelial migration of neutrophils is highly important in the pathogenesis of ALI because neutrophils are the key players in the acute inflammatory response. During leukocyte transmigration, the epithelial barrier needs to temporarily open in order for neutrophils to gain passage (Zemans, Colgan et al. 2009). Under pathological conditions, excessive transmigration of neutrophils can cause damage to the epithelium (Nusrat, Parkos et al. 1997) due to the release of harmful neutrophil-derived mediators and the mechanical stress exerted by the neutrophil pseudopods (Nash, Stafford et al. 1987). The damage caused by neutrophils can directly affect epithelial integrity, thereby promoting edema.

In the early phase of transepithelial migration, “scout” neutrophils initially cross the epithelium individually. Over time, neutrophils begin to migrate in very large numbers, following the tracks of the leading leukocytes (Ginzberg, Cherapanov et al. 2001). The mechanical force applied by the multitude of migrating neutrophils to the epithelial cells at the intercellular junction injures the epithelium (Zemans, Colgan et al. 2009). Such wounds can “denude” the epithelium, thus leading to the development of ulcerated lesions that characterize ALI/ARDS (Parkos 1997).

Neutrophils release various proteinases upon activation, such as elastase. Elastase is a serine proteinase stored in azurophilic granules. The bronchoalveolar lavage fluid (BALF) and plasma of ARDS patients contain high levels of elastase, which are associated with the severity of the disease (Suter, Suter et al. 1992, Donnelly, Macgregor et al. 1995). In animal models, administration of elastase has been shown to cause lung injury, while elastase inhibition has a
protective effect. Intratracheal administration of human neutrophil elastase caused a massive lung hemorrhage, as shown by increased BALF hemoglobin content in hamsters (Tremblay, Vachon et al. 2002). In contrast, intratracheal administration of an elastase-specific inhibitor, trappin-2, prior to human neutrophil elastase administration, dose dependently inhibited lung injury in hamsters (Tremblay, Vachon et al. 2002).

Evidence suggests that elastase contributes to lung injury by promoting degradation of the basement membrane and endothelium, neutrophil migration, and apoptosis of lung epithelial cells. Elastase is known to degrade the basement membrane. When soluble laminin, which are a major component of the basal lamina of the basement membrane, was incubated with supernatants from stimulated human neutrophils, cleaved laminin fragments were detected (Heck, Blackburn et al. 1990). Treatment of the supernatant with diisopropylfluorophosphate (DFP), which is a serine protease inhibitor, effectively blocked laminin digestion (Heck, Blackburn et al. 1990). In addition, elastase facilitates paracellular transmigration of neutrophils by cleaving epithelial-cadherin and vascular endothelial cadherin (Carden, Xiao et al. 1998), which may contribute to increased epithelial permeability. Several studies suggest a role for elastase in lung epithelial cell apoptosis. Intratracheal administration of elastase caused lung epithelial cell and vascular endothelial cell apoptosis in animal models (Yang, Kettritz et al. 1996). Furthermore, elastase activates proteinase activated receptor-1 (PAR-1), which in turn leads to increased mitochondrial permeability, release of cytochrome c to cytosol, and activation of caspase-9 and -3, thereby promoting epithelial cell apoptosis (Suzuki, Morales et al. 2005).

In addition to elastase, activated neutrophils release matrix metalloproteinases (MMPs) and defensins, which also aggravate the inflammatory condition. MMPs are found in high concentrations in BALF and plasma of ALI/ARDS patients (Torii, Iida et al. 1997), and MMP levels correlate with the severity of the disease (Fligiel, Standiford et al. 2006). MMPs mediate tissue injury and facilitate neutrophil transmigration along with elastase (Donnelly, Haslett et al. 1997). High levels of defensins are also detected in the BALF and plasma of ARDS patients (Ashitani, Mukae et al. 2004). Defensins are cationic, antimicrobial peptides that enhance permeability in cultured epithelia and cause injury to the endothelium (VanWetering, MannesseLazeroms et al. 1997, Aarbiou, Rabe et al. 2002). Further, defensins cause lung injury in a dose-dependent manner, upon intratracheal administration into mice (Zhang, Porro et al. 2001).
Oxidants are also released by neutrophils and are key in the pathogenesis of ALI. Mortality in ALI patients is associated with increased levels of plasma and lung oxidants (Quinlan, Lamb et al. 1997). In animal models, neutrophil-derived reactive oxygen species (ROS) have been shown to induce lung injury by increasing permeability and causing histological damage (Folz, Abushamaa et al. 1999). Gao et al. demonstrated the effect of neutrophil ROS production on lung injury using a sepsis model (Gao, Standiford et al. 2002). When mice lacking the NADPH oxidase complex, which is needed for the respiratory burst of neutrophils, were challenged with Escherichia coli, neutrophils were recruited to the lungs but the lung microvascular permeability did not increase. Thus, superoxide radical production by neutrophils is needed to induce lung injury (Gao, Standiford et al. 2002).

1.5 Current treatments

The main focus of current ALI treatment is supportive care, to prevent complications and to treat the underlying cause, while ensuring adequate oxygenation. There is no specific therapy for ARDS. Mechanical ventilation is required for all ALI/ARDS patients and is often accompanied by prone positioning, fluid management, and use of pharmacological agents.

Traditional methods of mechanical ventilation involved 10 to 15 mL/kg of body weight, which are greater than the tidal volumes in healthy adults at rest (7-8 mL/kg). Such high volumes are often necessary to achieve normal values in the partial pressure of arterial carbon dioxide and pH. However, in animal models, mechanical ventilation with large tidal volumes caused excessive distention of the lung and, therefore, aggravated lung inflammation (Brower, Matthay et al. 2000). In addition, mechanical ventilation led to disruption of the pulmonary endothelium and epithelium, hypoxemia, and release of proinflammatory cytokines, as reviewed by Brower and colleagues (Brower, Matthay et al. 2000). The National Heart, Lung, and Blood Institute ARDS Clinical Trials Network conducted a multicenter, randomized controlled trial of 861 ALI/ARDS patients, where they compared traditional ventilation (12 mL/kg) to lower tidal volume ventilation treatment (6 mL/kg) (Parsons, Eisner et al. 2005). Significantly lower mortality, as well as more ventilator-free days during the first 28 days after randomization were observed in the lower volume ventilation group compared to the group that received the traditional treatment (Parsons, Eisner et al. 2005). Lower tidal volume ventilation treatment is
associated with attenuation of inflammation, as evidenced by reduced plasma IL-6 and IL-8 levels (Parsons, Eisner et al. 2005).

Other non-ventilation strategies include prone positioning and fluid management. Prone positioning is thought to promote redistribution of blood flow and ventilation to increase oxygenation and improve dorsal lung function (Lamm, Graham et al. 1994). Fluid management is an issue because pulmonary edema results from increased lung vascular permeability, which worsens as intravascular hydrostatic pressure increases and oncotic pressure decreases. A randomized controlled study conducted by the ARDS network involving 1000 patients investigated a conservative approach to fluid management, where fluid intake was minimized and diuresis was encouraged (Wiedemann, Wheeler et al. 2006). This conservative approach led to improved lung function and more ventilation-free days without increasing non-pulmonary organ dysfunction when compared to an unrestricted method of fluid management (Wiedemann, Wheeler et al. 2006).

Pharmacological strategies are also used to reduce inflammation. β2-adrenergic receptor agonists are of interest because both human and animal studies have reported their effectiveness in the resolution of pulmonary edema by reducing inflammation. A retrospective study of ARDS patients observed that inhalation of salbutamol, a β2 agonist, was associated with a shorter duration and less severity of ALI (Manocha, Gordon et al. 2006). Similarly, pretreatment with terbutaline, another β2 agonist, lowered serum TNF-α levels, increased plasma IL-10, and improved survival in LPS-induced ALI rats (Wu, Liao et al. 2000). However, a recent multicenter, randomized controlled clinical trial showed that intravenous administration of salbutamol was poorly tolerated, could aggravate the condition, and was associated with increased mortality in ARDS patients (Smith, Perkins et al. 2012). Routine use of the drug was not recommended (Smith, Perkins et al. 2012).
2 Acute lung injury and acute respiratory distress syndrome

Animal models should ideally be able to mirror the clinical manifestations and underlying mechanisms seen in ALI patients. Various models of ALI exist, and each one has its own advantages and disadvantages.

2.1 Acid aspiration model

Aspiration of gastric contents is a risk factor for ARDS (Hudson, Milberg et al. 1995). Many studies have used hydrochloric acid (HCl) to mimic the low pH of gastric content in animals. However, gastric contents also contain other substances that may contribute to the pathogenesis of aspiration-related ALI, such as bacterial cell wall products, proinflammatory cytokines, and food particles (Knight, Rutter et al. 1993). Acids with a lower pH than the physiological pH are often used to cause lung injury in animal models (Matute-Bello, Frevert et al. 2008).

Intratracheal instillation of low pH acid causes damage mainly to the lung epithelium, affecting type I epithelial cells, which in turn results in proliferation of type II epithelial cells as a repair response (Folkesson, Matthay et al. 1995). Acid aspiration has been shown to reduce the ability to transport alveolar epithelial fluid and compromise alveolar fluid clearance, despite normal levels of pulmonary blood flow and vascular filtration (Modelska, Pittet et al. 1999). It also appears to cause damage to the vascular endothelium through neutrophil activation (Folkesson, Matthay et al. 1995). However, the exact mechanism of action through which acid aspiration causes lung injury has not been elucidated. Since bodily fluids are able to rapidly neutralize the acid with the protein and bicarbonate buffering system, it is difficult to conclude whether acid aspiration causes direct injury to the epithelium (Matute-Bello, Frevert et al. 2008).

HCl is directly injected into the trachea while an animal is under mechanical ventilation. The concentration of acid determines the severity of damage. The most common concentration is 0.1 N of HCl. Acid instillation causes acute inflammation in the lungs that involves neutrophil infiltration, alveolar hemorrhage, accumulation of proteinaceous debris, pulmonary edema, and defective alveolar fluid clearance (McAuley, Frank et al. 2004). Fibrosis is also seen at approximately 7 days following instillation (Yano, Deterding et al. 1996). Acid aspiration physiologically affects lung compliance, which in turn leads to lower functional residual capacity.
It also increases pulmonary vascular resistance and pulmonary arterial pressure (Matute-Bello, Frevert et al. 2008). Very reproducible results can be obtained from the acid aspiration model, and this model is useful for examining the physiological changes associated with ALI and mechanisms of neutrophil infiltration. It can produce a more clinically relevant scenario when used with mechanical ventilation (Folkesson, Matthay et al. 1995).

The disadvantages of the acid aspiration model involve the difference between the actual composition of the gastric content and pure acid. As mentioned, lack of other gastric substances may result in a different biological response. Furthermore, a fine line exists between effective and ineffective doses/concentrations; therefore, it is difficult to find an appropriate concentration for a particular model (Matute-Bello, Frevert et al. 2008).

### 2.2 Bleomycin model

Bleomycin is a commonly used mouse model of ALI, although it not only causes inflammation as in the case of ALI, but also induces severe pulmonary fibrosis. Bleomycin is an antineoplastic antibiotic derived from *Streptomyces verticillus* (Umezawa 1965). It associates with oxygen and metal ions, forming a complex that leads to oxygen radical production and subsequent DNA cleavage and cell death (Burger, Peisach et al. 1981).

The intratracheal bleomycin model requires only a single dose of bleomycin, where it mainly targets the pulmonary epithelium to cause lung injury and fibrosis (Moore and Hogaboam 2008). Intravenous administration of bleomycin requires multiple doses and yields less reproducible results, so the intratracheal model is favoured. Intratracheal administration of bleomycin causes a number of inflammatory responses. Within 24 hours of administration, neutrophil infiltration into the alveolar space is observed, then subsides by day 11 (Cavarra, Carraro et al. 2004). Fibrosis is seen in tissues by day 11 and persists until day 20 (Cavarra, Carraro et al. 2004). Proinflammatory cytokines, such as IL-1β, IL-6, and TNF, reach maximum expression between 6 and 63 hours (Cavarra, Carraro et al. 2004). Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), and IL-4 achieve maximum expression at day 14 (Cavarra, Carraro et al. 2004). The acute phase of bleomycin-induced lung injury involves inflammation in the alveolar compartment in small, isolated areas, largely due to an influx of neutrophils. The late phase involves an uneven distribution of fibrosis (Shen, Haslett et al. 1988).
The bleomycin model partly reflects the pattern of ALI, which can be characterized by early inflammation and late fibrosis in the process of resolution (Matute-Bello, Frevert et al. 2008).

Unlike what is observed in ARDS patients, bleomycin does not cause hyaline membrane formation. Another disadvantage is that bleomycin only causes lung injury in certain species. Species that express high levels of bleomycin hydrolase, a protease capable of inactivating bleomycin (Sebti, Mignano et al. 1989), are less susceptible. However, species that express low levels of this protease, such as C57BL/6 mice, are more prone to bleomycin-induced injury (Lazo and Humphreys 1983). Despite all the advantages that are associated with this model, its clinical relevance remains questionable.

### 2.3 LPS model

#### 2.3.1 LPS signaling

LPS is an immunostimulatory component of Gram-negative bacteria, found on the outer membrane. LPS is comprised of three parts: lipid A, a core oligosaccharide, and an O side chain (Raetz and Whitfield 2002, Miller, Ernst et al. 2005). Lipid A is the part that is recognized by LPS-binding protein (LBP), which facilitates the transfer of LPS to the Toll-like receptor 4 (TLR4)/lymphocyte antigen-2 (MD-2) receptor complex along with CD14 (Tobias, Soldau et al. 1986, Wright, Tobias et al. 1989). CD14 is a glycosylphosphatidylinositol-anchored protein, mainly expressed in macrophages, which also exists in a soluble form. LPS signaling is mediated by the TLR/MD-2 receptor complex (Wright, Ramos et al. 1990). Upon LPS binding, TLR4 undergoes oligomerization and binds to downstream adaptors through interactions with the Toll/interleukin-1 receptor (TIR) domains, which are critical for signal transduction (Poltorak, He et al. 1998).

TLRs interact with five different adaptor molecules that contain the TIR domain: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α and HEAT-Armadillo motifs-containing protein (SARM) (O’Neill and Bowie 2007). TLR4 is known to interact with all five of these adaptor proteins. TLR4 signaling can be divided into MyD88-dependent and MyD88-independent pathways. The
MyD88-dependent pathway mediates proinflammatory cytokine production, while the MyD88-independent pathway is associated with Type 1 interferon expression (Lu, Yeh et al. 2008).

In addition to TIR domain, MyD88 also has a death domain that is able to interact with other death domain-containing molecules, such as IL-1 receptor-associated kinase-4 (IRAK-4). IRAK-4 is a kinase that is important for the transmission of TLR signals, including induction of proinflammatory cytokines (Suzuki, Suzuki et al. 2002). It also recruits and activates IRAK-1, which goes on to activate TNF receptor-associated factor 6 (TRAF6). TRAF6 interacts with ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 isoform A (UEV1A), and activates transforming growth factor-β-activated kinase 1 (TAK1) (Lomaga, Yeh et al. 1999, Gohda, Matsumura et al. 2004), which then activates downstream IκB kinase (IKK) and p38 mitogen-activated protein kinase (MAPK) pathways (Sato, Sanjo et al. 2005). IKKα, β and γ form a complex and phosphorylate inhibitor of κ light chain gene enhancer in B cells (IκB) proteins, leading to IκB protein degradation and subsequent release and nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the master regulator of proinflammatory cytokine expression (Lu, Yeh et al. 2008). Activation of MAPK promotes expression of another transcription factor activator protein 1 (AP-1), which also acts to increase expression of proinflammatory cytokines (Maitra, Deng et al. 2012).

LPS recognition by the TLR4 complex also leads to increased expression of Type I interferons, which raise anti-bacterial and anti-viral responses (Bowie and Haga 2005). This is mediated through the MyD88-independent pathway that relies largely on TRIF. TRIF interacts with TRAF3, which binds to TRAF family member-associated NFκB activator (TANK), TANK binding kinase 1 (TBK1), and IKKi. The resulting complex promotes translocation of IRF3 (Hemmi, Takeuchi et al. 2004), which induces transcription of Type I interferons (Moynagh 2005, Honda and Taniguchi 2006), collaborating with NFκB that has been activated through the TRIF-dependent activation of receptor-interacting protein 1 (RIP1) (Cusson-Hermance, Khurana et al. 2005).

### 2.3.2 Role of macrophages in LPS-induced inflammation

As part of the innate immune system, macrophages express TLR4 on the cell surface, making them one of the first local detectors of danger. Due to high plasticity, macrophages are able to change their phenotype depending on the stimulus. Macrophages are classified on a continuum,
where proinflammatory M1 and anti-inflammatory M2 phenotypes define either end of the spectrum (Gordon and Taylor 2005, Mosser and Edwards 2008). LPS and IFN-γ can stimulate macrophages to take on the M1 phenotype. Classically activated M1 macrophages actively secrete inflammatory cytokines, such as TNF-α, IL-1β, and monocyte chemoattractant protein-1 (MCP-1), express high levels of inducible nitric oxide synthase (iNOS), and are more capable of antigen presentation (Benoit, Desnues et al. 2008). LPS stimulates macrophages to produce proinflammatory mediators through the activation of NFκB as mentioned, and these M1 macrophages are able to confront microorganisms to protect the host (Liao, Sharma et al. 2011). On the other hand, IL-4 and IL-13 stimulation activates M2 macrophages that have anti-inflammatory characteristics. M2 macrophages are involved in tissue remodeling, have increased phagocytic capacity, and express high levels of IL-10 and the mannose receptor (Sica and Mantovani 2012).

2.3.3 Leukocyte transmigration cascade

2.3.3.1 Endothelial transmigration

1) Recruitment: Neutrophils may be recruited from the bloodstream and from the bone marrow(Phillipson and Kubes 2011). The major players involved in neutrophil recruitment from the bone marrow include leukotriene B4, C5α, and IL-8. IL-8 is a CXC chemokine, where CXC represents the two N-terminal cysteines that are separated by one amino acid, “X”. In rodents, the key chemokines are chemokine (C-X-C) motif ligand 1 (CXCL1) and CXCL2 (also called macrophage inflammatory protein 2; MIP-2) (Furze and Rankin 2008). IL-8 in humans and CXCL1, 2, and 5 in mice are ELR-CXC chemokines, where ELR indicates the presence of a glutamate-leucine-arginine motif immediately preceding the N-terminal CXC motif (Sadik, Kim et al. 2011). ELR-CXC chemokines play an important role in neutrophil activation, as they bind to CXCR2 to promote neutrophil activation and adhesion to the endothelium (McDonald, Pittman et al. 2010, Williams, Azcutia et al. 2011). These positively charged chemokines bind to negatively charged heparin sulphates present on endothelial cell surface, which act as anchors to establish and maintain the intravascular chemotactic gradients on the apical side of the endothelium (Massena, Christofferson et al. 2010). The chemotactic gradient is lost when the chemokines are taken up by the endothelial cells, possibly limiting neutrophil recruitment (Hillyer and Male 2005). As neutrophils roll along the endothelium, they come in contact with
the chemokines presented on the endothelial cells. The activation of neutrophil G-protein-coupled receptors (GPCRs) that recognize chemokines triggers changes in integrin conformation on the cell surface, allowing them to acquire higher affinity for their ligands (Kolaczkowska and Kubes 2013). As previously mentioned, binding of lymphocyte function-associated antigen 1 (LFA-1) to intercellular adhesion molecule 1 (ICAM-1) is very important to achieve solid adhesion (Phillipson, Heit et al. 2006).

2) Tethering: Localized tissue infection activates various immune cells, such as dendritic cells, macrophages, mast cells, endothelial cells, and likely all cells of the infected tissue. These immune cells secrete inflammatory mediators that establish chemotactic gradients and upregulate adhesion molecules, which help to recruit neutrophils. The initial stages of the leukocyte transmigration cascade involve neutrophil capture and rolling (Ley, Laudanna et al. 2007). Adhesion molecules, such as selectins, are initially upregulated by the stimuli. Translocation of P-selectin, which is stored in Weibel-Palade bodies of endothelial cells, and E-selectin, synthesized de novo, to the apical cell membrane allows the endothelium to bind to neutrophil ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1) and E-selectin ligand 1 (ESL-1), CD44, as well as other glycosylated ligands (Phillipson and Kubes 2011). L-selectin expressed on neutrophils seems to help with secondary tethering (Kolaczkowska and Kubes 2013).

3) Rolling: A ‘sling’ model has been proposed for rolling of neutrophils along the endothelium. In order to overcome shear forces, long membranes protrude from the rear of the cell (Schmidtke and Diamond 2000, Sundd, Gutierrez et al. 2010) and ‘sling’ to the front of the rolling neutrophil (Sundd, Gutierrez et al. 2012). PSGL-1 is expressed along the protruding membrane and binds to endothelial selectins to maintain its attachment to the endothelium. Sequential dissociation of selectins from their ligands helps to move neutrophils along the endothelium (Sundd, Gutierrez et al. 2012).

4) Arrest and adhesion: Additional adhesion molecules are expressed on the neutrophil, such as LFA1 (CD11a-CD18) and macrophage antigen-1 (MAC1; CD11b-CD18), which bind to ICAM-1 and ICAM-2 expressed on endothelial cells. Binding of these molecules contributes to the slow-down of neutrophils, allowing for arrest and firm adhesion (Zarbock, Ley et al. 2011, Kolaczkowska and Kubes 2013).
5) Intravascular crawling: Prior to transmigration, neutrophils that are now anchored in one location, probe the surroundings by extending pseudopods (Jenne, Wong et al. 2013). Neutrophils then crawl along the endothelium to find the cell junctions, moving in a straight line, perpendicular to the shear forces (Kolaczkowska and Kubes 2013). Some dissociation of adhesion molecules occurs simultaneously to allow for movement, and this crawling is mediated by endothelial ICAM-1 and neutrophil MAC-1 (Phillipson, Heit et al. 2006).

6) Endothelial transmigration: Crossing the endothelium takes about two to five minutes, then crossing the basement membrane takes five to fifteen minutes (Ley, Laudanna et al. 2007). Transmigration involves various integrins, ICAM-1, ICAM-2, vascular cell adhesion protein 1 (VCAM-1), platelet/endothelial cell adhesion molecule 1 (PECAM-1), and other adhesion molecules (Ley, Laudanna et al. 2007). Endothelial transmigration occurs through either a paracellular or transcellular route. Neutrophils prefer the paracellular route, as it is more efficient and less time-consuming compared to the transcellular route, which involves movement through an endothelial cell (Phillipson, Heit et al. 2006). Paracellular transmigration involves movement between endothelial cells, which requires breakage of bonds formed by intercellular junctional proteins, such as vascular endothelial (VE)-cadherin. In vitro studies have shown that neutrophils prefer to migrate at corners, where three endothelial cells are in contact, likely because it is relatively less ordered and there are fewer junctional proteins (Burns, Bowden et al. 2000). Transcellular transmigration involves formation of endothelial projections that cover the adhering neutrophil, thereby forming a dome-like structure. ICAM-1 and VCAM-1 are highly expressed on these projections, which bind to LFA-1 on neutrophils (Carman and Springer 2004). Formation of the dome-like structure is dependent on the localization of lymphocyte-specific protein 1 (LSP-1), an intracellular F-actin binding protein, to the F-actin filament containing cytoskeleton upon endothelial cell activation (Petri, Kaur et al. 2011). Vesiculo-vacuolar organelles (VVOs) are also seen in endothelial cells at sites of transmigration. VVOs are small continuous channels that may act as a passageway for neutrophil migration (Dvorak and Feng 2001). Ezrin, radixin, and moesin (ERM) proteins positioned around the channel may act as a bridge between ICAM-1 and the cytoskeleton to provide support during transmigration (Barreiro, Yanez-Mo et al. 2002).

In summary, neutrophils have to sequentially cross the layer of endothelial cells, the basement membrane, and pericytes that surround the endothelial cells. Once the neutrophil is anchored at
an endothelial junction through interaction of LFA-1 and MAC-1 with ICAM-1, it begins to migrate through the endothelial layer with the help of various adhesion molecules. The basement membrane is partially degraded with neutrophil-derived proteases to facilitate movement, but neutrophils prefer to move through areas of lower extracellular matrix (ECM) density (i.e. low expression of collagen). Neutrophils finally exit the vasculature by crawling along the cells and migrating between pericytes, which is again mediated by MAC-1 and LFA-1 binding to ICAM-1 (Kolaczkowska and Kubes 2013).

2.3.3.2 Epithelial transmigration

After leaving the vasculature and moving through the interstitium, neutrophils must cross the epithelial barrier in order to reach the alveolar space. Epithelial transmigration has not been investigated as extensively as endothelial transmigration, and many gaps exist in the identification of molecules that participate in the transmigration cascade.

Epithelial transmigration also involves initial adhesion to the epithelium and migration through the epithelial cell junctions. In contrast to the endothelium, neutrophil adhesion does not occur on the apical side, but on the basolateral surface of the epithelium (Zemans, Colgan et al. 2009). Initial adhesion of neutrophils to the basolateral epithelial surface occurs through β2 integrins. In the case of lung epithelial transmigration, CD11b/CD18 is the most important molecule for neutrophil adhesion (Zen and Parkos 2003). Selectins do not play a role in epithelial transmigration (Jaye and Parkos 2000). CD11b/CD18 ligands have not been clearly identified in the basolateral adhesion of neutrophils. Although ICAM-1 expression has been shown to be upregulated on the surface of lung epithelial cells in response to inflammatory stimuli, ICAM-1 is ruled out, as it is expressed exclusively on the apical side of the epithelium, and not on the basolateral surface (Kang, Crapo et al. 1993). Instead, transmembrane junctional adhesion molecules (JAMs) have been identified as more important CD11b/CD18 ligands for neutrophil adhesion to the epithelium. In particular, JAM-C directly binds to neutrophil CD11b/CD18 to mediate adhesion and migration (Zen, Babbin et al. 2004). However, JAM-C inhibition does not prevent initial adhesion and leads to only partial suppression of neutrophil migration, which suggests that there may be other unidentified epithelial ligands that are more crucial for initial adhesion (Zemans, Colgan et al. 2009).
Carbohydrates are also potential CD11b/CD18 ligands for adhesion (Diamond, Alon et al. 1995). A study has shown that pretreatment with different polysaccharides are able to inhibit epithelial transmigration of neutrophils (Colgan, Parkos et al. 1995). It is likely that the administered polysaccharides interfere with the interaction between CD11b/CD18 and the carbohydrate ligands expressed on the epithelium (Zen, Liu et al. 2002). A potential carbohydrate candidate is the fucosylated (a type of glycosylation) proteoglycans, as treatment with fucoidin, which binds to CD11b/CD18, and fucosidase treatment were able to inhibit neutrophil adhesion (Zen, Liu et al. 2002).

Neutrophils migrate through the epithelium in a paracellular fashion, and this tunnel is much longer compared to what is seen in the endothelium (Parkos 1997). There is no evidence of transcellular epithelial migration (Zemans, Colgan et al. 2009). The transmigration step involves CD47, which is expressed on the basolateral epithelial surface as well as neutrophils (Parkos, Colgan et al. 1996). Its expression is upregulated in response to inflammatory signals (Liu, Merlin et al. 2001), and CD47 deficiency has been shown to impair neutrophil migration to an extravascular site of infection and increase mortality following intraperitoneal administration of *E. coli* in mice (Lindberg, Bullard et al. 1996). Its exact mechanism of action has not been elucidated. CD47 inhibition did not prevent neutrophil adhesion but did decrease transmigration and cause neutrophils to be jammed in the inter-epithelial passageway (Parkos, Colgan et al. 1996). Furthermore, the role of signal-regulatory protein alpha (SIRPα) has also been implicated in neutrophil transmigration, as it appears to bind to CD47 in *cis* (i.e. both are expressed on the neutrophil) and regulate transmigration via phosphoinositide 3-kinase (PI3K)-dependent mechanisms. Its inhibition is associated with inhibition of neutrophil transmigration (Liu, Buhring et al. 2002).

Finally, once the neutrophil has reached the apical side of the epithelium, it adheres to the apical surface to prevent being swept over by the pulmonary edema fluid (Zemans, Colgan et al. 2009). ICAM-1, which is highly expressed on the apical side of the epithelium under inflammatory conditions, appears to mediate neutrophil adhesion to the epithelium via ligation with CD11b/CD18 (Huang, Eckmann et al. 1996).
2.3.4 High- and low-dose LPS model

In the clinic, patients experience a wide range of endotoxemia, ranging from less than 1 ng/mL to 10-300 ng/mL in septic patients. LPS has been shown to have differential effects on the cell depending on the concentration. Two distinct pathways are activated by low versus high doses of LPS. Studies of murine bone marrow-derived macrophages (BMDMs) revealed that a low dose of LPS (50 pg/mL) led to weak, leaky expression of proinflammatory markers, such as IL-6 and MCP-1. Sustained IkBα expression and impaired NFκB translocation were seen with the low-dose treatment. By contrast, a high dose of LPS (100 ng/mL) gave rise to degradation of IkB and significantly promoted NFκB nuclear translocation (Maitra, Gan et al. 2011). LPS not only activates the NFκB pathway, but also activates IKKe, which in turn phosphorylates and activates CCAAT/enhancer-binding protein delta (C/EBPδ) (Kravchenko, Mathison et al. 2003). C/EBPδ is able to enhance transcription of proinflammatory agents along with NFκB (Litvak, Ramsey et al. 2009). Although low-dose LPS treatment could not activate the NFκB pathway, it was able to increase the expression of C/EBPδ in cells. A number of nuclear receptors, such as peroxisome proliferator-activated receptor alpha (PPARα) and retinoic acid receptor alpha (RARα), are involved in the repression of proinflammatory cytokines by preventing recruitment of proinflammatory transcription factors (Glass and Saijo 2010). The authors showed that a low dose of LPS (50 pg/mL) removed PPARα and RARα transcriptional repressors from the promoter regions of proinflammatory genes, which may explain the feeble expression of proinflammatory cytokines (Maitra, Gan et al. 2011). Thus, different molecular pathways appear to be activated depending on the dose of LPS.

As mentioned, mice are very resistant to LPS compared to humans and require much higher doses to develop lung injury and sepsis (over 10 mg/kg for mortality) (Matute-Bello, Frevert et al. 2008). Our model of ALI uses a low dose of 800 µg/kg to look at pulmonary inflammation.

2.3.5 Advantages and disadvantages of LPS model

One of the advantages of using LPS to model ALI is that it is readily available and easy to administer. It also yields reproducible results. Moreover, LPS is able to induce a neutrophilic inflammatory response and proinflammatory cytokine production in the lung, similar to clinical ARDS. However, the changes in alveolar-capillary permeability are subtle compared to what is seen in ARDS patients (Wienerkronish, Albertine et al. 1991). Another disadvantage is that LPS
preparations can be contaminated with other bacterial substances, which may cause signaling through other TLRs and give rise to different biological responses (Matute-Bello, Frevert et al. 2008). Furthermore, the responses to LPS are species- and strain-dependent. Small doses of LPS can cause pulmonary inflammation in sheep, calves, and pigs; however, much higher doses are needed to produce the same effect in dogs and rodents (Winkler 1989). BALB/c mice are highly susceptible to LPS, whereas C57BL/6 mice are more resistant (Matute-Bello, Frevert et al. 2008).

3 Ischemic preconditioning (IPC)

The idea of ischemic preconditioning (IPC) first emerged in 1986, when Murry et al. reported that 4 cycles of 5-minute coronary ischemia and 5-minute reperfusion immediately before a sustained coronary occlusion that lasted 40 minutes followed by reperfusion reduced infarct size in dogs (Murry, Jennings et al. 1986). Its cardioprotective effect was greater than what could be seen with pharmacological treatment (Heusch 2015). IPC is thus known to attenuate ischemia/reperfusion (I/R) injury.

IPC has two protective phases. The first window involves immediate protection, where the effect of IPC occurs within minutes of the preconditioning stimulus and lasts for approximately 6 hours. The second window of protection is when the effect, less powerful than the acute effect, reoccurs within 24 hours of preconditioning and lasts up to 96 hours (Marber, Latchman et al. 1993, Saxena, Newman et al. 2010). The acute phase of IPC makes use of readily available signaling molecules, whereas the late phase involves changes in gene expression that lead to increased production of protective proteins.

Clinical application of IPC is highly limited, as it can only be applied prior to challenge, as in the cases of cardiac surgery and organ transplantation. In order to overcome such limitations, ischemic post-conditioning (POC) was established. The group of Vinten-Johansen reported in 2003 that 3 cycles of 30-second reperfusion and 30-second reocclusion at the start of reperfusion following 60-minute coronary occlusion reduced infarct size to the same extent as IPC in dogs (Zhao, Corvera et al. 2003). Although POC is effective only in the early minutes of reperfusion, it produced the same cardioprotective effects as IPC.
4 Remote ischemic conditioning (RIC)

A less invasive form of ischemic conditioning was developed based on the hypothesis that these potentially protective signaling molecules could travel through circulation. It was first demonstrated by Przyklenk et al. that 4 cycles of 5-minute ischemia and 5-minute reperfusion of the left circumflex coronary artery applied prior to a 60-minute occlusion of the left anterior descending coronary artery with reperfusion reduced infarct size in dogs (Przyklenk, Bauer et al. 1993). It was a novel finding, as transient I/R in one vascular bed was able to protect a different part of the myocardium from subsequent sustained occlusion of the artery, away from the site of preconditioning. The authors concluded that the protective effect of preconditioning might be due to factors being activated, produced, or transported through the heart during brief episodes of I/R (Przyklenk, Bauer et al. 1993). Similarly, Birnbaum et al. found that remote ischemic preconditioning (RIPC) could be induced by applying cycles of transient I/R to the skeletal muscle of the hind limb. They observed that when brief episodes of I/R were applied to the skeletal muscle of the lower limb, prior to an acute coronary artery occlusion, myocardial infarct size was reduced in the rabbit heart (Birnbaum, Hale et al. 1997). However, hindlimb I/R was induced by reducing blood flow through the femoral artery, a rather invasive intervention. A non-invasive method of preconditioning was introduced by Oxman et al. in 1997, where they used a tourniquet and a clamp to occlude blood flow to the hindlimb (Oxman, Arad et al. 1997). Remote ischemic conditioning (RIC) was later successfully translated to humans. Clinical studies began to use a standard blood pressure cuff placed around the upper arm to inflate and deflate in order to induce I/R (Candilio, Malik et al. 2013). Since then, RIC has been developed as a non-invasive, protective intervention that works not only in the heart, but also in the vasculature as well as other organs (Murry, Jennings et al. 1986). RIC may be applied before challenge (pre-conditioning; RIPC), during challenge (per-conditioning; RIPerC), or following challenge (post-conditioning; RIPostC).

4.1 Molecular basis of ischemic conditioning

4.1.1 Signaling components of ischemic conditioning

Numerous studies using various models have attempted to identify the signaling molecules responsible for the protective effect. As recently reviewed by Heusch, the current notion of IPC is that preconditioning releases a trigger that activates a mediator to relay the organ-protective
signal during the sustained ischemic challenge onto an effector, which ultimately exerts its protective effects and reduces damage (Heusch 2015). Various endogenous molecules are possible candidates for exerting the initial protective effect via neural and humoral transmission, such as reactive oxygen species (ROS), nitric oxide (NO), protein kinase C (PKC), ATP-sensitive $K^+$ (KATP) channel, and hypoxia-inducible factor 1-alpha (HIF-1α).

4.1.1.1 Reactive oxygen species (ROS)

ROS are products of normal cellular metabolism defined as molecules that contain one or more unpaired electrons. At high concentrations, these free radicals cause biological damage, which is referred to as oxidative stress (Valko, Leibfritz et al. 2007). The role of ROS in conditioning is rather controversial. Although excess ROS can cause detrimental effects that may lead to irreversible organ injury, when expressed at low levels (e.g. in response to mitochondrial KATP channel activation) ROS may contribute to protection through oxidation of protective kinases (e.g. extracellular signal-regulated kinases, ERKs) (Otani 2004). It is very similar to the idea of conditioning, in that mild I/R could confer protection, whereas I/R to a greater extent could cause damage.

In 1997, Baines and colleagues observed that oxygen radicals produced during IPC contribute to cardioprotection in the rabbit heart. In this study, all rabbits underwent 30 minutes of coronary occlusion and 180 minutes of reperfusion. The IPC group was subjected to either 1 or 4 cycles of 5-minute ischemia and 10-minute reperfusion prior to prolonged ischemia. A single or four cycles of IPC were both cardioprotective, reducing infarct size compared to the control. Pretreatment with N-2-mercaptopropionylglycine (MPG), a free radical scavenger, abolished the protective effect seen with a single cycle of IPC, but not that with four cycles of preconditioning. The authors speculated that multiple cycles of IPC might lead to accumulation of other factors that play a role in preconditioning, suggesting that free radicals are not the sole trigger. Similar findings were observed in isolated rabbit hearts, where 5-minute global ischemia followed by 10-minute reperfusion prior to prolonged regional ischemia led to cardioprotection, which was abrogated with MPG pretreatment. Treatment with a protein kinase C (PKC) inhibitor, polymixin B, also abolished the protection. Therefore, the group concluded that oxygen radicals take part in IPC in the rabbit via PKC activation (Baines, Goto et al. 1997).
Another study published in 1997 demonstrated that oxygen radicals could reproduce the protective effects of IPC in isolated rabbit hearts. Five minutes of exposure to a low concentration of oxygen radicals prior to 30 minutes of global ischemia and 45 minutes of reperfusion significantly enhanced post-ischemic recovery of left ventricular contractile function in rabbit hearts. In a substudy, myocardial infarction was induced with 30 minutes of coronary occlusion and 2.5 hours of reperfusion. Likewise, oxygen radical infusion prior to prolonged ischemia significantly reduced infarct size compared to the control. Polymixin B abrogated the protection. The group hypothesized that reperfusion following brief preconditioning ischemia may generate low, non-cytotoxic levels of ROS, which could affect cellular activities and contribute to preconditioning (Tritto, Dandrea et al. 1997).

4.1.1.2 Nitric oxide (NO)

NO and its synthases have also been implicated in preconditioning. There are three distinct isoforms of nitric oxide synthases (NOS): nNOS in neuronal tissue, inducible NOS (iNOS), and endothelial NOS (eNOS). Generally, nNOS and eNOS are calcium-dependent and constitutively expressed, whereas iNOS is independent of calcium and is induced when required (Alderton, Cooper et al. 2001). In 1992, Vegh et al. were the first to demonstrate that administration of NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, inhibited the anti-arrhythmic effect of IPC (2 cycles of 5-minute coronary occlusion and reperfusion) in a prolonged myocardial I/R injury model of dogs, suggesting a role for NO in early preconditioning (Vegh, Szekeres et al. 1992).

Numerous studies have also demonstrated its role in late preconditioning (i.e. second window of protection). In 1998, Takano and colleagues showed that NOS mediates late preconditioning against myocardial infarction in rabbits. In this study, conscious rabbits were subjected to sham treatment or IPC (6 cycles of 4-minute coronary occlusion and reperfusion) 24 hours prior to a 30-minute coronary occlusion and 3-day reperfusion. IPC significantly reduced infarct size compared to control; this protection was abrogated by pretreatment with a non-selective NOS inhibitor or a selective iNOS inhibitor (nitroarginine or aminoguanidine, respectively). The NOS inhibitors also impaired late IPC-induced myocardial functional improvements (Takano, Manchikalapudi et al. 1998).
In 1999, Guo et al. suggested that iNOS in particular assumes an important role in the late phase of preconditioning. The study involved IPC (6 cycles of 4-minute coronary occlusion and reperfusion) of mice 24 hours prior to a 30-minute coronary occlusion followed by 24-hour reperfusion. IPC significantly reduced I/R-induced infarct size and was associated with marked upregulation of myocardial iNOS expression and activity, as well as nitrite (an NO donor under hypoxic/acidic conditions) and nitrate levels in the tissue. Intriguingly, nNOS and eNOS protein expression and activity were unaffected by IPC in the ischemic heart. In contrast, the cardioprotective effect of IPC was absent in iNOS knockout mice (Guo, Jones et al. 1999).

NOS has also been implicated in preconditioning of organs other than the heart. Abu-Amara and colleagues studied the role of eNOS in RIPC of the mouse liver. In this I/R model, animals were subjected to lobular hepatic ischemia (70%) for 40 minutes followed by 2 hours of reperfusion. RIPC (6 cycles of 4-minute hindlimb ischemia and reperfusion) significantly decreased histopathological hepatic injury and plasma aminotransferase levels, reduced ultrastructural damage (i.e. extensive mitochondrial damage, extravasation of erythrocytes into liver parenchyma), and potently improved hepatic microcirculatory blood flow (MBF) compared to the sham group in I/R-induced wild type mice. None of these liver-protective effects of RIPC were observed in the eNOS knockout mice. The authors, therefore, concluded that eNOS (hindlimb, hepatic, or both) is an essential component of RIPC-induced liver protection (Abu-Amara, Yang et al. 2011).

A recent study conducted by Rassaf et al. demonstrated that circulating nitrite contributes to RIPC-induced cardioprotection. Previous studies from the same group showed that RIPC affects circulating NO/nitrite levels in humans (Rassaf, Heiss et al. 2006). Nitrite is the oxidation product of NO but is also an important reservoir of NO, as it can revert to NO. The group observed that RIPC (4 cycles of 5-minute ischemia and reperfusion) significantly increased plasma nitrite levels in humans and mice. Administration of cPTIO, an NO scavenger, blocked the effect by preventing the conversion of NO to nitrite. RIPC also significantly increased nitrite levels in the myocardium, which suggests that nitrite may be generated in the preconditioned organ/tissue and then transferred to the heart through blood. In eNOS knockout mice, RIPC could not upregulate nitrite expression either in the heart or plasma. Another set of experiments demonstrated that RIPC reduced I/R-induced (30-minute regional myocardial ischemia and 24-hour reperfusion) infarct size, and that the protection was abrogated with cPTIO treatment in
wild type mice. The RIPC-induced protection was again absent in the eNOS knockout animals. Administration of nitrite recapitulated the effects of RIPC in both the wild type and knockout mice. Previously it was shown that myoglobin acts as a functional nitrite reductase and converts nitrite to NO (Hendgen-Cotta, Merx et al. 2008). Thus, Rassaf et al. showed in the myoglobin knockout mice that RIPC increased both plasma and myocardial nitrite levels but failed to confer protection against I/R-induced infarction. Nitrite/NO affected mitochondrial respiration, as well. RIPC significantly promoted inhibitory S-nitrosation of mitochondrial complex I, thereby decreasing complex I activity in the myocardium of wild type mice. As a result, there was a marked reduction in the formation of ROS in the myocardium. These effects were absent in the myoglobin knockout mice. Furthermore, RIPC-induced human plasma containing increased nitrite reduced infarct size in the isolated mouse heart. Pretreatment with sulfanilamide, a nitrite scavenger, abrogated the protective effect of the plasma. Conditioned human plasma could not confer protection in the myoglobin-deficient mouse heart. In conclusion, their results collectively imply that eNOS-derived nitrite plays a role in RIPC-induced cardioprotection, as it travels from a remote organ/tissue to the heart to contribute to infarct size reduction, which involves myoglobin-mediated reduction into NO (Rassaf, Totzeck et al. 2014).

4.1.1.3 Protein kinase C (PKC)

As briefly aforementioned, an important intracellular mediator of conditioning is PKC. In 1994, Armstrong et al. were the first to suggest PKC as a mediator of IPC. In their study, rabbit cardiomyocytes were subjected to 15-minute oxygenated, glucose-free pre-incubation prior to prolonged ischemia. Although the preconditioning was not triggered by transient ischemia in this case, the absence of substrate was expected to produce metabolic stress, and thus a similar preconditioning effect. The pre-incubation led to a 40% reduction of ischemia-induced cell death. Calphostin C, a competitive inhibitor of PKC activation, abrogated the protective effect of preconditioning when added to the cells during prolonged ischemia (Armstrong, Downey et al. 1994). Similarly, Downey et al. have shown that PKC inhibitors, staurosporine and polymyxin B, were able to abolish the protective effects of preconditioning in rabbit hearts. They also observed that treatment with PKC activators mimicked preconditioning in the heart (Ytrehus, Liu et al. 1994). As reviewed by Heusch, in larger mammals, PKCα is the most important isoform with respect to protection, whereas PKCε is the essential isoform in rodents (Heusch 2015). PKC appears to be a “point of convergence” for various preconditioning signaling molecules that act
via GPCRs, such as adenosine and bradykinin (Hausenloy and Yellon 2006). GPCR ligand binding causes activation of phospholipase C, which cleaves phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) (Hausenloy and Yellon 2006). DAG is involved in PKC activation (Hausenloy and Yellon 2006).

4.1.1.4 ATP-sensitive K$^+$ (KATP) channel

A key component of cardioprotective conditioning is the KATP channel. The KATP channel used to be a cardioprotective drug target and is now considered a signaling element of conditioning. The KATP channels in the inner mitochondrial membrane are the major players involved in conditioning (Liu, Sato et al. 1998), as they are targeted by NO and PKC. In 2000, Pain et al. suggested that opening of mitochondrial KATP channels induces preconditioning by generating free radicals. Thirty minutes of coronary branch occlusion and 2 hours of reperfusion led to infarction in isolated rabbit hearts. The group showed that 5 minutes of ischemic preconditioning was able to significantly reduce the infarct size, and that similar protection was conferred by treatment with diazoxide, a mitochondrial KATP channel opener. In contrast, treatment with KATP channel blockers, 5-hydroxydecanoate (5-HD) and glibenclamide, abolished the protection. Interestingly, the protective effect was abolished only when the KATP channel blockers were administered early (i.e. close to or during the time of ischemic preconditioning or treatment with KATP channel openers) but not when they were administered shortly before or during the prolonged ischemia. The role of free radicals was evident from the use of free radical scavengers, N-(2-mercaptopropionyl) glycine and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride, which mitigated the cardioprotective effect exerted by diazoxide (Pain, Yang et al. 2000). Thus, once activated, KATP channels produce ROS, which goes on to further activate the PKC, which in turn induces opening of more KATP channels in a positive feedback loop. This positive feedback loop may contribute to the late-phase protective effect of preconditioning (Costa and Garlid 2008).

The role of KATP channels in preconditioning was further validated in 2005 when Konstatinov et al. studied the effect of RIPC on myocardial infarction following heart transplant in pigs. The RIPC group was subjected to four cycles of 5-minute lower-limb ischemia and reperfusion following heart transplantation with the support of hypothermic cardiopulmonary bypass. Then myocardial infarction was induced by a 30-minute occlusion of the left anterior descending
(LAD) artery, followed by 2 hours of reperfusion. Cardioprotection was seen in the recipients that underwent RIPC, as preconditioning significantly decreased the mass of myocardial infarction and myocardial infarction to area at risk mass ratio. Pretreatment with glibenclamide, a nonselective KATP channel inhibitor, abrogated the cardioprotective effect (Konstantinov, Li et al. 2005).

Furthermore, Loukogeorgakis et al. in 2007 studied the effect of RIPC and RIPerC in humans on endothelial dysfunction caused by sustained I/R. They first showed that 2 or 3 cycles of inflation and deflation of the blood pressure cuff around the upper arm prior to (RIPC) or during (RIPerC) 20-minute ischemia on the contralateral arm were able to prevent reduction in flow-mediated dilation (FMD) of the brachial artery, a measure of endothelial dysfunction. In order to investigate the role of KATP channels, glibenclamide was orally administered to a group of healthy volunteers before the study. The same protocol was repeated in these individuals, and results showed that the protective effects of RIPC and RIPerC on endothelial dysfunction were abrogated with the KATP channel blocker, suggesting that KATP channels contribute to the protection conferred by RIC. The mechanism of action remains unclear; however, much evidence links the activation of PKC, mitochondrial KATP channel activation, and subsequent ROS production with IPC and RIC (Loukogeorgakis, Williams et al. 2007).

4.1.1.5 Neural and humoral transmission

RIPC requires transfer of protective molecules from the remote organ to the target. In the case of cardioprotection, sensory fibers in the remote organ that are activated by I/R, trauma, or chemical/electrical stimuli can activate the autonomic cardiac innervation through the central nervous system, thereby releasing a humoral factor(s) that still needs to be identified.

Gho and colleagues have demonstrated that neurogenic pathways are involved in transmitting protective signals produced by RIC. Male rats were subjected to coronary artery occlusion (CAO) for 60 minutes, followed by 180 minutes of reperfusion. When rats were preconditioned by CAO 15 minutes prior to prolonged CAO, myocardial infarct size was significantly reduced. The same protective effect was seen with a 15-minute anterior mesenteric artery occlusion (MAO) performed prior to sustained CAO. The cardioprotective effect seen with MAO was fully abolished with hexamethonium, a ganglion blocker, which suggests a neurogenic component to the delivery of RIC factors. Ganglion inhibition was ineffective in abolishing protection exerted
by 15-minute CAO, indicating that different pathways might be involved in local ischemic conditioning and RIC. Moreover, sustained MAO was unable to provide cardioprotection against 60-minute CAO, suggesting a need for reperfusion for RIC to have an effect. The authors postulated that reperfusion allows for the delivery of molecules secreted from the preconditioned tissue/organ to other target sites (Gho, Schoemaker et al. 1996). An in vivo study using a mouse model of myocardial infarction has been used to elucidate the pathways involved in RIC signal transmission. In this study, mice were subjected to 30 minutes of myocardial ischemia by blocking the LAD artery, followed by 120 minutes of reperfusion (I/R). Prior to LAD artery ligation, RIPC was performed, which involved three cycles of 5-minute femoral artery occlusion and reperfusion. RIPC was able to significantly reduce infarct size. The positive effect was completely abrogated with resection of both the femoral and sciatic nerves prior to RIPC. Resecting just one of the two nerves resulted in a partial decrease in the protective effect. Occluding the femoral vein prior to RIPC and I/R failed to reduce infarct size. Furthermore, the cardioprotective effect of RIPC disappeared with the combination of occlusion of the femoral vein and resection of the femoral and/or sciatic nerves (Lim, Yellon et al. 2010). Therefore, both humoral and neural pathways appear to be required for successful delivery of protective factors to remote tissues and organs.

The role of adenosine has been implicated in cardioprotection exerted by RIC through neurogenic pathways. Similar to the results reported by Gho and colleagues, RIPC with 15-minute MAO resulted in reduced infarct size following a 60-minute CAO. The protective effect was lost with hexamethonium or adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-SPT). Pretreatment with specific doses of MRS-1191, another adenosine receptor antagonist, that target either A₁ or A₃ subtypes was able to reduce the protective effect of MAO preconditioning, which indicate their role in cardioprotection. Administration of adenosine to the intramesenteric artery showed similar cardioprotective effects seen with MAO. Inhibition of the ganglia with hexamethonium at 5 minutes of reperfusion following MAO failed to abolish the protective effect, likely because the neurogenic pathway has already been activated to produce the protective effect by that time. In contrast, 8-SPT administration at the same time point was able to reduce the effect. The authors suggested that RIPC applied to the small intestine leads to local release of adenosine that activates a neurogenic pathway in the first few minutes of reperfusion following preconditioning. The neurogenic pathway eventually transmits the signals and
prepares the heart for prolonged CAO and results in reduced myocardial infarction (Liem, Verdouw et al. 2002).

A rabbit study further demonstrated that the cardioprotective effect of RIPC on myocardial infarction is exerted through both neural and humoral pathways. RIPC involved four cycles of 5-minute ischemia and reperfusion with a tourniquet placed on a limb. Plasma obtained from preconditioned rabbits reduced infarction in isolated, naïve hearts following 30 minutes of global ischemia and 120 minutes of reperfusion. A similar protective effect was also seen when the isolated hearts were treated with plasma obtained from rabbits that received intra-arterial injection of adenosine into the femoral artery. The cardioprotective effect was lost when the femoral nerve was resected prior to RIPC or administration of adenosine. This suggests that adenosine may be an important factor in producing the protective effect seen with RIPC, and that preconditioning with limb I/R or adenosine leads to release of a humoral factor(s) that act on isolated, naïve hearts. Intact neural pathways appear to be required for the release of humoral factors following RIPC (Steensrud, Li et al. 2010).

There is also evidence for humoral transmission of RIC factors. Studies have shown the importance of humoral transfer of protective molecules with preconditioned plasma or dialysate exerting protective effects in another individual, even across species (Heusch 2015). It was observed by Leung et al. that zebrafish injected with plasma or dialyzed plasma containing factor(s) greater than 14 kDa in molecular weight from ischemic conditioned mice had reduced neutrophil migration toward the site of injury following tailfin transection, compared to what was seen in zebrafish injected with control mouse plasma (Leung CH, Caldarone CA et al. 2015). NO, IL-10, microRNA-144 (miR-144), adenosine, and many more have been proposed as the key humoral factors involved in protection; however, none of these factors seem to be solely responsible for the protective effects of RIC (Heusch 2015).

4.1.1.6 Bradykinin

Along with adenosine, bradykinin has been proposed as one of the autacoids that act as a trigger for IPC signal transduction. In 1995, Goto and colleagues studied the effect of IPC on rabbit hearts in situ and in vitro. Thirty minutes of regional ischemia was induced by coronary ligation, which was followed by 180 minutes of reperfusion. A single episode of preconditioning of hearts in situ with 5 minutes of ischemia and 10 minutes of reperfusion prior to coronary occlusion
significantly decreased infarct size compared to the I/R group. Pretreatment with a bradykinin B₂ receptor inhibitor, HOE-140, abrogated the protective effect of IPC. Interestingly, administration of HOE-140 following IPC was unable to abolish the protective effect, possibly because the bradykinin released in response to IPC acts as a trigger, not a mediator of protection. It was also observed that pretreatment with HOE-140 could not mitigate the protective effect of IPC when the preconditioning stimulus was enhanced with four cycles of 5-minute ischemia and 10-minute reperfusion. The group proposed that since bradykinin is not the sole contributor of IPC protection, a more intense IPC stimulus could overpower the bradykinin receptor inhibition, possibly by promoting the release of other signaling factors, such as adenosine (Goto, Liu et al. 1995).

The role of bradykinin in preconditioning has also been demonstrated in a rodent model by Schoemaker et al. Rats were subjected to brief CAO or MAO (a single episode of 15 minutes of ischemia and 10 minutes of reperfusion) prior to 60-minute CAO followed by 180-minute reperfusion. Preconditioning induced by brief CAO and MAO significantly reduced infarct size compared to the control. Intravenous administration of HOE-140 prior to preconditioning abolished the protective effect. Five minutes of intramesenteric infusion of bradykinin prior to prolonged CAO remarkably decreased infarct size, reproducing the preconditioning effect of MAO. Pretreatment with hexamethonium, a ganglion blocker, abrogated the preconditioning effect of bradykinin, which suggests that bradykinin likely acts via a neuronal, rather than humoral pathway (Schoemaker and van Heijningen 2000).

In 2001, Cohen et al. showed that bradykinin, along with acetylcholine (ACh), opioids, and phenylephrine, can reproduce the preconditioning effect of IPC. The group used isolated rabbit hearts to induce infarction with 30 minutes of coronary artery occlusion followed by 120 minutes of reperfusion. A 5-minute infusion of bradykinin prior to ischemia significantly reduced infarct size compared to the control. This protection was abolished by treatment with MPG, a ROS scavenger, or 5-HD, a mitochondrial KATP channel blocker. The agonists tested were thus shown to trigger preconditioning cascades that involve ROS production and opening of mitochondrial KATP channels (Cohen, Yang et al. 2001).

However, a more recent randomized, double-blind study conducted on human subjects suggested otherwise. I/R injury was induced in the non-dominant arm of 20 healthy male volunteers by
placing a cuff for 20 minutes. The RIPC group received preconditioning (3 cycles of 5-minute ischemia and reperfusion in the contralateral arm) prior to prolonged ischemia. Vasomotor function was assessed following induction of I/R injury by measuring forearm blood flow after an intra-arterial administration of ACh. I/R injury impaired ACh-induced vasodilation in the infused arm, and this inhibition was mitigated by RIPC. HOE-140 was ineffective in abolishing the protection provided by RIPC, which led the authors to conclude that endogenous bradykinin does not mediate RIPC in humans (Pedersen, Schmidt et al. 2011).

Effect on neutrophils

RIPC also affects neutrophils. Five healthy adult volunteers were subjected to three cycles of 5-minute ischemia and reperfusion using a blood pressure cuff on a forearm. This intervention was applied daily for 10 days. Neutrophils were isolated from blood obtained from the volunteers prior to start of the protocol (day 0) and following preconditioning. On days 1 and 10 following RIPC, neutrophil adhesion was significantly reduced. Neutrophil surface expression of CD11b and oxidant production were unaffected. Nevertheless, significant changes in cytokine production were reported. When neutrophils were treated with LPS, TNF-α secretion increased on day 10, at 6 and 24 hours following LPS stimulation. Interestingly, the significant increase in TNF-α secretion was not seen in neutrophils harvested prior to RIPC stimulus. Likewise, IL-6 and IL-10 expression showed a similar trend. In addition, IL-1β expression significantly increased in cells 10 days after LPS exposure. A decreasing trend in neutrophil apoptosis was also observed on day 1 and 10 (Shimizu, Saxena et al. 2010).

4.1.1.7 Heme oxygenase-1 (HO-1)

One other possible candidate for providing the protective effect of RIC is HO-1. Heme oxygenase (HO)-1 is an anti-inflammatory, anti-apoptotic, and anti-proliferative enzyme (Otterbein, Soares et al. 2003) that catalyzes the rate-limiting step of the degradation of heme into biliverdin, carbon monoxide (CO), and free iron (Fe^{2+}) (Ryter, Alam et al. 2006). There are two isoforms of HO: an inducible isozyme, HO-1, and a constitutively expressed isozyme, HO-2. HO-1 is highly expressed in tissues that degrade aged erythrocytes, such as the spleen (Ryter, Alam et al. 2006). HO-1 is also known as heat shock protein 32, which is mostly expressed at low levels under normal conditions in other tissues. Various types of stress are able to induce its transcriptional activation. It is commonly induced by oxidative stress that involves generation of reactive oxygen species (ROS) (Ryter, Alam et al. 2006). The products of heme degradation that
form as a result of HO-1 action mediate the anti-inflammatory effects of HO-1 (Otterbein, Soares et al. 2003).

A number of studies have identified a link between induction of HO-1 and ischemic conditioning. A group in 2009 showed that the protective effect of IPC on brain ischemic injury is associated with HO-1. Mice were subjected to IPC by ligating common carotid arteries for 1 minute and reopening for 5 minutes, for a total of three cycles. Twenty-four hours following preconditioning, either transient or permanent focal ischemia was induced by occluding the middle cerebral artery for 90 minutes and 48 hours, respectively. The group demonstrated that IPC attenuated both transient and permanent ischemia-induced brain infarct volume and lowered neurological deficit scores in the wild type mice. However, the protective effect of IPC was lost in the HO-1 knockout mice and mortality was increased compared to the wild type mice upon induction of permanent focal ischemia. IPC alone did not cause infarction in mice at 24 hours. HO-1 expression was significantly increased in mice subjected to RIPC compared to shams at 24 hours (Zeynalov, Shah et al. 2009).

In 2007, Tamion et al. showed that HO-1 might be implicated in preconditioning using a rodent model of endotoxin shock. Rats were subjected to either sham surgery or four cycles of 1-minute mesenteric artery occlusion and 4-minute reperfusion. Twenty-four hours later, the rats received 10 mg/kg of LPS intravenously. At 6 hours following LPS challenge, the preconditioned group had significantly reduced levels of both systemic and mesenteric TNFα, as well as protein expression of intestinal and lung TNF and intercellular adhesion molecule-1 (ICAM-1). Moreover, preconditioning reduced fluid requirements, intestinal lactate production and injury, as well as pulmonary edema. All of these protective effects were markedly abolished by treatment with zinc-protoporphyrin, a specific HO-1 inhibitor. The protective effect observed with preconditioning in the LPS-challenged animals was closely mimicked by bilirubin administration. Bilirubin is produced from conversion of biliverdin, which is formed from the cleavage of heme by HO-1, as mentioned previously. In addition, intestinal HO-1 mRNA expression was shown to be highly upregulated with preconditioning compared to the control (Tamion, Richard et al. 2007). Thus HO-1 may play a key role in providing protection against inflammation that is not limited to I/R injury.
The role of HO-1 has also been highlighted in a model of ischemic post-conditioning. Rats were subjected to unilateral lung ischemia, by clamping the left pulmonary artery, bronchus, and pulmonary vein for 40 minutes, followed by 105 minutes of reperfusion. In the post-conditioning group, three cycles of 30-second occlusion and 30-second release were applied in the same way, starting immediately after the 40-minute ischemia. Following lung I/R, significantly lower partial pressure of arterial oxygen (PaO\textsubscript{2}), higher lung wet-to-dry (W/D) ratio, lung injury score and plasma malondialdehyde (MDA) levels, and higher lung HO-1 expression were observed in the I/R group compared to the sham. Post-conditioning significantly reduced the magnitude of injury seen in the I/R group and led to an even higher expression of HO-1 in the lung. When rats were treated with a specific HO-1 inhibitor, zinc protoporphyrin IX, 24 hours prior to the experiment, the protective effect of post-conditioning was abrogated, as seen by low PaO\textsubscript{2}, high lung W/D ratio, lung injury score and plasma MDA levels, similar to those of the I/R group (Xia, Gao et al. 2010).

### 4.1.1.8 Antioxidant defense

Antioxidant defense refers to the defense mechanisms against oxidative stress caused by free radicals. Enzymes involved in antioxidant defense include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and others (Valko, Leibfritz et al. 2007). Non-enzymatic components to antioxidant defense include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), and other antioxidants (Valko, Leibfritz et al. 2007). Studies have suggested enhanced antioxidant defense with RIC in various organs.

In 2013, Czigany et al. reported positive effects of RIPerC on hepatic tissue injury and redox homeostasis in a rodent model of liver I/R injury. The study involved rats subjected to 60-minute hepatic ischemia, followed by 1, 6, or 24 hours of reperfusion. Twenty minutes into the prolonged ischemia, the RIPerC groups received four cycles of 5-minute bilateral lower limb ischemia and reperfusion, applied by infrarenal aortic cross-clamping. Hepatic histological damage was evident in the control I/R group, as necrotic zones were pronounced in the liver sections. The overall damage was significantly reduced in the RIPerC group with all three durations of reperfusion. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were also reduced in the preconditioned animals, indicative of less hepatocellular damage. In addition, serum TNF-α levels were significantly decreased with preconditioning at 1
hour into reperfusion. The group also found that free radical content in the liver was significantly decreased in the RIPerC group, and the liver tissue global antioxidant capacity (i.e. presence of thiol groups that act as reducing agents) was notably preserved at 24 hours of reperfusion compared to the control (Czigany, Turoczi et al. 2013).

Similar results were shown by Jiang and colleagues in 2015 using a rodent model of renal I/R injury. To examine the protective effect of RIC on renal function, RIC was performed on rats during a 60-minute prolonged renal ischemia (RIPerC; four cycles of 5-minute right hindlimb ischemia and reperfusion), at the start of 24-hour renal reperfusion following ischemia (RIPostC; four cycles of 5-minute hindlimb ischemia and reperfusion), or both during and following prolonged renal ischemia (RIPerC+RIPostC; two cycles of 5-minute hindlimb ischemia and reperfusion each). Although serum creatinine and blood urea nitrogen levels were still significantly elevated in all of the I/R groups compared to shams, which is indicative of impaired renal function, all three RIC protocols markedly attenuated the increases in response to prolonged renal I/R. More importantly, all RIC+I/R groups demonstrated a significant reduction in MDA levels compared to the I/R group at 24 hours into reperfusion, values comparable to the sham. In addition, SOD activity in the RIC groups was restored to sham levels, whereas the I/R group showed a significant reduction in SOD activity. Thus, the authors postulated that the protective mechanism of RIC may involve promotion of endogenous antioxidant activity (Jiang, Chen et al. 2015).

Findings from an in vitro study conducted by Chen et al. also supported the idea of antioxidant defense enhancement. Rats were subjected to sham or RIPC (3 cycles of 5-minute right hindlimb ischemia and reperfusion) at 20 minutes or 24 hours following preconditioning. The sham or preconditioned sera were used to pretreat HUVECs before incubation with H$_2$O$_2$ for 2 hours. Firstly, the preconditioned sera, regardless of the time of collection, significantly increased cell viability compared to the HUVECs that received only H$_2$O$_2$. Secondly, the preconditioned sera significantly reduced intracellular ROS. Rat RIPC sera also significantly attenuated lipid peroxidation, as indicated by decreased medium MDA concentration, and strongly upregulated antioxidant mRNA expression (e.g. CAT, GPx, SOD) and enhanced their intracellular activity. Additionally, the preconditioned sera promoted nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation in H$_2$O$_2$-injured HUVECs (Chen, Zhang et al. 2015). Nrf2 is a ubiquitous transcription factor that plays an important role in inducing the expression of proteins involved in
the response to oxidative stress (Bryan, Olayanju et al. 2013). In summary, the results demonstrated that RIPC protects HUVECs against H$_2$O$_2$-induced oxidative stress and suggested that Nrf2 may be implicated in enhancing antioxidant defense.

A recent in vivo study conducted by Costa et al. likewise showed that RIC transiently enhances antioxidant defense. In this study, RIC was performed on the rats in three cycles of 5-minute left hindlimb ischemia and reperfusion. At 10 or 60 minutes following RIC, liver and kidney tissues were harvested for assessment of antioxidant defense. At 10 minutes, RIC significantly improved the antioxidant capacity in both the liver and kidney. The enhancement was lost by 60 minutes. The authors suggested that RIC prepares tissues distant from the preconditioned organ/tissue for subsequent oxidative stress. However, since the enhancing effect is only temporary, RIC likely produces a narrow window of protection (Costa, Teixeira et al. 2015). Therefore, RIC appears to confer organ protection by strengthening antioxidant defense.

### 4.1.1.9 Opioid receptors

Studies have suggested that opioids may be closely involved in the preconditioning-induced protection against ischemia in various organ systems. There are 4 major types of opioid receptors: mu (µ), delta (δ), kappa (κ), and opioid receptor-like subtype 1 receptors. Opioid receptors are inhibitory GPCRs that cause membrane hyperpolarization upon activation, leading to presynaptic neuronal inhibition. Opioid receptor activation can also inhibit adenylyl cyclase, leading to a decrease in cyclic adenosine monophosphate (cAMP) production, another mode of reducing neuronal action potential (Dragasis, Bassiakou et al. 2013).

In 1995, Schutz and colleagues presented a study that investigated the involvement of opioid receptors in IPC. Rats were subjected to 30 minutes of coronary occlusion and 2 hours of reperfusion. Prior to prolonged ischemia, three cycles of 5-minute occlusion and reperfusion were applied to the IPC group, which strongly reduced infarct size. This protective effect was abolished by treatment with naloxone, a non-selective opioid antagonist. Naloxone did not have an effect on ischemia-induced infarct size in sham-RIPC rats. It was the first study to suggest that opioid receptor activation takes part in myocardial preconditioning (Schultz, Rose et al. 1995).
Miki et al. showed in 1998 that the opioid receptor is involved in IPC via PKC activation in rabbits. The study involved induction of myocardial infarction by 30-minute coronary occlusion and 2 or 3 hours of reperfusion. IPC (5-minute ischemia and 10-minute reperfusion) was able to significantly reduce ischemia-induced infarction. The protection was inhibited by naloxone when a single episode of IPC was applied but was not blocked with 3 cycles of IPC. Morphine recapitulated the protective effect of IPC. Treatment with chelerythrine, a PKC inhibitor, or naloxone could abolish morphine-induced cardioprotection, whereas the treatment was ineffective in non-preconditioned hearts subjected to I/R (Miki, Cohen et al. 1998).

Opioid receptors have also been implicated in preconditioning of non-cardiac organs. In 2002, Dickson et al. demonstrated that myocardium-derived preconditioning factors could protect against I/R injury in a non-cardiac organ. In this study, coronary effluent was collected from rabbit hearts that were subjected to normoxic perfusion or 5 cycles of 5-minute ischemia and 10-minute reperfusion. The effluent was then used to pretreat isolated jejuna, where opioid receptors are highly expressed, in preparation for 1-hour simulated ischemia followed by 30-minute reoxygenation. In IPC concentrate-treated jejunal segments, maximal contractile force following sustained I/R was significantly improved compared to the control. This protective effect was moderately reduced with naloxone or glibenclamide treatment. The authors speculated that it might be due to the particular dose or duration of inhibitor administration, or because many other mediators are also involved in preconditioning (Dickson, Tubbs et al. 2002). Nevertheless, the results indicate that opioid receptor and KATP channel activation may contribute to preconditioning-induced organ protection.

A study published in 2004 also showed an association between opioid receptor activation and IPC in a rodent model of myocardial I/R injury. In this study, rats were subjected to 30 minutes of coronary occlusion and 2 hours of reperfusion to induce myocardial infarction. IPC (3 cycles of 5-minute occlusion and reperfusion) and administration of remifentanil, a potent, short-acting opioid analgesic agent, both led to a significant reduction in infarct size compared to the sham. In the IPC group, naltrindole (NTD), a selective δ-opioid receptor blocker, abrogated the protection; whereas nor-binaltorphimine (nor-BNI), a selective κ-opioid receptor inhibitor, moderately mitigated protection. Treatment with (CTOP), a µ-opioid receptor antagonist, did not have an effect on IPC-induced protection. Pretreatment with CTOP and NTD remarkably abolished the cardioprotective effect of remifentanil, whereas nor-BNI only mildly attenuated the effect. Since
only δ- and κ-opioid receptors have been well characterized in the heart, the authors suggested that intracardiac μ-opioid receptor may not be involved in transient ischemia- and remifentanil-induced myocardial preconditioning. Perhaps μ-opioid receptor of non-cardiac origin may be involved in the action of remifentanil (Zhang, Irwin et al. 2004).

4.1.1.10 MicroRNA-144

MicroRNAs are small non-coding RNA molecules that play a role in regulating post-transcriptional gene expression (Wang, Zhu et al. 2012). miR-144 and miR-451 originate from a single gene locus that is regulated by GATA-binding factor 1 (GATA-1), a stress-responsive erythroid transcription factor, and form a cluster (Zhang, Wang et al. 2010). A number of studies have implicated miR-144 in preconditioning.

Zhang et al. in 2010 presented that miR-144 and miR-451 work synergistically to confer protection against I/R-induced cardiomyocyte death. The group hypothesized that the miR-144/451 cluster may be involved in cardioprotection, given that IPC is known to modulate GATA-4, which regulates cardiomyocyte survival (Suzuki, Nagase et al. 2004). Rat H9c2 (ventricular cell line) cells were subjected to simulated ischemia for 8 hours, followed by 3 hours of reoxygenation. Overexpression of miR-144 and miR-451 significantly enhanced cell survival, whereas miR knockdown with inhibitors significantly compromised cell viability. Co-transfection of cells with both miR-144 and miR-451 mimicked further improved survival rate compared to cells that were transfected with a single miR mimic, which is indicative of a synergistic effect (Zhang, Wang et al. 2010).

In a subsequent study, Wang and colleagues used a murine model of IPC to investigate the functional role of miR-144/451 cluster in the myocardium. The study involved preconditioning (3 cycles of 5-minute I/R) of the miR-144/451 knockout or wild type hearts prior to 20 minutes of global no-flow ischemia and 40 minutes of reperfusion. IPC significantly improved cardiac functional recovery and protected against necrotic cell death following I/R injury compared to the sham. However, the protective effect of IPC was absent in the knockout hearts. In vivo findings were consistent with the ex vivo results, where IPC-induced protection against infarction was ablated in the knockout animals. Surprisingly, further studies showed that knockdown of miR-451, but not miR-144, abolished IPC-induced cardioprotection (i.e. aggravation of myocardial damage, impaired contractile function, elevated ROS levels) (Wang,
Zhu et al. 2012). Perhaps with respect to local IPC, miR-144 may not play an essential role in preconditioning.

A more recent study conducted by Li et al. demonstrated that miR-144 is actually a circulating effector of RIPC. In this set of experiments, RIPC was induced in mice by 4 cycles of 5-minute hindlimb ischemia and reperfusion with a tourniquet. Primarily, myocardial miR microarray expression profiling in mice showed a significant upregulation in miR-144 levels following RIPC. Intriguingly, miR-451 was not greatly affected by RIPC. Secondly, isolated mouse hearts underwent 30 minutes of no-flow global ischemia and 60 minutes of reperfusion to induce infarction. RIPC significantly improved functional recovery and reduced infarct size compared to the sham, and this protective effect was abolished with antagomiR-144 administration. Lastly, the group studied the effect of RIPC on plasma microparticle (MP) levels, since exosomes and microvesicles are known to be responsible for the transport of miRs, which are susceptible to degradation by plasma RNase (Diehl, Fricke et al. 2012). Nullifying the hypothesis, MP levels were unaffected by RIPC. However, miR-144 precursor levels were significantly increased in the plasma exosome, and miR-144 levels were markedly elevated in the exosome-free serum. Additional experiments suggested that Argonaute-2 (Ago2), an extracellular carrier of miR, might be responsible for miR-144 transport, as RIPC significantly promoted Ago2 binding to miR-144. Thus, the authors postulated that RIPC upregulates miR-144 precursor expression in circulating MPs, which releases miR-144 into the serum, which in turn makes use of Ago2 as a mode of transport to induce protective effects in the heart, and perhaps in other organs, as well (Li, Rohailla et al. 2014).

4.1.1.11 Intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 also has been mentioned as a potential target for ischemic conditioning, since ICAM-1 is involved in neutrophil transmigration. An in vitro study performed preconditioning on rat aortic endothelial cells using an anaerobic chamber. Cells were preconditioned with 1 hour of anoxia and 1 hour of reoxygenation, immediately before 6 hours of prolonged anoxia and reperfusion (A/R). ICAM-1 mRNA levels increased over time following A/R, reaching a maximum at 6 hours of reoxygenation. ICAM-1 protein levels were also increased in a similar manner, peaking at 9 hours of reoxygenation. This significant increase in ICAM-1 expression was attenuated with preconditioning. A/R also drastically increased neutrophil adherence to the
endothelial cells. The increase in adhesion was abolished with preconditioning (Beauchamp, Richard et al. 1999).

### 4.1.2 Signal transduction of ischemic conditioning

Signal transduction of RIC in the heart in particular has been the focus of research in various animal models. RIC activates numerous intricate molecular signaling pathways, namely reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways.

#### 4.1.2.1 RISK pathway

The IPC-induced RISK pathway involves activation of GPCRs and prosurvival kinases, phosphatidylinositol-3-OH kinase (PI3K), protein kinase B (Akt), and extracellular-signal-regulated kinases 1 and 2 (ERK-1/2). The RISK pathway appears to be potently activated during preconditioning ischemia, as well as during reperfusion following sustained ischemia. As reviewed by Hausenloy in 2005, to date, various factors, such as insulin, urocortin, bradykinin, and opioid receptor ligands, have been identified as triggers for the RISK pathway (Hausenloy, Tsang et al. 2005).

In 2000, Tong et al. showed that IPC induces PI3K activation, which has been reported to activate PKC and eNOS. The study examined the effect of IPC (4 cycles of 5-minute ischemia and reperfusion) on the recovery of contractile function (i.e. left ventricular developed pressure) in isolated rat hearts following 20 minutes of global ischemia. Measured at 30 minutes into reperfusion, ventricular contractile function was significantly improved with IPC. Treatment PI3K inhibitors, wortmannin (WM) or LY-294002, throughout preconditioning abrogated the IPC-induced improvement in post-ischemic myocardial function. The group also observed that PI3K acts upstream of PKC. Administration of 1,2-dioctanoyl-sn-glycerol (DOG), a PKC activator, significantly enhanced post-ischemic functional recovery compared to the control. WM was unable to mitigate the protective effect of DOG but decreased the preconditioning-induced PKC translocation. Moreover, DOG treatment did not significantly change Akt phosphorylation, which was elevated with IPC and abrogated with WM+IPC. These results collectively indicate that PKC is downstream of PI3K/Akt (Tong, Chen et al. 2000).
In 2004, Hausenloy and colleagues demonstrated that IPC-induced protection involves prosurvival kinase activation at reperfusion following prolonged ischemia. In this study, isolated rat hearts were subjected to IPC (two cycles of 5-minute global ischemia and 10-minute reperfusion) prior to 35 minutes of sustained ischemia and 2 hours of reperfusion. A biphasic increase in Akt, ERK-1/2, and p70S6K (the kinase downstream of Akt and ERK-1/2) phosphorylation was observed following IPC: an immediate increase and a second increase at reperfusion following prolonged ischemia. Treatment with LY-294002 during the early phase of reperfusion following sustained ischemia abolished the IPC-induced phosphorylation of Akt and p70S6K. Similarly, administration of PD-98059, an inhibitor of mitogen-activated protein kinase kinase 1 and 2 (MEK-1/2) that acts on ERK-1/2, during the first 15 minutes of reperfusion also blocked the IPC-induced ERK-1/2 phosphorylation and p70S6K. Individual inhibitor treatment alone did not have an effect on Akt or ERK-1/2 phosphorylation in the sham heart. Furthermore, the cardioprotective effect of IPC on infarct size was abrogated with LY-294002 or PD-98059 treatment early during reperfusion. The authors concluded that IPC leads to PI3K-Akt and MEK-1/2-ERK-1/2 pathways during reperfusion following sustained ischemia. Therefore, activation of the RISK pathway is required during preconditioning as well as at reperfusion to produce cardioprotective effects (Hausenloy, Tsang et al. 2005).

In 2012, Hausenloy and colleagues reported that activation of the RISK pathway is also involved in RIPC-induced cardioprotection. The authors subjected pigs to in situ 60-minute coronary occlusion followed by 3 hours of reperfusion. Both RIPC (4 cycles of 5-minute lower limb ischemia and reperfusion) prior to induction of prolonged ischemia and RIPerC (same protocol as RIPC) applied 1 minute before the onset of I/R significantly decreased infarct size. WM (PI3K inhibitor) attenuated the protective effect of RIPC when administered 30 seconds prior to sustained reperfusion, but did not significantly affect RIPerC. RIPC also was shown to significantly increase both myocardial PI3K and Akt phosphorylation compared to the control. This effect on phosphorylation was abrogated with WM and 8-SPT (adenosine receptor inhibitor). Interestingly, 8-SPT could not block the protective effect of RIPC nor RIPerC against infarction. The group suggested that RIPC-induced cardioprotection involves activation of the RISK pathway at the time of reperfusion within the heart, whereas RIPC confers protection independent of PI3K/Akt activation (Hausenloy, Iliodromitis et al. 2012). They also speculated that RIPC might not require adenosine receptor activation during reperfusion, although their
findings on the role of adenosine in RIPC were rather ambiguous and contradict results from another study that demonstrated the protective role of endogenous adenosine in IPC of the heart via Akt activation during the early phase of reperfusion (Solenkova, Solodushko et al. 2006).

As reviewed by Hausenloy in 2005, pharmacological activation of the RISK pathway during reperfusion leads to phosphorylation of various downstream proteins such as p70S6K, eNOS, BAD, and glycogen synthase kinase 3 beta (GSK3β). Various studies suggested that the RISK pathway may confer protection via inhibition of mitochondrial permeability transition pore (mPTP) opening (Hausenloy, Tsang et al. 2005). In 2004, Hausenloy and colleagues demonstrated that preconditioning protects the myocardium by reducing the probability of mPTP opening, which normally occurs in response to I/R-induced oxidative stress. In this study, pore opening was induced in rat myocardioocytes subjected to oxidative stress. Pretreating cells with hypoxic or pharmacological preconditioning was able to increase the time taken to induce opening, thereby conferring protection against oxidative stress-induced rigor contracture (Hausenloy, Yellon et al. 2004). Tsuruta et al. showed in 2002 that activation of the PI3K-Akt pathway inhibits translocation of Bcl2-associated X protein (Bax), a proapoptotic member of the Bcl-2 family that promote cytochrome c release through mPTP, from the cytoplasm to mitochondria in serum-stimulated HeLa cells (Tsuruta, Masuyama et al. 2002). It was also reported that preconditioning involves inhibition of GSK3β via PI3K-Akt pathway activation, which in turn mediates inhibition of mPTP opening in rat myocardioocytes (Juhaszova, Zorov et al. 2004).

### 4.1.2.2 SAFE pathway

Activation of the SAFE pathway, which involves Janus kinases (JAKs; tyrosine kinases associated with membrane receptors) and signal transducer and activator of transcription 3 (STAT-3), is triggered by TNFα. As reviewed by Lecour in 2009, various studies have shown the paradoxical protective effect of TNFα (Lecour 2009). Controversial findings have been reported, but the discrepancy may result from activation of different TNFα receptor types: TNFα receptor 1 activation is associated with damaging effects, whereas TNFα receptor 2 activation is implicated in cardioprotection (Monden, Kubota et al. 2007). IPC-induced protection was observed in wild type mice; however, in TNFα knockout mice, IPC (4 cycles of 5-minute ischemia and reperfusion) could not confer protection against I/R-induced myocardial infarction
(Smith, Suleman et al. 2002). It was also shown that TNFα-induced cardioprotection against I/R injury is independent of p38-MAPK activation (Tanno, Gorog et al. 2003).

During I/R, ligand binding (e.g. TNFα, IL-6) causes activation of membrane receptors, which leads to transphosphorylation of JAKs, which subsequently activate STAT proteins through tyrosine phosphorylation. Activated STAT transcription factors form dimers and translocate into the nucleus, resulting in transcription of stress-response genes (Lecour 2009). In 2001, Xuan et al. reported that IPC (6 cycles of 4-minute coronary occlusion and reperfusion) increased JAK1/2 phosphorylation, which led to activation and nuclear translocation of STAT1 and STAT3 in mice. The authors also observed that pretreatment with AG-490, a JAK inhibitor, prior to preconditioning inhibits the IPC-induced increase in JAK1/2 and STAT1/3 phosphorylation. AG-490 also abrogated late IPC-mediated protection against myocardial infarction and upregulation of iNOS expression and increased activity, which is seen 24 hours following IPC without JAK inhibition (Xuan, Guo et al. 2001). Similar results were observed by Hattori et al., who showed that extensive IPC-induced phosphorylation of JAK2 and STAT3 was strongly abrogated by AG-490 in isolated rat hearts. AG-490 also abolished IPC-induced cardioprotection against sustained I/R (i.e. improved contractile function, reduced infarct size and cardiomyocyte apoptosis). Further, STAT3 activation led to an increase in anti-apoptotic Bcl-2 and a decrease in proapoptotic Bax gene expression (Hattori, Maulik et al. 2001). Furthermore, activation of the JAK/STAT pathway has been implicated in opioid-mediated cardioprotection via phosphorylation and inhibition of GSK3β in rats (Gross, Hsu et al. 2006).

A recent study conducted by Skyschally and colleagues demonstrated that activation of both the RISK and SAFE pathways is involved in inter-species transfer of RIPC-induced cardioprotection against I/R injury. In this study, pigs were subjected to RIPC (4 cycles of 5-minute hindlimb ischemia and reperfusion) or sham treatment prior to 60-minute coronary occlusion and 3-hour reperfusion. RIPC significantly reduced infarct size compared to the sham and also promoted STAT3 phosphorylation during the early phase of reperfusion. The effects of RIPC were abolished with AG-490 (SAFE blockade), whereas WM treatment (RISK blockade) was ineffective. Treating isolated rat hearts with RIPC-induced pig plasma prior to sustained I/R (30-minute global ischemia and 2-hour reperfusion) resulted in reduced infarct size. This protective effect was abolished with either RISK or SAFE pathway inhibition. The group suggested that both SAFE and RISK pathways are involved in RIPC-mediated cardioprotection in rodents,
whereas SAFE, but not RISK activation, is required in pigs, which contradicts the findings reported in 2012 by Hausenloy et al. Differences between species may exist with respect to the signaling pathways activated by preconditioning (Skyschally, Gent et al. 2015).

4.2 Effect of RIC on various inflammatory conditions

4.2.1 Effect of RIC on hemorrhagic shock and resuscitation (S/R)

Hemorrhagic shock and resuscitation (S/R) experienced by trauma patients gives rise to the development of systemic inflammation and dysfunction in various organs as a form of I/R injury. S/R introduces oxidative stress, which is the culprit for activating neutrophils, promoting an inflammatory response in the lungs, and causing organ damage. Jan and colleagues showed that bilateral RIC prior to S/R reduced histological damage in the rat lung and pulmonary edema. Furthermore, BALF protein concentration, malondialdehyde, and plasma IL-6 levels were also decreased, suggesting that RIPC is able to alleviate the detrimental effects of S/R on the lung (Jan, Chen et al. 2011).

Our group recently observed that remote ischemic conditioning prevents lung and liver injury following S/R. Using a mouse model of S/R, Leung and colleagues reported that serum ALT levels and liver mRNA expression of IL-1β and TNF-α were substantially decreased with RIC (RIPC, RIPerC, and RIPostC). Liver histology was also improved compared to the control. Moreover, reduced lung injury and inflammation were seen with RIPC in particular (Leung CH, Caldarone CA et al. 2015). Therefore, RIC exerts its protective effects in cases of S/R, as well.

4.2.2 Effect of RIC on systemic inflammatory response

Few studies have investigated the effect of RIC on inflammation in particular. A 2014 study used a mouse model of sepsis to test for RIC protection. Kim et al. observed that application of RIC, both before and after intraperitoneal injection of lipopolysaccharide (LPS), increased survival and suppressed increases in inflammatory cytokines, such as IL-1β, IL-6, and TNF-α following LPS administration. Moreover, an anti-inflammatory cytokine, interleukin-10 (IL-10), was significantly increased in the LPS-injected, post-conditioned mice compared to the mice that received LPS alone. Neutrophil accumulation within the sinusoid of the liver was significantly reduced in the RIC-LPS groups, compared to those that received only LPS. The authors
concluded that both RIPC and RIPostC produced similar protective effects in the sepsis model (Kim, Yoon et al. 2014).

The effect of RIC has not yet been investigated in a model of acute lung injury. Our study will examine whether or not RIC can exert its protective effects in other inflammatory conditions, as well.

### 4.3 Ischemic post-conditioning

Ischemic conditioning studies often examine the effect of ischemic post-conditioning in addition to IPC. Post-conditioning is a more translational approach, as preconditioning can only be performed when the occurrence of an inflammatory response is predictable.

In 2003, Zhao and colleagues studied the effect of ischemic post-conditioning on myocardial infarction in dogs. In the control group, dogs were subjected to LAD artery occlusion for 60 minutes, followed by 3 hours of reperfusion. In the preconditioning group, the LAD artery was occluded transiently for 5 minutes followed by 10 minutes of reperfusion, prior to prolonged 60-minute occlusion. In the post-conditioning group, immediately after 60-minute LAD artery occlusion and brief 30-second reperfusion, the dogs were subjected to three cycles of 30-second ischemia and 30-second reperfusion, followed by prolonged 3-hour reperfusion. Myocardial infarct size was significantly reduced in both the preconditioned and post-conditioned groups compared to the control. Myocardial edema and neutrophil accumulation in the myocardium following I/R were significantly reduced in both conditioned groups. Neutrophil adhesion to the LAD arterial endothelium following I/R was also decreased with preconditioning and post-conditioning when compared to the control group. The data suggest that post-conditioning is as effective as IPC in protecting the myocardium from I/R challenge (Zhao, Corvera et al. 2003). Also, the protective effect of RIPostC has been demonstrated in a rat model of brain focal ischemia. Focal cerebral ischemia (stroke) was induced in male rats by occluding the bilateral common carotid arteries (CCAs) for 30 minutes and permanently occluding the left distal middle cerebral artery (MCA). RIPostC was performed by applying three cycles of 15-minute ischemia and reperfusion to the left femoral artery. RIPostC was either applied immediately, or at 3 or 6 hours following CCA release. The authors reported that RIPostC applied immediately after stroke induction caused a significant reduction in infarction at 2 days after stroke. RIPostC applied at 3 hours was still able to reduce infarct size significantly; however, when performed at
6 hours, the positive effect was not observed (Ren, Yan et al. 2009). Therefore, post-conditioning appears to have a protective effect in reducing inflammation and injury, similar to pre-conditioning.

Repeated application of post-conditioning has also been shown to be effective in providing cardioprotection in rats. Myocardial infarction was induced by ligating the LAD artery for 45 minutes, which was followed by reperfusion. The RIC protocol involved application of four cycles of 5-minute ischemia and reperfusion to the hindlimb. One group received a single application of conditioning during the 45-minute LAD artery ischemia (RIPerC), another group received RIPostC daily for 28 days (D-1PostC), and the last group received RIPostC every 3 days for 28 days (D-3PostC). All three conditioned groups showed greater survival rates over the course of 84 days compared to the control. Myocardial infarct size was significantly reduced in all the preconditioned groups at days 4 and 28 from the onset of infarction. Leukocyte infiltration into the infarct was dose-dependently attenuated with RIPerC and RIPostC. MDA levels and activation of NF-κB subunit p65 and IκBa were significantly increased in rats subjected to myocardial infarction without conditioning. The response was significantly attenuated with both RIPerC and RIPostC. TNF-α and IL-1β levels were attenuated in the post-conditioned group only at day 28. Both per- and post-conditioning improved left ventricular function and fractional shortening compared to the control; the greatest improvement was observed in the D-1PostC group. Thus, RIPerC and RIPostC both reduce myocardial infarct size in rats; however, chronic, repeated remote post-conditioning was associated with further dose-dependent improvements in cardiac function compared to a single administration of RIC (Wei, Xin et al. 2011).

### 4.4 Combined conditioning protocols

A number of clinical trials have been conducted to test whether the combination of RIPC and RIPostC can improve the clinical outcomes of patients subjected to cardiac surgery. Since numerous animal studies and a number of clinical trials have shown the protective effect of RIPC on various organs, such as the heart, lungs, kidneys, spinal cord, and skeletal muscles, groups have postulated that combining different RIC protocols may be able to augment the protective effect (Min, Bae et al. 2015).
4.4.1 Animal studies

In 2010, Xin et al. showed that combining local ischemic postconditioning with RIPerC produced a similar protective effect, as posed by IPC. Rats were subjected to prolonged ischemia induced by 45-minute coronary ligation followed by 2-hour reperfusion. In the IPC group, rats underwent IPC, which involved four cycles of 5-minute coronary ligation and reperfusion, prior to prolonged I/R. In the combined RIC group, rats received RIPerC at the end of the 45-minute ischemia by occluding blood flow to a hindlimb with a tourniquet in four cycles of 5-minute ischemia and reperfusion, as well as ischemic postconditioning involving six cycles of 30-second occlusion and reperfusion of the coronary artery at the beginning of the 2-hour reperfusion. It was found that the optimal combination of per-conditioning and post-conditioning resulted in lower infarct size compared to what was seen with per-conditioning or postconditioning alone. However, the combination was only able to produce protective effects that were comparable to IPC alone. Thus, the combined effect of different RIC protocols has been suggested (Xin, Zhu et al. 2010).

In 2011, Wei et al. repeated RIPostC combined with RIPerC protects against adverse left ventricular remodeling and enhances survival in a rodent model of myocardial infarction. The study involved induction of myocardial infarction by 45-minute coronary occlusion and reperfusion in rats. Different RIC protocols were tested in these rats. The RIPerC group was subjected to a single episode of RIPerC during prolonged ischemia, which involved four cycles of 5-minute hindlimb ischemia and reperfusion. D-1RIPostC group underwent RIPostC (four cycles of 5-minute hindlimb ischemia and reperfusion) daily for 28 days, in addition to RIPerC. D-3RIPostC group received RIPostC every 3 days for 28 days, on top of RIPerC. All three RIC groups demonstrated improved survival rate compared to the control, where the D-1RIPostC group was associated with the most improvement. Moreover, neutrophil and macrophage infiltration into the infarct border zone was significantly attenuated in the RIPerC rats, which was further attenuated in the D-3 and D-1RIPostC groups. All RIC groups showed remarkable protection against adverse LV remodeling compared to the control myocardial infarction group; however, further improvement was seen in the combined RIC groups, especially in the D-1PostC group (Wei, Xin et al. 2011). Surprisingly, although infarct size was significantly reduced in all RIC groups, no significant difference between the conditioning groups was observed. The group,
therefore, suggested clinical implications of combined RIC protocols for enhanced protection against myocardial infarction.

In 2012, Song et al. showed that combined preconditioning and postconditioning provides a synergistic protective effect against liver I/R injury. In this study, mice were subjected to prolonged hepatic ischemia for 30 minutes, followed by 1-hour reperfusion. Various combinations of RIC protocols were applied to assess the degree of I/R injury (e.g. preconditioning/postconditioning alone, preconditioning plus postconditioning). The group found that all RIC groups demonstrated a significant reduction in the plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared to the control, indicative of reduced hepatic injury. The reduction was much more remarkable with the combination of RIPC (3 cycles of 10-minute hindlimb ischemia and reperfusion) or local IPC (3 cycles of 5-minute hepatic ischemia and reperfusion) with local ischemic postconditioning (3 cycles of 30-second hepatic ischemia and reperfusion at the start of 1-hour reperfusion).

Furthermore, all RIC protocols decreased MDA levels and enhanced total antioxidative capacity, which is representative of reduced tissue oxidative stress. The decrease in oxidative stress was more pronounced in the combined RIC groups compared to individual RIC groups. The data suggested that combinations of pre- and postconditioning produce better outcomes than individual RIC treatments (Song, Zhang et al. 2012).

4.4.2 Clinical trials

Despite all of the promising data obtained from animal studies, results from clinical trials on combined RIC have not been as convincing. In 2012, Kim et al. reported findings from a small randomized, placebo-controlled, double-blind trial that attempted to investigate the effect of combined RIPC and RIPostC on pulmonary function in patients undergoing valvular heart surgery. Fifty-four patients were enrolled in the study, where the patients were randomly assigned to control or RIPC plus RIPostC groups. The combined RIC protocol involved three cycles of 10-minute right upper-leg ischemia (250 mmHg) and reperfusion using a cuff. RIPC was performed 10 minutes following induction of anesthesia and RIPostC 10 minutes after weaning from cardiopulmonary bypass. The data showed that the \( \text{PaO}_2/\text{FiO}_2 \) oxygenation ratio was significantly reduced 24 hours following surgery compared to baseline. However, there was no significant difference in the oxygenation ratio, incidence of ALI, and serum inflammatory
cytokine levels between the two groups. Therefore, the authors concluded that RIPC in combination with RIPostC was ineffective in providing pulmonary benefit in the case of valvular cardiac surgery (Kim, Shim et al. 2012).

A study by Hong et al., referred to as the “RISPO (Remote Ischemic Preconditioning with Postconditioning Outcome) trial”, demonstrated similar results. Patients undergoing elective cardiac surgery (1280 in total) were randomized to receive control or combined RIPC and RIPostC therapy. In the combined RIC group, four cycles of 5-minute ischemia and reperfusion were applied to the upper limb, once following anesthesia induction (RIPC) and once more following cardiopulmonary bypass (RIPostC). No significant differences were found in the incidence of major adverse outcomes, such as death, myocardial infarction, arrhythmia, stroke, coma, and multiorgan failure. Furthermore, neither the duration of care in the intensive care unit nor hospitalization significantly differed between the groups. Therefore, it was concluded that the combination of RIPC and RIPostC did not improve clinical outcomes in patients subjected to cardiac surgery (Hong, Lee et al. 2014).

However, a subgroup study of the RISPO trial that focused on the protective effect on the pulmonary function of combined RIPC and RIPostC in patients undergoing cardiac surgery revealed some benefit. Pulmonary function is often compromised following cardiopulmonary bypass used in cardiac surgery, due to pulmonary I/R injury caused by the arrest of arterial blood flow to the lungs during the bypass. Sixty-five patients were enrolled and randomized into control or combined RIC groups. The combined RIC group received four cycles of 5-minute upper limb ischemia and reperfusion before and after cardiopulmonary bypass. Twenty-four hours following surgery, the combined RIPC and RIPostC group had a significantly higher mean PaO₂/FiO₂ oxygenation ratio compared to the control group. Moreover, the use of mechanical ventilation for more than 48 hours was significantly decreased in the combined RIC group. Although no significant differences were seen with respect to other measures of pulmonary function or duration of stay in the intensive care unit, the group suggested that combined RIC protocol improves pulmonary function to an extent following cardiac surgery involving cardiopulmonary bypass (Min, Bae et al. 2015). The efficacy of combined RIC protocols remains unclear, as results vary depending on the model and endpoint of choice.
4.5 Protection of other organs by RIC

4.5.1 Acute kidney injury (AKI)

AKI or acute renal failure (ARF) is associated with significant morbidity and mortality, as it often emerges following operative procedures, such as cardiac surgery. It can affect up to 30% of patients that have undergone a major cardiac surgery, resulting in renal dialysis in 1-2% of cases, and often leads to death (Rosner and Okusa 2006). Patients also commonly develop AKI following major vascular surgeries involving the abdominal aorta, occurring in up to 10% of patients (Candilio, Malik et al. 2013). Although several clinical studies have been conducted to investigate the effect of RIC in preventing renal dysfunction and the development of AKI that may arise from various surgical procedures, not all of them have reported positive results. Wever et al. reported in 2011 that rat hindlimb RIPC prior to 25-minute ischemia in the right kidney improved renal function by 30-60% after 24 hours of reperfusion. Preconditioning was applied either on both hindlimbs or on one hindlimb. The protective effect was seen in both groups. Renal tubule damage and kidney injury molecule-1 expression were also reduced in the RIPC groups (Wever, Warle et al. 2011). Larger multicenter randomized controlled clinical trials are being conducted to investigate the actual efficacy of RIC on acute renal I/R injury (Candilio, Malik et al. 2013).

4.5.2 Acute lung injury (ALI)

As previously mentioned, ALI is a disorder of acute lung inflammation that eventually leads to acute hypoxemic respiratory failure. Mainly triggered by trauma, infection, lung transplantation, and cardiac surgery, ALI is a significant source of morbidity and mortality to patients in critical conditions. ALI has a high incidence of approximately 200 000 cases per year in the United States, and overall mortality rates remain high (Johnson and Matthay 2010). One of the earlier studies to examine the effect of RIC on ALI was conducted by Peralta et al. in 2001. Preconditioning of the rat liver reduced systemic inflammation as well as neutrophil infiltration into the lung and other organs following a sustained liver ischemia (Peralta, Fernandez et al. 2001). As reviewed by Candilio, the majority of the in vivo and clinical studies evaluate the effect of RIC on the heart, and pulmonary function is usually examined as a secondary endpoint (Candilio, Malik et al. 2013). More studies that focus on ALI as the primary insult still need to be conducted.
4.5.3 Acute liver ischemia/reperfusion (I/R) injury

Acute liver I/R injury may develop as a consequence of major liver surgery, as vascular clamping and unclamping of the liver during the procedure can cause accumulation of oxygen-derived free radicals in the liver (Candilio, Malik et al. 2013). Lai et al. were among the first to observe the beneficial effects of RIC on acute liver I/R injury. In 2006, the authors reported that application of 4 cycles of 10-minute ischemia and reperfusion to the hind limb of rats attenuated the increase in the serum liver enzyme ALT. They also observed that increased expression of HO-1 was associated with RIC protection (Lai, Chang et al. 2006). Since then, various RIC protocols have been evaluated in several animal models of liver I/R injury. Most studies have reported positive outcomes, such as decreased expression of inflammatory markers (Candilio, Malik et al. 2013).

4.5.4 Cerebral ischemia

Numerous studies have also investigated the effect of RIC on neuroprotection using various models. A study published in 2011 used a rat model of cerebral ischemia to observe the effect of RIPC and RIPerC. The conditioning stimulus included 4 cycles of 5-minute left hindlimb ischemia and reperfusion. Before or after conditioning, focal cerebral ischemia was achieved for 120 minutes, followed by reperfusion. In accordance with other studies, brain infarct size at 24 hours was significantly reduced in the conditioned groups compared to control, suggestive of neuroprotection (Hahn, Manlhiot et al. 2011).

Likewise, the protective effect of RIC has been observed in other organs, such as the brain and the gastrointestinal system (Candilio, Malik et al. 2013). Thus, RIC is considered a non-invasive and effective therapeutic intervention that is able to confer global organ protection, and more large multicenter randomized controlled clinical trials are needed to test its efficacy and feasibility in various clinical scenarios.

4.6 Clinical trials

Numerous clinical trials have been conducted to determine the efficacy of RIC in patients. Most large trials mainly focused on the cardiac application of RIC.
A study published in 2010 investigated the cardioprotective effect of RIPC in patients with evolving myocardial infarction in a single-centre randomized, controlled trial. Individuals diagnosed with ST-segment elevation and chest pain prior to maximum of 12 hours of hospital admission were included in the study (333 patients total). The preconditioning group received the intervention prior to percutaneous coronary intervention (PCI), mostly during transport to hospital. Preconditioning was performed on the upper arm with 4 cycles of 5-minute inflation and deflation of a standard blood pressure cuff. An elevated median salvage index of 0.69 was noted in the group that received preconditioning prior to PCI, compared to the control group with a median index of 0.54 (p=0.13). Salvage as a percentage of left ventricle was significantly higher in the preconditioned group than the control group. However, there were no significant differences in final infarct size, left ventricular ejection fraction at admission and after 30 days, peak troponin-T release and other parameters between the groups. Nonetheless, it was observed that for a given area at risk, measured as % of left ventricle, smaller infarcts developed in the preconditioned group. The authors concluded that RIPC is associated with increased myocardial salvage and more favourable outcomes (Botker, Kharbanda et al. 2010).

In 2014, the same group of authors reported their findings on patient follow-up from the study that was conducted on patients undergoing myocardial infarction. They were interested in the long-term clinical outcome, and it led to a median follow-up period of 3.8 years. The primary endpoint was occurrence of major adverse cardiac and cerebrovascular events (MACCE), which encompass all-cause mortality, myocardial infarction, readmission for heart failure, and ischemic stroke. According to the results, MACCE, including only cardiac mortality rather than all-cause mortality, occurred in 15 patients from the intervention group and in 25 patients from the control group. The hazard ratio for MACCE was 0.56, indicative of the effectiveness of the preconditioning intervention. The hazard ratios for the secondary endpoints, such as myocardial infarction, readmission for cardiac complications, and ischemic stroke, were all recorded below 1, in favour of preconditioning. Thus the data suggest that RIC prior to PCI may improve long-term clinical outcomes in patients with ST-elevation myocardial infarction (Sloth, Schmidt et al. 2014). Numerous in vivo studies and clinical trials reported that RIC is highly associated with favourable physiological outcomes in cases of I/R injury.

However, more recent clinical trials contradict the positive reports regarding the efficacy of RIC. A double-blind, multicenter, randomized, controlled clinical trial involving a total of 1403
patients undergoing elective cardiovascular surgery under propofol-induced anesthesia was recently conducted. The trial investigated the effect of upper-limb RIPC on clinical outcomes. Patients who were already anesthesized underwent four cycles of 5-minute upper-limb ischemia with blood pressure cuff inflation (≥ 200 mmHg) and 5-minute reperfusion with deflation of the cuff. The primary endpoints involved death, nonfatal myocardial infarction, stroke, and acute renal failure during the time of hospitalization, up to 14 days of hospital stay. The results showed no significant difference in the rate of the primary end points between the RIPC and sham-RIPC groups. Similar insignificant results were seen with respect to secondary endpoints involving troponin levels, duration of mechanical ventilation, and intensive care unit care. Although RIPC was not harmful to patients, it did not have a positive effect on clinical outcomes (Meybohm, Bein et al. 2015).

Similar results were reported in a recent multicenter, randomized, placebo-controlled trial that studied the effect of RIPC in patients subjected to coronary-artery bypass graft (CABG) surgery. The study involved a total of 1612 adults at increased surgical risk who were undergoing on-pump CABG with blood cardioplegia. Conducted after induction of anesthesia and before surgical incision, RIPC involved four cycles of 5-minute inflation (200 mmHg) and deflation applied to the upper arm with a standard blood pressure cuff. Twelve months following surgery, there was no significant difference between the experimental groups with respect to the combined primary endpoint, including death from cardiovascular complications, nonfatal myocardial infarction, coronary revascularization, and stroke. Moreover, RIPC did not have a significant effect on secondary clinical outcomes (i.e. perioperative myocardial injury, AKI, duration of hospitalization) or the incidence of adverse events. Therefore, the authors concluded that RIPC was ineffective in providing cardioprotection in higher-risk patients undergoing on-pump CABG (Hausenloy, Candilio et al. 2015).

Although RIC has been shown to have protective effects in various animal models as well as a number of clinical trials, its efficacy is still quite controversial. More studies are needed to uncover the exact underlying mechanisms in order for it to be actively implemented as an intervention in clinical settings.
Chapter 2 Objectives and Hypotheses

1 Objectives

ALI causes a heavy burden on public health due to high incidence, as it can be developed from various stimuli. An intervention that can alleviate the symptoms and improve clinical outcomes would be of great value to society. The objective of the present study was to investigate the effect of RIC on a novel inflammatory disease model. The effect of RIC on acute lung inflammation/injury has not yet been studied in the literature. We postulated that if RIC exerts its beneficial effects by releasing a protective factor(s), it would be capable of providing protection in various inflammation models, and not be limited to I/R injury. Intratracheal administration of LPS was used to induce lung inflammation, and a relatively low dose of 800 µg/kg was used in mice, in order to model the physiological response reported in patients and to avoid overwhelming the host inflammatory response.

2 Hypotheses

2.1 Hypothesis I: RIPC attenuates LPS-induced pulmonary inflammation.

Rationale for hypothesis: As previously discussed, preconditioning has been shown to be effective in various models of I/R injury and systemic inflammation. RIPC is thought to release protective factors into circulation, through which they travel and affect tissues that are distantly located from the preconditioned area of the body. By 4 hours following challenge, the first window of protection is expected to occur. RIPC is expected to reduce leukocyte influx into the alveolar space and damage to the alveolar-capillary barrier, which is seen with LPS stimulation. We also hypothesize that ICAM-1 expression will be decreased in the preconditioned lungs, since ICAM-1 is an essential factor in neutrophil transmigration. Lung HO-1 levels are expected to increase with preconditioning, as its upregulation has been shown to be associated with RIPC in the studies mentioned previously. RIPC is expected to reduce lung TNF-α and IL-1β expression, as Leung and colleagues reported that RIC is associated with decreased proinflammatory cytokine levels in the liver following liver I/R (Leung CH, Caldarone CA et al.
2015). Similar results were seen in a model of systemic inflammation, where RIPC reduced serum proinflammatory cytokine levels upon systemic LPS stimulation (Kim, Yoon et al. 2014). However, it is also possible that the proinflammatory cytokine expression might be increased, as was the case in the preconditioned neutrophils following LPS challenge, although it is somewhat paradoxical (Shimizu, Saxena et al. 2010). Structural histological damage is unexpected at this early time point.

2.2 Hypothesis II: RIPC provides sustained protection against LPS-induced pulmonary inflammation.

**Rationale for hypothesis:** Numerous studies have observed the effect of RIPC at later time points. In the model of systemic inflammation, RIC was able to maintain high percentage of survival in LPS-challenged mice over the course of 5 days (Kim, Yoon et al. 2014). Moreover, the 24-hour time point may fall under the second window of protection (Saxena, Newman et al. 2010). Preconditioning is expected to reduce pulmonary inflammation and injury measured 24 hours following challenge with LPS.

2.3 Hypothesis III: RIPostC prevents LPS-induced pulmonary inflammation.

**Rationale for hypothesis:** RIPostC has been shown to lead to reduced inflammation and injury, and subsequently better outcomes, as previously described. We hypothesized that post-conditioning would be able to attenuate leukocyte infiltration into the lungs and improve alveolar-capillary barrier integrity upon LPS exposure, similar to what was expected with RIPC.

2.4 Hypothesis IV: Dual conditioning exerts protection against LPS-induced pulmonary inflammation at 24 hours after challenge.

**Rationale for hypothesis:** Repeated RIPostC was expected to have a positive effect on LPS-induced pulmonary inflammation. Repeated, chronic RIPostC has been shown to give rise to dose-dependent improvements in infarct size and measures of inflammation in a rat model of myocardial infarction (Wei, Xin et al. 2011). Similar results are anticipated with dual conditioning.
Chapter 3 Materials and Methods

1 Reagents

Sodium pentobarbital (anesthetic) was purchased from Ceva. Lipopolysaccharide (LPS; *E. coli* O111:B4) was purchased from Sigma (Canada). The RNeasy Mini Kit was purchased from QIAGEN. Ethylenediaminetetraacetic acid (EDTA, 50 mM), DNase I, and 10X reaction buffer for DNase treatment were purchased from Fermentas (Thermo Fisher Scientific). iScript™ Select cDNA Synthesis Kit for reverse transcription was purchased from Bio-Rad. SYBR Green master mix was purchased from Applied Bio Systems. EDTA (0.5 M, pH 8.0) and phosphate-buffered saline (PBS, 1X, pH 7.4) used for bronchoalveolar lavage (BAL) was purchased from BioShop Canada Inc. DC™ Protein Assay kit was purchased from Bio-Rad. Hemacolor® rapid staining kit was purchased from EMD Millipore. Temgesic® (buprenorphine) was purchased from RB Pharmaceuticals Limited. All other reagents and primers were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2 Animal model of pulmonary inflammation

The animal experimental protocol (#635) was reviewed and approved by St. Michael’s Hospital Animal Care Committee. Male 9- to 13-week-old C57BL/6 mice from Charles River (Quebec, Canada) were used for the experiments.

2.1 Instrument preparation

All survival surgery procedures were held in the Research Vivarium at St. Michael’s Hospital, under aseptic conditions. A heating pad and lamp were used throughout the entire procedure to maintain a body temperature of approximately 37 °C. A rodent rectal thermometer was used to monitor temperature. Veterinary surgical tape, eye lube, gauze, 5-0 surgical suture (SOFTSILK), a tourniquet, and a timer were prepared for the surgery. A surgical clamp, scissors, forceps, and a needle driver were dry heat-sterilized using Germinator 500TM.
2.2 Surgical procedure

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg). The surgical plane of anesthesia was reached within 5 minutes of administration. The anesthetized animals were then transferred onto a heating pad in the supine position. Their forelimbs were fixed with loosely looped tape for natural positioning of the body.

2.2.1 Remote ischemic pre-conditioning (RIPC)

A tourniquet was applied to the right hindlimb of the mouse, 10 minutes following pentobarbital administration. The toes changed colour from light pink to purple-red. The tourniquet was released after 5 minutes; a total of 4 cycles of 5-minute ischemia and reperfusion were applied to the right hindlimb (Leung CH, Caldarone CA et al. 2015). Sensorcaine (bupivacaine, approximately 200 µL, diluted 1:6 with saline) was administered subcutaneously in the neck region 30 minutes after anesthesia. Temgesic® (buprenorphine, 0.2 mL) was also administered subcutaneously for mice that weigh 20-30 g. Following preconditioning, the neck incision site was shaved and the remaining fur was removed using hair removal cream. Betadine and alcohol were applied to the neck and a midline incision was made. The membrane and glands covering the trachea were blunt dissected. LPS (800 µg/kg) was dissolved in PBS, achieving a total volume of 50 µL. As outlined in Figure 1, the sham group received 50 µL of sterile PBS. Air (200 µL), followed by the PBS/LPS solution, was aspirated into a 1 mL syringe with a 30-gauge needle. The aspirated fluid was forcefully expelled through the needle into a tube in order to fill the dead space inside the syringe. Air (200 µL) was aspirated again with the same needle, followed by another PBS/LPS solution of equal concentration. The fluid was then injected forcefully into the trachea to create aerosol for even distribution. The site of incision was sutured, and the animals were given 500 µL of saline subcutaneously using a 27 gauge-needle before being placed in a recovery cage.
2.2.2 Remote ischemic post-conditioning (RIPostC)

Sodium pentobarbital was administered intraperitoneally to induce general anesthesia. After 30 minutes, 50 µL of PBS or LPS (800 µg/kg) was injected into the trachea (Figure 2). After another 15 minutes, RIPostC was performed by applying a tourniquet to the right hindlimb of the mouse with 4 cycles of 5-minute ischemia and 5-minute reperfusion. Following RIPostC, mice were placed in the recovery cage. Four hours from the time of PBS/LPS instillation, mice were sacrificed for sample collection and analysis.
Figure 2. Schematic of the remote ischemic post-conditioning (RIPostC) protocol of LPS-induced pulmonary inflammation.

2.2.3 Remote Ischemic Conditioning (RIC): pre- and post-conditioning

The procedures outlined in sections 2.2.1 Remote Ischemic Preconditioning were repeated for remote ischemic pre- and post-conditioning (dual conditioning). After having fully gained consciousness, the mice were returned to the animal room in cages with food and water. Then 4 hours before sacrifice (24-hour time point), mice were again subjected to general anesthesia, given only 50 mg/kg of sodium pentobarbital to achieve a surgical plane of anesthesia without death. After 10 minutes, RIPostC was performed to the RIC group using a tourniquet to control blood flow to the right hindlimb in 4 cycles of 5-minute ischemia and 5-minute reperfusion. The sham-RIC groups did not receive the intervention. Following ischemic conditioning, mice were placed in a recovery cage. Three hours and 10 minutes later, mice were sacrificed for sample collection and analysis.
2.3 Tissue harvest

Animals were sacrificed either at 4 hours or 24 hours from the time of intratracheal injection. A second dose of Buprenex (0.2 mL) was required before sacrifice for 24-hour studies.

2.4 Bronchoalveolar lavage (BAL)

Mice were placed in an induction chamber and exposed to 3% isoflurane to induce anesthesia. The animals were then transferred onto a heating pad and the surgical plane of anesthesia was maintained with a nose cone. The abdomen was opened and approximately 500 µL of blood was drawn from the inferior vena cava to obtain serum. The diaphragm was also cut to expose the lungs. The lungs were then perfused with cold PBS containing 0.5 mM EDTA, using a 20-gauge Angiocath to deliver the solution through the trachea. BAL was performed 4 times in 700 µL aliquots, and approximately 2.5 mL of BAL fluid (BALF) was obtained from each mouse.
3 Assessment of pulmonary inflammation

3.1 Analysis of BALF

3.1.1 Cell count

BAL samples were centrifuged (300 g, 7 minutes, 4°C), and the supernatant was collected and frozen at -80 °C. The pellet was treated with 1 mL of ammonium-chloride-potassium lysis buffer (ACK) for 3 minutes, then the buffer was inactivated with 3 mL of the lavage solution. Samples were centrifuged again (300 g, 7 minutes, 4°C), and the supernatant was discarded and the pellet was broken in 100 µL of the lavage solution. Total cell count was determined on a grid hemocytometer. The sample was further diluted with 300 µL of PBS. For cytospin, 100 µL of sample was loaded onto the apparatus and was spun onto microscope slides at 800 rpm for 5 minutes. Slides were then stained with the Hemacolor® staining kit. Cells were imaged at 40x magnification with Olympus Upright BX50. Five fields of view were imaged per sample, and differential immune cell populations were quantified with ImageJ software.

3.1.2 Protein analysis

Total protein concentration in the BALF was quantified using the DC™ Protein Assay kit. Sample (10 µL) was loaded onto each well of a 96-well plate, along with 25 µL of reagent A and S and 200 µL of reagent B. Bovine serum albumin (BSA) standards (0.25 mg/mL - 2.5 mg/mL) were also loaded onto the plate to obtain a standard curve. After 10 minutes of incubation, a plate reader was used to measure the absorbance at 690 nm. The linear trendline equation obtained from a scatter plot of the BSA standards was used to calculate the total BALF protein concentration. The values were divided by 5 in order to account for the excess amount of sample.

3.2 Myeloperoxidase (MPO) activity assay

The Myeloperoxidase (MPO) activity assay was performed to measure neutrophil infiltration as previously described(Leung CH, Caldarone CA et al. 2015).

3.2.1 Buffers

Buffers for the MPO activity assay were prepared as follows:

- Working buffer (WB): 50 mM potassium phosphate (pH 6.0)
• Lysis buffer (LB): WB + 0.5% hexadecyl trimethyl ammonium bromide (need heat to dissolve)
• Assay buffer (AB): WB + o-dianisidine + H₂O₂ (final 0.005%; prepared immediately before conducting assay)

3.2.2 Sample preparation

Approximately 3 mm³ of frozen lung tissue was placed in a glass dounce homogenizer containing 1 mL of the LB. Following homogenization, the mixture was poured into a microcentrifuge tube and was sonicated (level 3.5, 30%, 30 pulses). The tube containing the sonicated mixture was frozen in liquid nitrogen and thawed. Sonication and freezing steps were repeated for a total of 3 times each. Lastly, the tube was centrifuged (10 000 rpm, 10 minutes, 4 °C), and the supernatant was transferred into a new tube.

3.2.3 Assay

The assay was performed in duplicates. LB and samples were diluted (1:5) with WB. Either buffer control or sample (50 µL) was added into each well of a clear 96-well plate. AB (150 µL) was added to each well, and the absorbance was measured immediately with a plate reader at 460 nm for 15 minutes.

3.2.4 Analysis

Two time points at which the absorbance values lie in the linear range were chosen for analysis (time 1:00 and 2:00). The difference between the two absorbance values was determined and was divided by a constant, 0.0113. The values were then normalized to corresponding protein concentrations. The dilution factor of 5 was accounted for in the calculation. MPO activity is expressed as units of MPO per milligram of lung protein, whereby 1 unit of MPO is defined as the amount of enzyme that is required to degrade 1 nmol of H₂O₂ per minute.

3.3 Histology

3.3.1 Tissue preparation and staining

The left lobe of the lung inflated with 400 µL of formalin was fixed in 3 mL of formalin for more than 48 hours. Fixed lung samples were processed with a Leica Tissue Processor, and were
embedded in paraffin using Leica Tissue Embedder. Paraffin blocks were cut into 5 µm sections using a Leica RM2145 Microtome. Hematoxylin and eosin (H&E) staining of tissue sections was performed using a Leica Autostainer. Stained sections were imaged with an Olympus Upright BX50.

3.3.2 Lung injury score

Five fields of view were imaged for each sample at a 40X magnification, and were assessed by a blinded individual. The lung injury score ranges from 0 to 1, and the scoring system was based on neutrophil infiltration into the interstitium and alveolar compartment, thickening of the alveolar wall, and presence of proteinaceous debris and hyaline membrane (Matute-Bello, Downey et al. 2011).

3.4 Real-time quantitative polymerase chain reaction (RT-qPCR)

3.4.1 RNA preparation and reverse transcription

A RNeasy Mini Kit was used to extract RNA from approximately 30 mg of frozen lung tissue according to manufacturer’s instructions. A NanoDrop 2000 (Thermo Scientific) was used to determine RNA concentration as well as purity. The measured RNA concentration was used to calculate the volume of sample needed for 0.85 µg of RNA. RNA was then deoxyribonuclease (DNase)-treated to remove genomic DNA. Then reverse transcription was conducted to synthesize cDNA using iScript™ Select cDNA Synthesis Kit. The resulting cDNA was diluted with 80 µL of ribonuclease (RNase)-free water (1:5 dilution).

3.4.2 qPCR and analysis

RT-qPCR was performed using the SYBR green master mix. Forward and reverse primers (0.3 µM) were used for amplification. The cDNA was further diluted (1:8) for qPCR. All experiments were conducted in triplicates. The fold change of expression over sham was calculated by two-step normalization. First, the Ct (cycle threshold) value of the gene of interest was normalized against the corresponding Ct value of GAPDH. The resulting ΔCt value was normalized against the corresponding mean ΔCt value of the sham group. The ΔΔCt value was used to calculate the fold change of mRNA expression over sham by using the $2^{-ΔΔCt}$ formula.
\[ \Delta C_t = C_t(\text{gene of interest}) - C_t(\text{housekeeping/reference gene}) \]

\[ \Delta \Delta C_t = \Delta C_t(\text{gene of interest}) - \Delta C_t(\text{sham}) \]

\[ 2^{-\Delta \Delta C_t} = \text{fold change of expression over sham} \]

### 3.4.3 Primer sequences

Primer sequences are summarized in **Table 1**.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>Sense: ACGGAGCCAATTTTCATGC</td>
</tr>
<tr>
<td></td>
<td>Anti-sense: TGTCGAGCTTTGGGATGGTA</td>
</tr>
<tr>
<td>HO-1</td>
<td>Sense: TAG CCC ACT CCC TGT GTT TCC TTT</td>
</tr>
<tr>
<td></td>
<td>Anti-sense: TGC TGG TTT CAA AGT TCA GGC CAC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sense: TATGGCTCAGGGTCCAACCTC</td>
</tr>
<tr>
<td></td>
<td>Anti-sense: CTCCCTTTGCGAGAACTCAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: AGAAACCTGCCAAGTATGATGACA</td>
</tr>
<tr>
<td></td>
<td>Anti-sense: TGAAGTCGCAGGAGACAACCT</td>
</tr>
</tbody>
</table>

**Table 1.** Primer sequences for qPCR experiments.

### 3.5 Statistical analysis

Data are expressed as mean ± standard deviation. Pairwise comparisons between two groups were performed by *t*-test using Microsoft Excel. Multiple group comparisons were conducted by a 1-way analysis of variance, followed by the Tukey post-hoc test using IBM SPSS software. A *P* value of less than 0.05 was considered significant.
Chapter 4 Results

1. Remote ischemic pre-conditioning (4 hours)

1.1 Immune cell infiltration was reduced by RIPC

BALF total cell counts were performed on animals 4 hours following PBS vehicle or LPS exposure. Although LPS administration caused a massive influx of immune cells into the alveolar space compared to the sham group (Figure 4, \( P < 0.01 \), PBS vs. LPS), significantly lower cell counts were observed in animals that were subject to RIPC prior to exposure \( P < 0.05 \), LPS vs. RIPC-LPS).

![Figure 4](image.png)

Figure 4. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) total cell count at 4 hours (n=8-10 per group).
Similarly, BALF neutrophil counts were determined at 4 hours following LPS administration (Figure 5). Administration of LPS promoted neutrophil influx into the alveolar space ($P < 0.01$, sham vs. LPS). Preconditioning prior to LPS exposure strongly attenuated neutrophil infiltration ($P < 0.05$, LPS vs. RIPC-LPS).

Figure 5. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) neutrophil count at 4 hours (n=8-10 per group).
LPS administration lowered BALF macrophage counts in both LPS and RIPC-LPS groups in comparison to sham (Figure 6, $P < 0.05$, PBS vs. LPS/RIPC-LPS). No significant difference in macrophage counts was observed between LPS and RIPC-LPS groups.

Figure 6. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) macrophage count at 4 hours (n=8-10 per group).
The immune cell populations that were predominant in each experimental group are shown in Figure 7. The majority of the cells in the sham group were alveolar macrophages, whereas in the LPS group, neutrophils were observed at a much greater percentage. RIPC appears to have attenuated the inflammatory response following LPS exposure, as there were much fewer cells - especially neutrophils - in the RIPC-LPS group.

Figure 7. Representative images of bronchoalveolar lavage fluid (BALF) cytospin at 4 hours following LPS injection. Black arrows indicate macrophages and red arrows indicate neutrophils (40X magnification).
At 4 hours post-injection, although there were signs of neutrophil infiltration, slight hemorrhaging, and accumulation of proteinaceous debris in the alveolar space, the damage was quite modest and no obvious difference was observed between the LPS and RIPC-LPS groups (Figure 8).

Figure 8. Representative images of hematoxylin and eosin (H&E) stained sections of lung tissue at 40X magnification, harvested at 4 hours following PBS vehicle/LPS exposure. Red circles indicate polymorphonuclear leukocytes.
1.2 Total protein content was decreased by RIPC

Despite minimal histological damage, BALF total protein content was significantly increased with LPS treatment (**Figure 9**, *P* < 0.01, PBS vs. LPS/RIPC-LPS). There was a notable decrease in the total protein concentration with RIPC performed prior to LPS administration (*P* < 0.05, LPS vs. RIPC-LPS).

Figure 9. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) total protein content at 4 hours following LPS exposure.
1.3 HO-1 mRNA expression was not affected by LPS

Lung HO-1 mRNA levels were determined at 4 hours following LPS administration, as HO-1 is considered to have prominent anti-inflammatory properties. We initially hypothesized that the anti-inflammatory effect of RIPC may be partially exerted through HO-1 and the molecular pathways associated with it; however, results indicated that RIPC does not have a significant effect on HO-1 expression following LPS treatment (Figure 10). In fact, lung HO-1 mRNA expression was not significantly affected by LPS treatment itself at this time point.

![Figure 10. Effect of remote ischemic preconditioning (RIPC) on lung heme oxygenase-1 (HO-1) mRNA expression at 4 hours following LPS exposure (n=3-6).](image-url)
1.4 ICAM-1 expression slightly decreased following RIPC

Neutrophil infiltration into the alveolar compartment was significantly reduced with RIPC performed prior to LPS administration. Since ICAM-1 plays an important role in leukocyte-endothelial transmigration, we hypothesized that down-regulation of ICAM-1 may be associated with the protective effect of RIPC. ICAM-1 expression was highly elevated with LPS treatment (Figure 11, $P < 0.01$, PBS vs. LPS). The data showed a trend (not statistically significant) consistent with our hypothesis, lung ICAM-1 mRNA expression was reduced with RIPC applied prior to LPS administration ($P > 0.05$, LPS vs. RIPC-LPS).

Figure 11. Effect of remote ischemic preconditioning (RIPC) on lung intercellular adhesion molecule -1 (ICAM-1) mRNA expression at 4 hours following LPS administration (n=3-6).
1.5 TNF-α expression slightly increased following RIPC

We also wanted to examine the effect of RIPC on lung pro-inflammatory cytokine expression. We first looked at lung TNF-α mRNA expression, which was highly elevated with LPS treatment. Surprisingly, greater TNF-α levels were observed in the RIPC-LPS group, even though the difference between the LPS and RIPC-LPS groups was statistically insignificant.

![Figure 12: Effect of RIPC on lung TNF-α mRNA expression at 4 hours following LPS administration (n=3-7).](image)

*Figure 12.* Effect of RIPC on lung TNF-α mRNA expression at 4 hours following LPS administration (n=3-7).
1.6 IL-1β expression slightly increased following RIPC

Lung IL-1β levels showed a similar trend, although its response to LPS was smaller in magnitude compared to TNF-α levels. There was no significant difference between the experimental groups.

Figure 13. Effect of remote ischemic preconditioning (RIPC) on lung IL-1β mRNA expression at 4 hours following LPS administration (n=3-7).
2 Remote ischemic pre-conditioning (24 hours)

At 24 hours following LPS exposure, histological damage was evident with LPS treatment compared to the control; however, RIPC did not appear to have a histological effect on lung tissue exposed to LPS (Figures 14 and 15, $P < 0.05$, LPS vs. RIPC-LPS).

Figure 14. Hematoxylin and eosin (H&E) stained lung histology imaged at 20X magnification, 24 hours following PBS vehicle/LPS exposure.
Figure 15. Lung injury score determined at 24 hours following PBS/LPS instillation (n=3-4).
BALF total cell counts were performed on animals 24 hours following PBS vehicle or LPS exposure. LPS administration caused a massive influx of immune cells into the alveolar space compared to the sham group (Figure 4, $P < 0.01$, PBS vs. LPS and PBS vs. RIPC-LPS). However, no difference in total cell numbers was observed with RIPC intervention ($P > 0.05$, LPS vs. RIPC-LPS).

Figure 16. Effect of remote ischemic preconditioning (RIPC) on bronchoalveolar lavage fluid (BALF) total cell count at 24 hours (n=3 per group).
MPO activity assay was conducted to assess the effect of RIPC on neutrophil infiltration into the lung. There was very little MPO activity in the sham lungs at 24 hours (Figure 17). LPS administration remarkably promoted neutrophil influx, as can be seen by highly elevated MPO activity ($P < 0.01$, sham vs. LPS; $P < 0.05$, sham vs. RIPC-LPS). However, at 24 hours, preconditioning did not have a significant effect on neutrophil infiltration following LPS treatment ($P > 0.05$, LPS vs. RIPC-LPS).

Figure 17. Effect of remote ischemic preconditioning (RIPC) on myeloperoxide (MPO) activity in the lung at 24 hours following LPS injection (n=3-4).
Although total protein concentrations were increased further compared to the values seen at the 4-hour time point, no difference could be observed between the group that received only LPS and the group that was subject to RIPC prior to LPS instillation (Figure 18, $P < 0.05$).

Figure 18. Bronchoalveolar lavage fluid (BALF) total protein concentration measured at 24 hours following LPS exposure (n=3-4 per group).
3 Remote ischemic post-conditioning (4 hours)

At 4 hours post-LPS injection, RIPC exerted a protective effect on pulmonary inflammation. We wanted to investigate whether remote ischemic post-conditioning (RIPostC) would have the same effect on LPS-induced pulmonary inflammation, as post-conditioning would be more clinically relevant. According to the data, post-conditioning did not have a significant effect on BALF total cell counts, as no statistically significant difference was noted between the groups (Figure 19).

![Bar graph showing effect of post-conditioning on BALF total cell count](image)

Figure 19. Effect of post-conditioning on bronchoalveolar lavage fluid (BALF) total cell count at 4 hours following LPS injection (n=3-5).
Similarly, RIPostC did not have a significant effect on BALF neutrophil counts (Figure 20) at 4 hours following LPS instillation. LPS was able to cause a massive influx of neutrophils into the alveolar space, thereby significantly increasing BALF neutrophil percentage ($P < 0.01$ PBS vs. LPS/P-LPS); however, no statistically significant difference was seen between the LPS and P-LPS groups.

Figure 20. Effect of post-conditioning on bronchoalveolar lavage fluid (BALF) neutrophil count at 4 hours following LPS injection (n=3-5).
LPS caused a significant increase in protein leakage (Figure 21, $P < 0.05$, PBS vs. LPS); however, the decrease in total protein content seen in the LPS-treated, post-conditioned group, compared to the LPS-only group, was negligible.

Figure 21. Effect of post-conditioning on bronchoalveolar lavage fluid (BALF) total protein concentration at 4 hours following LPS injection (n=3-5).
4 Remote ischemic pre- and post-conditioning (24 hours)

The data so far suggest that RIPC has a protective effect against LPS-induced pulmonary inflammation at 4 hours, but that the effect is lost by 24 hours post-injection. Our next hypothesis was that repetition of RIC may prolong its anti-inflammatory effect. Although there was a decreasing trend in BALF total cell count with dual ischemic conditioning in the LPS-treated animals, the difference was not statistically significant (Figure 22, \( P > 0.05 \), LPS vs. RIC-LPS).

![Figure 22](image-url) Effect of pre- and post-conditioning on bronchoalveolar lavage (BALF) total cell count at 24 hours following LPS injection (n=3-5).
Pre- and post-dual conditioning had no significant effect on BALF neutrophil counts in the LPS-treated animals. Even though a decreasing trend, similar to that observed with BALF total cell counts, was also seen in BALF neutrophil count with dual ischemic conditioning, the difference was minimal and not statistically significant (Figure 23, $P > 0.05$, LPS vs. RIC-LPS).

![Graph showing neutrophil counts in BALF](image)

**Figure 23.** Effect of pre- and post-conditioning on bronchoalveolar lavage fluid (BALF) neutrophil count at 24 hours following LPS injection (n=3-5).
Similarly, there was no significant difference in protein leakage with dual conditioning at 24 hours following LPS administration (Figure 24, $P > 0.05$, PBS vs. LPS vs. RIC-LPS). These data collectively indicate that application of another RIC, hours after the protective effect of RIPC has subsided, did not help to prolong the anti-inflammatory effect on LPS-induced pulmonary inflammation.

Figure 24. Effect of pre- and post-conditioning on bronchoalveolar lavage fluid (BALF) total protein concentration at 24 hours following LPS injection (n=3-5).
Chapter 5 Discussion

In the present study, we aimed to investigate the effect of remote ischemic conditioning (RIC) in a model of acute lung injury (ALI). RIC has been shown to improve outcomes when performed before, during, or after challenge in different inflammatory disease models. This was the first study to examine the therapeutic potential of RIC in the context of LPS-induced ALI.

Intratracheal instillation of LPS was performed to induce pulmonary inflammation in mice. Remote ischemic preconditioning (RIPC), post-conditioning (RIPostC), or dual conditioning were performed and mice were studied at 4 and 24 hours after the challenge in order to examine both the first and second windows of protection. RIPC suppressed the inflammatory response to endotoxin in the acute phase; however, as the protective effect of preconditioning subsided over time, the inflammatory response seemed to escalate accordingly and become full-blown by 24 hours.

1 RIPC provides protection against LPS-induced pulmonary inflammation at 4 hours following challenge

1.1 Leukocyte recruitment

We observed that application of four cycles of 5-minute ischemia and reperfusion to the right hindlimb of the mouse immediately before an intratracheal administration of LPS was able to attenuate the inflammatory response in the lungs at 4 hours following LPS exposure. RIPC significantly reduced total cell counts and neutrophil counts in the BALF in LPS-treated mice.

Our results were also consistent with a study that investigated the effect of IPC on ALI caused by prolonged I/R in a porcine model. In this study, pigs were subjected to IPC by clamping and releasing the external iliac artery for 5 minutes each, in three cycles (Harkin, D'Sa et al. 2002). Following IPC, the animals underwent 120 minutes of ischemia and 150 minutes of reperfusion via occlusion and release of the same artery (Harkin, D'Sa et al. 2002). Edema, MPO activity (i.e. neutrophil infiltration), and IL-6 expression were significantly decreased and gas exchange improved in the lungs with RIPC performed prior to prolonged I/R of the hindlimb, at the end of...
prolonged reperfusion (Harkin, D'Sa et al. 2002). As we hypothesized, RIPC reduced inflammation in our model of LPS-induced ALI, as was previously observed in models of I/R-induced ALI.

Furthermore, the decrease in neutrophil influx into the alveolar space in the RIPC-LPS was consistent with the findings reported by a group that measured expression of numerous inflammatory genes by microarray. Healthy human subjects received RIPC in three cycles of 5-minute ischemia and reperfusion by placing a blood pressure cuff on an arm (Konstantinov, Arab et al. 2004). Genes involved in cytokine production, leukocyte chemotaxis, adhesion and migration, exocytosis, innate immunity signaling pathways, and programmed cell death all showed biphasic suppression; the suppression was more potent at 24 hours than at 15 minutes (Konstantinov, Arab et al. 2004). The authors postulated that the biphasic change in gene expression corresponded to the first and second window of protection of RIPC (Konstantinov, Arab et al. 2004). CD11b, which is a counter-receptor for ICAM-1, showed a modest decrease in gene expression at 15 minutes from the time of RIPC stimulus, which was further decreased at 24 hours, reaching statistical significance (Konstantinov, Arab et al. 2004). Of our interest, TLR4 and NFκB were also significantly downregulated at both of those time points (Konstantinov, Arab et al. 2004). Therefore, RIPC alone is able to modify leukocyte inflammatory gene expression. Reduced expression of leukocyte chemotaxis, adhesion and migration genes may contribute to the inhibition of leukocyte recruitment that we observed in our study. This effect may contribute to the protective effect of RIPC; for instance, if TLR4 has been downregulated prior to LPS challenge, the effect of LPS would be less powerful, because there is now a limited number of TLR4 expressed on cell surface.

RIPC did not restore macrophage count, which was significantly decreased in the BALF following LPS challenge. This decrease in macrophage count may be due to LPS-induced apoptosis. An in vitro study showed that LPS dose-dependently induced apoptosis in alveolar macrophages isolated from the BALF of healthy human volunteers (Bingisser, Stey et al. 1996). The authors speculated that the LPS-triggered apoptosis might be a regulatory mechanism to prevent excessive damage. IFN-γ treatment in addition to LPS stimulation further increased apoptosis of alveolar macrophages. Since both LPS and IFN-γ are able to cause M1 polarization of macrophages and increase production of proinflammatory cytokines (Liao, Sharma et al.
apoptosis may be initiated by macrophages as self-restraint to protect the surrounding environment from excessive inflammation and damage.

1.2 BALF total protein content

As expected in this model, BALF total protein content was significantly increased with LPS treatment. LPS activates the TLR4 pathway in alveolar macrophages, which eventually results in the release of proinflammatory cytokines, such as TNF-α and IL-1β, which induce chemotaxis and activate neutrophils locally. Neutrophils in turn produce oxidants, proteases, and other proinflammatory factors that damage the local environment (Ware and Matthay 2000). While transmigrating through the vascular endothelium and the alveolar epithelium, neutrophils exert mechanical stress and release proinflammatory molecules that disrupt the alveolar-capillary barrier, which results in pulmonary edema and protein leakage (Ware and Matthay 2000). As hypothesized, RIPC significantly reduced the BALF total protein content, indicating decreased protein leakage. RIPC likely mitigated the damaging effect of LPS on the vascular endothelium by upregulation of anti-inflammatory mediators, like IL-10 (Cai, Luo et al. 2013), and by reducing leukocyte adhesion and transmigration by decreasing the expression of adhesion molecules, such as ICAM-1 (Beauchamp, Richard et al. 1999).

1.3 Histological damage

At 4 hours following challenge, 800 μg/kg of LPS caused only minor histological damage. There were some signs of leukocyte recruitment, slight hemorrhaging, and accumulation of proteinaceous debris in the alveolar compartment. RIPC treatment did not appear to reduce this minor damage. Intratracheal instillation of LPS (200 μg/kg) in a rat model induced similar damage; there was marked neutrophil infiltration but very minimal interstitial edema at 4 hours following challenge (Delclaux, RezaiguiaDelclaux et al. 1997).

1.4 Gene expression in the lung: HO-1, ICAM-1, TNF-α, and IL-1β

LPS-treatment alone was unable to upregulate HO-1 mRNA expression, which contradicts previous literature. Song and colleagues demonstrated that intravenous LPS (minimum dose 0.5
mg/kg) significantly and dose-dependently increased HO-1 expression in the liver in mice. The LPS-induced increase in HO-1 was abolished in mice with defective TLR4, suggesting that LPS induces HO-1 expression by activating TLR4 (Song, Shi et al. 2003). Similar results were seen in human peripheral blood mononuclear cells (PBMCs) and monocytic THP-1 cells, HO-1 mRNA expression was increased by 4 hours and especially 8 hours following LPS treatment. The group also showed that in THP-1 cells, Nrf2 translocated to the nucleus and bound to the antioxidant response element (ARE) of the HO-1 promoter to increase HO-1 expression in response to LPS (Rushworth, Chen et al. 2005). As previously mentioned, IPC significantly reduced brain infarct volumes and increased HO-1 protein levels in the brain, and the protective effect was abolished in HO-1 knockout mice (Zeynalov, Shah et al. 2009). Furthermore, higher HO-1 expression in the liver was observed in mice that were subjected to hindlimb preconditioning prior to intraperitoneal administration of LPS (sepsis model; 20 mg/kg of LPS) at 1 hour post-injection (Kim, Yoon et al. 2014). We expected to see an increase in HO-1 expression with preconditioning based on these previous reports. It is possible that the dose of LPS administered in our model was insufficient to induce the same response reported in the literature. In addition, tissue-specific responses may be involved, leading to a different response in the lungs compared to other tissues, such as the brain and liver.

We observed that RIPC significantly reduced LPS-induced neutrophil influx into the alveolar compartment by 4 hours. We speculated that since ICAM-1 plays a major role in leukocyte transmigration, ICAM-1 expression might be affected by RIPC, resulting in decreased neutrophil infiltration. We found that ICAM-1 mRNA expression in the lung was significantly increased 4 hours after LPS challenge. Although statistically insignificant, the RIPC data showed a trend consistent with the hypothesis; ICAM-1 expression was reduced with RIPC treatment to some extent. When rat aortic endothelial cells were preconditioned with short-term anoxia and reoxygenation prior to prolonged anoxia and reperfusion, ICAM-1 mRNA expression decreased (Beauchamp, Richard et al. 1999). This was an in vitro model of I/R, however, so it is possible that the same effect might not be reproduced in our in vivo model of pulmonary inflammation. Perhaps molecules other than ICAM-1 that involved in leukocyte migration are more affected by RIPC. CD11b is a binding partner for ICAM-1. Shimizu and colleagues demonstrated that neutrophils isolated from blood from healthy volunteers that were subjected to RIPC showed decreased adhesion to tissue culture plates compared to neutrophils that were not preconditioned
(Shimizu, Saxena et al. 2010). However, neutrophil surface expression of CD11b was unaffected by RIPC over the course of the experiment (Shimizu, Saxena et al. 2010). If RIPC was unable to affect CD11b expression, it is likely that the observed decrease in adhesion with RIPC was not due to down-regulation of ICAM-1 expression.

Interestingly, RIPC increased proinflammatory cytokine mRNA levels in the lung 4 hours following LPS challenge. Lung TNF-α expression was highly elevated with LPS treatment, and RIPC further increased its expression, although the difference between the LPS-only and RIPC-LPS groups was insignificant. Although at a lower magnitude compared to TNF-α expression, LPS treatment also increased lung IL-1β mRNA expression and RIPC increased it further. This is rather paradoxical, as one would expect to see a decrease in proinflammatory cytokine production if RIPC reduced neutrophil influx and protein leakage. Contradicting data have been reported in the literature regarding TNF-α and IL-1β expression. Leung et al. demonstrated that lower lung mRNA levels of TNF-α and IL-1β were associated with RIPC prior to hemorrhagic shock and resuscitation in mice, compared to the group that did not receive preconditioning (Leung CH, Caldarone CA et al. 2015). In a BALB/c mouse model of sepsis, serum levels of TNF-α 1 and 4 hours following an intraperitoneal injection of LPS were significantly reduced with RIPC compared to the group that received only LPS (Kim, Yoon et al. 2014). IL-1β expression was slightly reduced (though not statistically significant) with RIPC 1 hour following LPS exposure (Kim, Yoon et al. 2014). By contrast, observations of preconditioned human neutrophils by Shimizu et al. were consistent with ours. LPS treatment elevated the extracellular levels of TNF-α and IL-1β in neutrophils obtained from preconditioned humans, compared to unconditioned neutrophils (Shimizu, Saxena et al. 2010). Expression of proinflammatory cytokines was not increased with RIPC alone; however, when RIPC was combined with LPS treatment, secretion significantly increased (Shimizu, Saxena et al. 2010). The authors postulated that the TNF-α might be upregulated in order to attenuate the subsequent expression of proinflammatory cytokines during I/R injury, as part of autoregulation (Shimizu, Saxena et al. 2010). This hypothesis is supported by a murine study, which demonstrated that preconditioning with low-dose TNF-α provides protection against liver I/R injury (Teoh, Leclercq et al. 2003). Similarly, other studies have suggested that TNF-α provides cytoprotection through upregulation of manganese superoxide dismutase (MnSOD) (Wong and Goeddel 1988). In addition, TNF-α protected rats against myocardial I/R injury (Eddy, Goeddel et al. 1992). It could be interpreted
from the results of our study that RIPC itself may be taken as a challenging stimulus to the host, which can amplify the inflammatory response to subsequent stimuli to some degree. Moreover, since TNF-α is a potent activator of the SAFE pathway, increased TNF-α expression in the RIPC group may activate the SAFE pathway, thereby conferring protection against LPS-induced inflammation.

2 Protective effect of RIPC on LPS-induced pulmonary inflammation is lost by 24 hours post-injection

2.1 Neutrophil infiltration

MPO activity assay was performed to assess neutrophil infiltration into the lungs at 24 hours following LPS exposure. LPS administration caused a significant increase in MPO activity, similar to what was shown in a rat model, where an intratracheal administration of LPS (200 µg/kg) promoted massive influx of inflammatory cells into the alveolar compartment, both at 4 and 24 hours (Delclaux, RezaiguiaDelclaux et al. 1997), and preconditioning had no effect on it.

2.2 BALF total protein content

At 24 hours following LPS challenge, vascular permeability increased further compared to 4 hours, as suggested by higher values of BALF total protein content. We initially hypothesized that the protective effect of RIPC would persist for a longer period of time, beyond 4 hours, under the influence of the second window of protection. Contrary to our hypothesis, RIPC did not attenuate the BALF total protein concentration.

2.3 Histological damage

Intratracheal administration of LPS caused histological damage to the lungs at 24 hours following injection; however, no significant difference was observed between the LPS-only and RIPC-LPS groups. It is possible that we could not observe a difference between the groups because the injury caused was too minimal. The LPS dose used for our study (800 µg/kg) may have been insufficient to cause histological damage to the lungs in C57BL/6 mice, which are more resistant to LPS. The lung injury scores assigned to each experimental group of our study are generally very low. The score for the LPS group was less than 0.1, which is very low
compared to other studies that used the same lung injury scoring system to assess LPS-induced lung damage. Intratracheal instillation of 3 mg/kg of LPS resulted in a lung injury score of approximately 0.6 in BALB/c mice (Gong, Wu et al. 2014). A score of approximately 0.8 was given to the C57BL/6 mouse lungs that were affected by inhalation of LPS (Grommes, Vijayan et al. 2012). There were no other studies that used this scoring system to test the effect of a lower dose of LPS via intratracheal instillation. We expect that a higher dose of LPS will increase the score, as observed by the studies that used higher doses of LPS.

The protective effect of RIPC against LPS-induced pulmonary inflammation was lost by 24 hours. The protective effective of RIPC is known to be biphasic. The early effect of ischemic conditioning occurs within the first few minutes of application and persists for about 6 hours, which is referred to as the first window of protection. The effect reappears within 24 hours of the preconditioning stimulus and lasts for up to 96 hours (Saxena, Newman et al. 2010). Initially we hypothesized that the 24-hour time point may fall under second window of protection, and that pulmonary inflammation may continue to be attenuated with a single application of RIPC. However, in the literature, the second window of protection actually emerged when the challenge was given at approximately 24 hours from the time of initiation of preconditioning. For example, myocardial infarct size was significantly reduced in rabbits when prolonged I/R was induced 24 hours following IPC (Marber, Latchman et al. 1993). Our study introduced the LPS challenge immediately after the RIPC stimulus, thus the inflammatory response to endotoxin may have been full-blown by the time the second window of protection emerged.

The degree of protection seems to depend on the duration and timing of preconditioning. The effect of the duration and timing of preconditioning was shown in a rat model of acute myocardial ischemia and reperfusion. Barbosa and colleagues tested various IPC protocols and observed that infarct size was reduced as preconditioning ischemia time increased in rats subjected to a single application of IPC (Barbosa, Sievers et al. 1996). In another study, rats were preconditioned by occluding the LCA, followed by either 1-minute or 30-second reperfusion, prior to prolonged I/R. Infarct size significantly decreased with the longer 1-minute reperfusion but not with the shorter 30-second reperfusion (Alkhulaifi, Pugsley et al. 1993). In a mouse model of sepsis, RIPC improved survival rate over the course of 5 days using three cycles of 10-minute ischemia and reperfusion applied to the right hindlimb (Kim, Yoon et al. 2014). Our RIC protocol differs from the one used by Kim’s group, as our protocol involves four cycles of 5-
minute ischemia and reperfusion. However, it should be noted that serum cytokines and neutrophil accumulation were not measured past 4 and 12 hours, respectively, so the effect of RIPC on systemic inflammation may not have lasted for more than 12 hours. Thus, it is possible that a small difference in the timing and duration of the preconditioning protocol can lead to a different outcome.

3 RIPostC does not confer protection against LPS-induced pulmonary inflammation at 4 hours following LPS challenge

After finding that RIPC protects against LPS-induced pulmonary inflammation at 4 hours following LPS injection, we examined the effect of RIPostC, as post-conditioning would be more clinically relevant. We hypothesized that RIPostC would provide similar protection against endotoxin-induced inflammation in the lungs. Instead, RIPostC did not significantly change inflammation upon LPS administration. Post-conditioning following LPS administration was unable to reduce BALF total cell count. Similarly, RIPostC did not have a significant effect on neither BALF neutrophil percentage nor BALF neutrophil count upon LPS treatment. LPS treatment increased vascular permeability and significantly increased BALF total protein content. RIPostC was unable to reduce protein leakage.

The results were unexpected, as many previous studies have supported the use of RIPostC in models of inflammation and injury. For example, ischemic post-conditioning significantly attenuated lung injury following prolonged ischemia for 40 minutes and reperfusion for 105 minutes in rats (Xia, Gao et al. 2010). In another rat model of myocardial infarction, RIPerC was induced by 5-minute occlusion of the renal artery and reperfusion immediately before the onset of 3 hours of reperfusion preceded by 30 minutes of LCA occlusion (Kerendi, Kin et al. 2005). RIPostC decreased myocardial infarct size by 50% compared to control (Kerendi, Kin et al. 2005). In a mouse model of sepsis, RIPostC performed immediately after an intraperitoneal injection of LPS was as effective as RIPC in attenuating the inflammatory response. Five-day survival was significantly higher, 4 hour serum TNF-α and IL-6 levels were lower, and neutrophil recruitment to the liver was remarkably reduced with post-conditioning compared to the unconditioned, LPS-treated group (Kim, Yoon et al. 2014). However, this was a model of
sepsis rather than pulmonary inflammation and their endpoints were mainly focused on damage to the liver. In sepsis, the protective effect of RIPostC may be exerted by activation of lung-independent pathways, which would not be relevant in our study of endotoxin-induced pulmonary inflammation.

4 RIPC combined with RIPostC (dual conditioning) did not prolong the protective effect against LPS-induced pulmonary inflammation at 24 hours following LPS administration

Given that we found a protective effect of RIPC against LPS-induced pulmonary inflammation at 4 hours that was lost by 24 hours post-injection of LPS, we next investigated whether a second application of RIC would prolong the protective effect. We hypothesized that dual conditioning would lengthen the duration of protection against inflammation. Although a decreasing trend was seen in BALF total cell count with RIPC and RIPostC combined in LPS-treated animals, the difference between the groups was statistically insignificant at 24 hours following challenge. Likewise, dual conditioning did not significantly change neutrophil percentage or numbers in the BALF at the same time point. Finally, dual conditioning did not lead to a significant difference in BALF total protein concentration at 24 hours post-injection. Overall, dual conditioning was unable to prolong the effect of RIPC, as no evidence of reduced inflammation was seen at 24 hours following LPS injection. However, it should be noted that statistical significance could not be achieved due to a small sample size. Being unable to reach statistical significance does not mean dual conditioning is ineffective in providing protection against LPS-induced pulmonary inflammation. More animals would be required to draw a solid conclusion. In a previous study, rats that received dual conditioning (RIPerC during prolonged coronary occlusion as well as RIPostC daily for 28 days) demonstrated reduced myocardial infarct size and improved survival (Wei, Xin et al. 2011). The protective effect of RIC was apparent in a dose-dependent manner (Wei, Xin et al. 2011). Whether the difference in the findings between this I/R study and our ALI study is due to the difference in diseases or due to the difference in RIPostC dosing (once vs. daily for 28 days) is unclear and warrants further study.
5 Limitations

5.1 Physiological relevance of LPS model

LPS-induced ALI is widely used as an insightful in vivo model that resembles many of the features of ALI/ARDS in patients. However, this is a “model” after all and has some weaknesses. Clinically, patients are exposed to live bacteria, not just the endotoxin. A study examined the effects of live bacteria, Pseudomonas aeruginosa, versus LPS on the lungs. Instillation of P. aeruginosa and LPS both caused ALI in rats but several differences were noted. All of the rats treated with the live bacteria that survived up to 24 hours showed signs of abnormally rapid breathing and were apathetic, whereas LPS rats were breathing normally (Delclaux, RezaiguiaDelclaux et al. 1997). Live bacteria instillation led to significant neutrophil recruitment, hemorrhage, and severe edema in the lungs (Delclaux, RezaiguiaDelclaux et al. 1997). Moreover, increased mortality at 24 hours, severe hypoxemia, and higher BALF protein concentration were observed in this group (Delclaux, RezaiguiaDelclaux et al. 1997). By contrast, even though LPS also caused a remarkable increase in neutrophil infiltration, it resulted in only mild interstitial edema, as indicated by significantly lower BALF protein concentrations compared to the bacteria model (Delclaux, RezaiguiaDelclaux et al. 1997). Live bacteria may release other cytotoxic compounds, such as Pyocyanin, which can cause further damage and inflammatory response in the host compared to LPS alone (Reszka, Denning et al. 2006). In addition, human ALI/ARDS is often developed through complex interactions between primary and additional risk factors rather than a single event. As previously mentioned, the huge variation between animal species and strains is another confounding factor (Chen, Bai et al. 2010). Therefore, administration of LPS by itself in an animal model may not be able to reproduce the same features of ALI/ARDS that are seen in patients.

5.2 Dose of LPS

LPS has been shown to trigger different intracellular signaling pathways depending on the dose. Most of the mechanistic studies use high doses of LPS (>10 ng/mL) that cause a robust inflammatory response. The TLR4 signaling pathway, through which LPS acts, was elucidated in studies that used high doses of LPS. Maitra and colleagues examined the effect of low-dose LPS on mouse BMDCMs and suggested that an NFκB-independent signaling pathway may be activated (Maitra, Deng et al. 2012). When a high dose of LPS (100 ng/mL) was used to treat cells,
expression of both proinflammatory and anti-inflammatory mediators were significantly increased (Maitra, Deng et al. 2012). The expression of proinflammatory mediators was robust but peaked at 4-6 hours and decreased significantly by 8-10 hours, which was largely due to activation of TLR4, IRAK-1/2/4 and NFκB (Maitra, Deng et al. 2012). The expression of anti-inflammatory mediators was induced by activation of the PI3K pathway (Maitra, Deng et al. 2012). By contrast, low-dose LPS (50 pg/mL) led to a slight induction of proinflammatory mediators that lasted throughout the course of the experiment, and anti-inflammatory factors were barely expressed (Maitra, Deng et al. 2012). The group also observed that low-dose LPS does not induce the classical NFκB and MAPK pathway (Maitra, Deng et al. 2012). Instead, low-dose LPS induced mitochondrial production of ROS via IRAK-1 and Toll-interacting protein (Tollip) (Maitra, Deng et al. 2012). Induction of activating transcription factor 2 (AFT2) required IRAK-1 and Tollip, as low-dose LPS was unable to increase AFT2 protein levels in cells lacking IRAK-1 and Tollip (Maitra, Deng et al. 2012). A rapid and powerful induction of AFT2 led to increased binding of ATF2 to the promoter region of IL-6 (Maitra, Deng et al. 2012). In addition, the low-dose treatment failed to activate the autoregulatory PI3K pathway, which involves degradation of IRAK-1 in the LPS-TLR4 signaling cascade (Maitra, Deng et al. 2012). The lack of negative regulation through activation of PI3K in cells may be the reason for the prolonged, leaky expression of proinflammatory mediators, which contradicts the robust response with high-dose LPS (Maitra, Deng et al. 2012). In this regard, the dose that we used (800 µg/kg) may have triggered alternative signaling pathways, giving rise to different outcomes from those seen in studies that used higher doses of LPS (e.g. 20 mg/kg). It is also possible that our dose was insufficient to cause enough inflammation or injury to the lungs for us to observe a notable difference between the LPS-only and RIC-LPS groups.

5.3 Protocol design

The results from the 24-hour dual conditioning showed a decreasing trend in BALF differential cell counts with combined pre- and post-conditioning upon LPS administration. It is possible that a change in the protocol may give rise to a more protective, statistically significant outcome. Since protection is evident at 4 hours, perhaps an application of RIPostC at 4 hours may be able to prolong the protective effect. However, we observed that a second dose of sodium pentobarbital caused death in mice even at 24 hours following the first dose; the dose of
anesthetic would need to be adjusted to prevent mortality in order for dual conditioning to be feasible.

Although our finding that RIPC is effective in a model of ALI is novel and important, RlPostC is a more clinically relevant treatment. RIPC could be used to prevent the development of secondary ALI in trauma patients that are highly susceptible to secondary challenge, such as infections. However, its clinical use as an intervention for ALI/ARDS would be highly limited, as it is difficult to predict the timing of the onset of ALI/ARDS.
Chapter 6 Conclusions

In summary, RIPC performed prior to LPS administration was able to significantly reduce leukocyte recruitment to the alveolar compartment at 4 hours following LPS challenge in our murine model of pulmonary inflammation. It also attenuated protein leakage from the vasculature, as seen by a significant reduction in BALF total protein concentrations in the RIPC-LPS group. At the 4-hour time point, LPS treatment did not cause a notable histological damage to the lungs. A decreasing trend in lung ICAM-1 mRNA expression was observed with RIPC upon LPS-treatment. Lung HO-1 mRNA expression did not change with either LPS or RIPC treatment. Lung TNF-α and IL-1β expressions were surprisingly (though not significantly) increased in the RIPC-LPS group compared to the LPS alone group. At 24 hours following exposure to LPS, RIPC did not have a significant effect on lung histology, leukocyte infiltration into the lungs, or BALF total protein concentration. Unlike RIPC, RIPostC performed 15 minutes following LPS instillation, was unable to significantly reduce leukocyte infiltration into the lungs or BALF total protein levels at 4 hours. Similarly, the combination of pre- and post-conditioning could not significantly attenuate leukocyte recruitment, namely neutrophils, to the lungs or decrease protein leakage in response to LPS at 24 hours following challenge.

In conclusion, RIPC protects against LPS-induced pulmonary inflammation in mice at 4 hours following LPS administration. The protective effect of RIPC diminishes over time and is lost by 24 hours. RIPostC was unable to significantly attenuate inflammation caused by LPS at 4 hours post-injection. Likewise, a second application of RIC (RIPostC) could not significantly prolong the protective effect of the initial RIPC.
Chapter 7 Future Directions

Our study is the first to provide evidence for an acute protective effect of RIPC against endotoxin-induced pulmonary inflammation. This novel finding inspires many questions. Further investigation is required to elucidate the underlying mechanisms and to design a clinically relevant strategy.

First, it would be worthwhile to increase the dose of LPS (e.g. 10 mg/kg) in order to cause severe injury in the lungs and make our study comparable to other LPS models. Also, it would be interesting to examine the effect of RIPC in a high-dose LPS model, for it may lead to activation of other signaling cascades and produce different outcomes.

Next, the protocols for post-conditioning and dual conditioning could be modified. Most post-conditioning studies involve application of post-conditioning immediately following challenge (e.g. LPS or prolonged I/R). Instead of having 15 minutes of wait-time, RIPostC could be performed immediately following LPS injection to examine its effect. Although that would be less clinically relevant, it would make our study comparable to other studies. The protocol for dual conditioning could be modified, so that post-conditioning could be performed at 4 hours, while the protective effect of the initial application of RIPC still persists. There is a decreasing trend in LPS-induced BALF immune cell infiltration with RIPC, which may reach statistical significance with a increased sample size. It would also be interesting to repeat RIPostC daily for several days to observe its effect, similar to the study conducted by Wei and colleagues (Wei, Xin et al. 2011). Modifying the number of cycles and/or duration of I/R in the RIC protocol itself could also be considered, as the standard RIC protocol was developed from I/R models rather than endotoxin-induced inflammation.

There was quite a lot of variability between samples with respect to the lung mRNA expression of ICAM-1, TNF-α, and IL-1β. qPCR requires only a small portion of a lobe, thus it might not reflect the actual expression in the whole lung. It might be due to a patchy distribution of LPS, since some parts of the lung could come in contact with LPS more so than the others. The inflammation inflicted by LPS may not be diffuse, due to the technical limitations associated with an intratracheal injection. BALF proinflammatory cytokine levels could be measured instead, through the enzyme-linked immunosorbent assay (ELISA). Since BALF involves lavage
of all five lobes, ELISA on the BALF would be more appropriate for the measurement of proinflammatory mediator expression in the lungs.

It would also be interesting to examine the effect of RIPC on circulating neutrophils. Blood samples collected from mice subjected to RIPC alone could be used to quantify circulating neutrophils through fluorescence-activated cell sorting (FACS). This would provide insight into whether the reduction in leukocyte infiltration into the alveolar compartment with RIPC occurs because RIPC somehow sequestered neutrophils in other tissues, thereby decreasing the number of circulating neutrophils available for neutrophil recruitment to the lungs upon LPS stimulation. In addition, CD11b expression on the surface of circulating neutrophils could be measured by FACS. Since ICAM-1 expression was reduced to some extent with RIPC, its counter-receptor may also be affected. For the assessment of neutrophil CD11b expression, it would best to collect blood from sham/RIPC-induced mice and stimulate the cells in vitro with LPS, as neutrophils are very sensitive to noxious stimuli.

In the present study, total protein concentrations were measured in the BALF to assess the effect of RIPC on pulmonary vascular permeability. However, according to Matute-Bello and colleagues, BAL total protein content can be affected by immune cells and lung epithelial cells in the alveolar compartment that release proteins upon stimulation. Specific quantification of high-molecular proteins should be performed to determine the source of the protein present in the BALF. It would be more informative to quantify the leakage of Evans Blue dye, a fluorescent, inert azo dye that binds to albumin, into the alveolar space (Matute-Bello, Downey et al. 2011).

In addition, the effect of RIPC on the production of ROS by immune cells could be examined. LPS is known to stimulate macrophages to release inflammatory cytokines and ROS (Hsu and Wen 2002). The anti-inflammatory effects of RIPC demonstrated in our study may encompass suppression of immune cell activation. ROS levels in plasma or serum could be fluorescently detected using in vitro ROS assays.

Functional studies should also be conducted to test the efficacy and clinical relevance of RIPC. Although neutrophil infiltration is an important measure of inflammation, it may be limited in predicting functional outcomes. Functional studies would examine lung injury rather than acute inflammation, and would need to be conducted within 24 hours of challenge, as maximal lung injury should be observed within that timeframe. Measuring lung compliance would be a feasible
option, as decreased lung compliance caused by pulmonary edema is an important feature of ALI/ARDS, which can easily be assessed in vivo during mechanical ventilation in rodents (Matute-Bello, Downey et al. 2011).

The results from the present study showed that lung TNFα mRNA expression was increased in the RIPC group 4 hours after LPS challenge, to an even greater extent than what was seen with LPS treatment alone. Despite the increase in TNFα levels, RIPC was shown to confer protection against LPS-induced inflammation at this time point. As mentioned, since TNFα can trigger the SAFE pathway during RIPC to confer protection (Smith, Suleman et al. 2002), it would be intriguing to examine the effect of SAFE pathway inhibitors, such as AG-490, a JAK inhibitor, in the mouse model of LPS-induced pulmonary inflammation. Additional studies could be conducted to test whether the RISK and SAFE pathways that are involved in RIPC-mediated cardioprotection are also activated in this model of pulmonary inflammation, through administration of RISK pathway inhibitors, such as WM.

Lastly, it would be also interesting to investigate whether the same intricate signaling cascades activated in preconditioning-induced cardioprotection are involved in the rodent model of acute pulmonary inflammation, since no previous studies have characterized RIPC signaling pathways in an ALI model. NOS, PKC, mitochondrial KATP channel, HIF-1α, ROS, miR-144, adenosine, and bradykinin have all been implicated in IPC/RIPC-induced cardioprotection in models of I/R injury. Treatment with inhibitors of these signaling components would provide insight into the actual mechanism of RIPC in the setting of pulmonary inflammation.
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