THE PHYSIOLOGICAL MECHANISMS UNDERLYING THE OSMOTIC EFFECTS ON NEUTROPHIL FUNCTION:
ROLE OF MAPK p38 AND THE OSMOTIC REMODELLING OF THE ACTIN CYTOSKELETON

by

Sandro Baleotti Rizoli

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

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Abstract

“The physiological mechanisms underlying the osmotic effects on neutrophil function: role of MAPK p38 and the osmotic remodelling of the actin cytoskeleton”

for the degree of Doctor of Philosophy, 2000

Sandro Baleotti Rizoli, Institute of Medical Science, University of Toronto

Trauma care has progressed significantly in 50 years and new challenges have arisen. Nowadays, patients often die of inflammatory complications rather than from the trauma/hemorrhage itself. Multiple organ failure is the commonest cause of death, and lung, the earliest and commonest organ to fail. Motivated by nascent concepts, we resuscitated rodents in a post-hemorrhage acute lung injury model, with hypertonic saline solution. The increased serum osmolarity attenuated lung damage, mostly by causing a profound, but transient suppression of neutrophil function, which are pivotal in the pathogenesis of post trauma lung failure.

In trying to understand the underlying mechanisms, we identified that cell shrinkage alone altered surface expression of adhesion molecules, and impaired adhesive, migratory, oxidative and exocytic neutrophil functions. Earlier studies demonstrating that neutrophil shrinkage caused tyrosine phosphorylation led us to investigate the involvement of stress-activated signaling pathways. We found that shrinkage activated p38, which regulated both the osmotically induced L-selectin shedding and apoptosis. We also identified other osmosensitive kinases, Hck, Syk and Pyk2, that did not mediate the functional outcomes described above.

Considering the relevance of the actin cytoskeleton in all suppressed neutrophil functions, we hypothesized its involvement. In fact, shrinkage markedly increased filamentous actin content while abolishing exocytosis of all granules, regardless of the stimulation used. Pharmacological inhibition of osmotic actin polymerization with latrunculin B, restored exocytosis in shrunken cells while induction of polymerization mimicked the effects of hypertonicity under isoosmotic conditions. Impaired chemotaxis,
rolling, adhesion, transmigration, respiratory burst and L-selectin shedding were all found to be fully or partially regulated by these cytoskeletal changes.

In conclusion, osmotically induced changes in both signaling transduction pathways and actin cytoskeleton are important and common mechanisms accounting for many neutrophil suppressive effects, which will be explored for the management of post-trauma organ failure in an upcoming clinical trial.
Acknowledgments

This thesis is dedicated to my wife Michele and my sons Lucas and Eric; for their tolerance, patience, advice, encouragement and actual help throughout this undertaking.

I would also like to express my many thanks to my supervisor Dr Ori Rotstein for his critical comments and support. Dr Andras Kapus has my sincere gratitude for sharing his enduring support, his scientific problem-solving skills and his friendship. I also extend my thanks to the members of my thesis committee: Dr Sergio Grinstein, Dr Miles Johnston and Dr Paul Walker.

Jean Parodo deserves a special mention for her technical expertise and assistance in so many experiments, but above all for her constant encouragement, willingness to help and to participate. I am also in debt to my colleagues from the laboratory, without their help and repeated blood donations, the neutrophil studies would never been done and the trips to many conferences would not have been so enjoyable: George Oreopoulos, Jie Fan, Yue Li, Ravi Taneja, Alice Wei, Chris Davreux, Julia Jones, Maria Jimenez, Ian McGilvray and Richard Bittar.
ABSTRACT................................................................................................................ II

ACKNOWLEDGMENTS..................................................................................................... IV

LIST OF FIGURES.......................................................................................................... VII

DISSEMINATION OF THESIS CONTENTS....................................................................... VIII

CONTRIBUTING PROJECTS............................................................................................. VIII

CHAPTER 1. INTRODUCTION.............................................................................................. 9

SECTION I. TRAUMA, HEMORRHAGIC SHOCK, SIRS/MODS AND ARDS ...................... 9
Objectives ......................................................................................................................... 9
Introduction ........................................................................................................................ 9
Hemorrhagic shock ........................................................................................................... 9
SIRS/MODS ....................................................................................................................... 10
Acute respiratory distress syndrome .............................................................................. 11
Risk factors for ARDS ...................................................................................................... 12
Pathological features ....................................................................................................... 12
ARDS mediators .............................................................................................................. 13
Neutrophils ...................................................................................................................... 13
Other cellular mediators ................................................................................................. 16
Other humoral mediators ............................................................................................... 17
Other important factors in the pathogenesis of ARDS .................................................. 20
Clinical management of ARDS ....................................................................................... 20

SECTION II. FLUID RESUSCITATION AND HYPERTONIC SALINE ........................................ 22
Objectives ......................................................................................................................... 22
Fluid resuscitation, therapeutic options ......................................................................... 22
Historical overview ......................................................................................................... 24
Hemodynamic effects ..................................................................................................... 25
Immune and anti-inflammatory effects ......................................................................... 26
Clinical benefits .............................................................................................................. 27
HSD side effects ............................................................................................................. 29

SECTION III. NEUTROPHILS .......................................................................................... 31
Objectives ......................................................................................................................... 31
Introduction ...................................................................................................................... 31
Granules .......................................................................................................................... 32
Determinant factors for neutrophil sequestration in the lungs ........................................ 33
Adhesion molecules: selectins ....................................................................................... 34
Adhesion molecules: integrins ....................................................................................... 39
Sequence of events in neutrophil extravasation .............................................................. 41
Exocytosis of granules in neutrophils ........................................................................... 41
Respiratory burst ............................................................................................................. 43

SECTION IV. SIGNAL TRANSDUCTION PATHWAYS IN NEUTROPHILS ......................... 45
Objectives ......................................................................................................................... 45
Introduction ...................................................................................................................... 45
Receptors ........................................................................................................................ 46
From chemoattractant receptors to MAPK .................................................................... 47
The small GTP-binding proteins .................................................................................... 51
Intracellular signaling pathways triggered by adhesion receptors ................................ 54
Signaling intermediates ................................................................................................. 56
Lipopolysaccharide ........................................................................................................ 58
Specificity of selected protein kinase inhibitors .......................................................... 59

SECTION V. VOLUME AND NEUTROPHIL FUNCTION ...................................................... 62
Objectives ......................................................................................................................... 62
List of Figures

**FIGURE 1:** SERUM OSMOLARITY ........................................................................................................ 93
**FIGURE 2:** TRANSPULMONARY ALBUMIN LEAK ........................................................................ 93
**FIGURE 3:** NEUTROPHIL SEQUESTRATION IN THE LUNGS ...................................................... 94
**FIGURE 4:** LUNG HISTOLOGY ....................................................................................................... 94
**FIGURE 5:** CINC mRNA EXPRESSION .......................................................................................... 95
**FIGURE 6:** EX VIVO MANIPULATION ........................................................................................... 95
**FIGURE 7:** CD11B EXPRESSION IN RAT NEUTROPHILS ............................................................. 99
**FIGURE 8:** L-SELECTIN EXPRESSION IN RAT NEUTROPHILS .................................................. 99
**FIGURE 9:** EXPRESSION OF ADHESION MOLECULES IN HUMAN NEUTROPHILS .............. 100
**FIGURE 10:** ICAM-1 EXPRESSION ............................................................................................... 101
**FIGURE 11:** TRANSIENT EFFECT ON NEUTROPHIL SEQUESTRATION ................................ 101
**FIGURE 12:** TRANSIENT EFFECT ON CD11B SURFACE EXPRESSION ..................................... 102
**FIGURE 13:** TRANSIENT EFFECT ON NEUTROPHIL SPREADING ........................................... 102
**FIGURE 14:** SHEDDING OF L-SELECTIN ..................................................................................... 111
**FIGURE 15:** SHEDDASE INHIBITOR AND L-SELECTIN SHEDDING ........................................... 111
**FIGURE 16:** CELL SHRINKAGE AND SHEDDING ......................................................................... 112
**FIGURE 17:** pH AND SHEDDING .................................................................................................. 112
**FIGURE 18:** TYROSINE PHOSPHORYLATION AND SHEDDING ................................................ 115
**FIGURE 19:** TYROSINE KINASE INHIBITORS AND SHEDDING ................................................... 116
**FIGURE 20:** NON-RECEPTOR TYROSINE KINASES ...................................................................... 117
**FIGURE 21:** P38 AND OSMOTIC L-SELECTIN SHEDDING ......................................................... 118
**FIGURE 22:** HYPERTONICITY AND APOPTOSIS ......................................................................... 120
**FIGURE 23:** GRANULE EXOCYTOSIS ......................................................................................... 127
**FIGURE 24:** HYPERTONICITY AND F-ACTIN CONTENT AND DISTRIBUTION ............................ 128
**FIGURE 25:** INVERSE RELATIONSHIP BETWEEN F-ACTIN AND EXOCYTOSIS ..................... 133
**FIGURE 26:** LATRUNCULIN B PREVENTS HYPERTONIC EFFECTS ........................................... 134
**FIGURE 27:** LATRUNCULIN B AND OTHER HYPERTONIC EFFECTS ......................................... 135
**FIGURE 28:** JASPLAKINOLIDE MIMICS HYPERTONIC EFFECTS ................................................ 140
**FIGURE 29:** CYTOSKELETON AND TRANSMEMBRANE FLOW ................................................ 141
**FIGURE 30:** TYROSINE KINASE INHIBITORS AND ACTIN POLYMERIZATION ......................... 142
**FIGURE 31:** IONIC STRENGTH AND ACTIN POLYMERIZATION ............................................... 143
**FIGURE 32:** LATRUNCULIN B AND RESPIRATORY BURST ......................................................... 143
**FIGURE 33:** CYTOSKELETON AND L-SELECTIN SHEDDING .................................................... 144
Dissemination of Thesis Contents

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Contributing projects


CHAPTER 1. Introduction

Section I. Trauma, Hemorrhagic Shock, SIRS/MODS and ARDS

Objectives

The objective of this section is to review current and relevant literature on trauma, hemorrhagic shock, the post-trauma systemic inflammatory response (SIRS) and multiple organ dysfunction syndrome (MODS) and finally acute respiratory distress syndrome (ARDS). The following are the most relevant issues:

1. trauma is the leading cause of death and permanent disability worldwide in the first 4 decades of life;
2. hemorrhagic shock is one the most common causes of death immediately after trauma;
3. hemorrhage also triggers a systemic inflammatory response that unabated, leads to sequential organ failure and eventually late death;
4. ARDS is the earliest and most common organ dysfunction associated with SIRS;
5. neutrophils are the most important inflammatory effectors of lung damage in ARDS, a lethal condition in up to 50% of the cases. There are currently no specific therapeutic measures of proven benefit for the treatment of ARDS.

Introduction

Throughout the world, trauma remains the leading cause of death in the first 4 decades of life. Including all age groups, only atherosclerosis and cancer surpass trauma as the major cause of death. In Ontario alone, about 4,000 patients die annually from trauma according to the Ontario Trauma Registry. These mortality figures however are just a fragment of the enormous amount of suffering and financial cost in hospitalization and lost productivity imposed by this condition. For each death, three survive with permanent disabilities, and over 70,000 Canadians in Ontario alone, require hospital admission for trauma care.

Hemorrhagic shock

Hemorrhage is the second leading cause of death in trauma, responsible for more than 30% of all deaths. Since the role of hemorrhage in triggering multiple organ failure and sepsis is not included in this figure, it underestimates the real importance of hemorrhage.

Hemorrhagic shock is defined as the condition where the blood loss results in inadequate organ perfusion and tissue oxygenation. The homeostatic responses to acute blood loss have been well described by generations of physiologists. These responses serve the primary purpose of maintaining perfusion to the brain and heart while striving to restore homeostasis. Hypermetabolic compensatory responses to hemorrhagic shock include a series of cardiovascular, neuroendocrine and inflammatory responses with activation of the coagulation, complement and fibrinolytic systems. At a cellular level, inadequate perfusion and failure to extract oxygen results in conversion to the less efficient anaerobic metabolism with excess lactate production and acidosis. When higher oxygen requirements are not met,
the cell membrane subsequently depolarizes and sodium, chloride and water leak into the cytosol. The profuse cellular edema that follows decreases even further the circulating volume adding to the deleterious effects of the initial blood loss. This cycle eventually progress toward death if not halted.2,3

In those patients surviving hemorrhagic shock, the hypermetabolic responses peak within 3 to 5 days and abate by 7 to 10 days.7 Extensive traumatic injuries or inadequate or delayed resuscitation are frequently followed by a pattern of non-resolution of the hypermetabolic inflammatory response, which is called systemic inflammatory response syndrome or SIRS. The clinical presentation of a continuous inflammatory response or SIRS, is often accompanied by varying degrees of acute respiratory failure, frequently progressing to a sequence of organ failure referred to as multiple organ dysfunction syndrome or MODS.

SIRS/MODS

History and definition

Fifty years ago patients in hemorrhagic shock would likely die early of blood loss or kidney failure and MODS was not observed. During the Vietnam War, improvements in the management of shock resulted in increased survival after hemorrhage. However, many of the survivors died later of what was then termed "shock lung". By that time it became clear that systemic processes such as hemorrhage could induce not only respiratory failure, but also a sequence of organ failures. Today in North America, with the advances in pre-hospital care and trauma resuscitation, most victims of traumatic hemorrhage survive long enough to reach a hospital, and MODS is the leading cause of death among those surviving trauma.2,4,5 MODS is currently diagnosed in more than 30% of all severely traumatized patients.6

In 1991, a consensus conference by the American College of Chest Physicians and the Society of Critical Care Medicine defined MODS and SIRS.7 It was proposed that the term SIRS should describe the massive systemic inflammatory response to a variety of severe insults while MODS was a sequence of altered organ function such that homeostasis can not be maintained without intervention. Therefore, SIRS is the adaptive response to disruption of homeostasis while MODS is the maladaptive consequence of SIRS.9

Pathophysiological mechanisms

In the trauma setting, the auto-destructive and excessive systemic inflammatory response results from complex interactions between hypoxemia, tissue hypoxia, direct organ injury, non-viable tissue, microorganisms/toxins, antigen/antibody complexes and iatrogenic effects of therapy plus cellular and humoral mediators.5 A widely accepted concept is that SIRS/MODS results from the imbalance between pro- and anti-inflammatory mediators. Bone proposed that the imbalance goes through 5 stages of development.4,9 The initial traumatic insult prompts the release of both pro- and anti-inflammatory mediators at the local site of injury. When local defenses are insufficient to correct local injury, then pro-inflammatory mediators are released into the systemic circulation, recruiting additional cells to the local
area, or stage 2. If systemic release of pro-inflammatory mediators is massive or the anti-inflammatory reaction insufficient, most patients develop evidence of SIRS as well incipient evidence of MODS, which most often is acute lung injury and characterizes stage 3. Stage 4 occurs when excessive anti-inflammatory response leads to immunosuppression with increased risk for infection. Stage 5 is the final stage of MODS, balance between pro- and anti-inflammatory mediators is not only lost but may ultimately be reinforced by the mediators themselves.

A massive traumatic insult can precipitate severe SIRS and early MODS independent of sepsis, by the so-called one-hit model. This tends to occur in patients so severely injured that they cannot be resuscitated into an early hyperdynamic state. Another hypothesis proposes that multiple sequential insults are required in order to produce clinically apparent organ dysfunction, the so-called “two-hit hypothesis”. Essentially a primary event such as hemorrhage, primes inflammatory cells and cascades, while a subsequent secondary event, usually mild and trivial, produces an overwhelming inflammatory response accompanied by extensive tissue damage. The wide range of timing and pattern for MODS, which may occur early after hemorrhage or 3 to 7 days later, supports this theory and reflects the complex and often overlapping network of interactions determining this pathology.

**Acute respiratory distress syndrome**

Acute respiratory distress syndrome or ARDS is the earliest and most frequent manifestation of uncompensated systemic inflammatory response and multiple organ dysfunction syndromes. ARDS remains one of the most important causes of mortality and morbidity among all patients admitted to an Intensive Care Unit. The modern description of the clinical syndrome was published in 1967 by investigators from the University of Colorado. It is estimated that approximately 150,000 cases occur per year in the United States alone or 75/100,000 population per year. Despite the vast improvements in our understanding of this disease process and refinements in the provision of supportive care, ARDS still carries a mortality rate that varies from 10 to 90%. These figures, though imprecise, disclose the tremendous human and financial cost of this condition.

**Definition**

ARDS was recently defined in a Consensus Conference as the syndrome of inflammation and increased permeability of the lungs that is associated with a constellation of clinical, radiological and physiological abnormalities that cannot be explained by left atrial or pulmonary capillary hypertension. The operational definition of ARDS proposed, is of an acute and persistent non-cardiogenic edematous lung injury, or pulmonary capillary wedge pressure of < 18 mmHg and no evidence of elevated left heart filling pressure, by arterial hypoxemia with PaO2/FiO2 ratio of < 200 mmHg and resistant to oxygen therapy alone and diffuse radiological infiltrates. Acute lung injury or ALI, refers to the initial changes in lung function characterized by less severe hypoxemia with PaO2/FiO2 ratio of < 300 mmHg, while the term ARDS is reserved for the extreme manifestation of a spectrum of pulmonary damage.
Risk factors for ARDS

Diverse insults have been linked to the pathogenesis of ARDS. While sepsis remains the most frequently encountered, hemorrhagic shock and trauma are among the leading causes of ARDS. Trauma may lead to ARDS through diverse mechanisms, pulmonary contusion has been associated with a 22% incidence of ARDS, long bone and pelvic fractures with an 11% incidence and multiple transfusions with 35%. The overall incidence of ARDS following trauma is 25.5%.

Table 1 presents the different risk factors. They have been separated into two groups, those directly harming the lung and those indirectly harming the lung via a systemic inflammatory response.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Indirect injury:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct injury:</td>
<td></td>
</tr>
<tr>
<td>Aspiration</td>
<td>Sepsis syndrome</td>
</tr>
<tr>
<td>Diffuse pulmonary infection</td>
<td>Hemorrhagic shock</td>
</tr>
<tr>
<td>Near-drowning</td>
<td>Severe non-thoracic trauma</td>
</tr>
<tr>
<td>Toxic inhalation</td>
<td>Massive blood transfusion</td>
</tr>
<tr>
<td>Lung contusion</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td></td>
<td>Pancreatitis</td>
</tr>
<tr>
<td></td>
<td>Burns</td>
</tr>
<tr>
<td></td>
<td>Drug exposure/overdose</td>
</tr>
<tr>
<td></td>
<td>Others (increasing age, chronic alcoholism)</td>
</tr>
</tbody>
</table>

The notion that diverse precipitating insults act to cause lung inflammation, microvascular injury and progressive hypoxemia through a final common pathway is controversial. Current evidence maintains that ARDS is not one single entity, which might explain why none of the anti-inflammatory or antioxidant therapy has ever produced any impressive result in ARDS. This discussion will focus on lung injury caused by trauma and hemorrhagic shock.

Pathological features

Despite the diversity of insults, the pathological features of ARDS are quite constant. The histological changes in the lung have been extensive described and vary according to the timing of the tissue sampling. Its temporal progression has been divided into three interrelated and overlapping phases, which correlate with the clinical evolution of this disease.

Exudative phase

This phase is characterized by extensive intra-alveolar and interstitial exudate, deposition of eosinophilic hyaline membranes on the alveolar ducts and increased permeability of the alveolar-
capillary membrane. The earliest histological changes precede any clinical evidence of respiratory failure, and include massive capillary congestion mostly of neutrophils, which so intense that results in the formation of neutrophil and platelet thrombi; marked interstitial and alveolar edema and intra-alveolar hemorrhage. Later, the neutrophils also infiltrate the intra-alveolar compartment extensively. Neutrophils comprise over 75% of the cells collected by bronchoalveolar lavage (BAL) during this phase, while macrophages and lymphocytes account for the remainder. Markers of intense neutrophil-mediated inflammatory response are also evident in the circulating blood. In severe cases there is cellular necrosis and denudation of the epithelial lining cells, disruption of the capillary basement membrane and intravascular fibrin deposition. Grossly the lungs are dusky, wet and heavy with a weight of 1000 g or more.

**Proliferative phase**

This phase is characterized by the organization of the exudate acquired during the initial phase and occurs between the first and 3rd week of clinical ARDS. There is extensive epithelial cell regeneration, formation and subsequent organization of granulation tissue by migrating fibroblasts and epithelial cells. The alveolar-capillary membrane permeability reduces progressively and macrophages are the predominant cell in the BAL fluid after day 14.

**Fibrotic phase**

This phase is characterized either by progressive lung fibrosis and reduction in lung compliance of restoration to normal or near-normal histology. Beyond the 3rd or 4th week, histological assessment of the lungs shows different degrees of fibrosis diffusely deposited in the lungs. The degree of fibrosis correlates with either prolonged ventilator dependence or clinical resolution of the process and some patients progress with pulmonary hypertension.

**ARDS mediators**

ARDS is a syndrome that results from complex interactions between cellular and humoral mediators and the role of each mediator clearly differ according to the many different etiologies. An exhaustive review of all ARDS mediators is beyond the scope of this dissertation. The following is a brief survey of soluble and cellular agents that might be responsible for lung dysfunction. The potential mediators are listed in Table 2.

**Neutrophils**

There is ample evidence implicating neutrophils as the final effector cell responsible for ALI/ARDS. Characteristically in acute lung injury, there is a massive accumulation of activated neutrophils in the lungs, very early within the vascular spaces and subsequently in the interstitial and alveolar spaces. In fact, the appearance of neutrophils precedes discernible lung injury in models of endotoxin-, hypovolemic shock- and ischemia/reperfusion-mediated lung injury. Blood and BAL fluid from ARDS patients can activate neutrophils in vitro and cause damage and fluid leakage in
isolated lungs and lung endothelial cell monolayers. Furthermore, neutrophil depletion significantly reduces ALI following ischemia-reperfusion, acute pancreatitis, and LPS administration \(^\text{31,35-37}\), while neutrophil activation with phorbol myristate acetate, platelet activating factor and complements fragments produce lung injury resembling ARDS \(^\text{38-40}\). There is abundant evidence of neutrophil activation after trauma and/or ischemia-reperfusion, both in trauma patients and laboratory animals. In humans, neutrophils from all 17 major torso trauma patients were primed and activated within 6 hours of injury \(^\text{41}\). There is consistent pattern of neutropenia, vascular sequestration and oxidative injury in post-trauma ALI \(^\text{42}\). In this context, neutrophil profile include increased superoxide production \(^\text{43}\), increased CD11b/CD18 surface expression \(^\text{42,44,45}\), increased adherence \(^\text{46}\) and impaired chemotactic ability \(^\text{47-48}\). These data clearly indicate that neutrophils have a pivotal role in the transition between the initial injury and subsequent acute lung injury.

Neutrophils might also have a role in enhancing and maintaining the inflammatory process since they are capable of expressing pro-inflammatory and immunoregulatory cytokines and chemokines such as IL-1\(\beta\), IL-8, IL-10, TGF-\(\beta\)1, MIP-2 and TNF-\(\alpha\) \(^\text{32,49,50}\). Despite their importance, neutrophils are certainly not the sole responsible for ARDS. ARDS has been described in profoundly neutropenic patients in preparation for bone marrow transplantation as well as in animal models of direct toxic effect on the alveolar-capillary membrane with oleic acid, which is unaffected by neutrophil depletion \(^\text{51,52}\).

**Neutrophil proteases**

Neutrophils do cause lung injury by releasing multiple cytotoxic products such as reactive oxygen species (ROS), eicosanoids, PAF, cytokines and proteolytic enzymes \(^\text{32}\). Patients with ARDS characteristically have above-normal amounts of oxidized antiproteases in the BAL fluid, and increased quantities of hydrogen peroxidase on their breath \(^\text{53}\). Proteases such as elastase and metalloprotease breakdown extracellular matrix components, damaging the alveolar-capillary basement membrane and increasing permeability \(^\text{54}\). Elastase is toxic only in the presence of ROS, which inactivates \(\alpha\)1-antiprotease \(^\text{55}\). Trauma severity correlates positively with elastase levels in plasma and BAL fluid of patients \(^\text{56}\), which in turn, correlate with ARDS severity \(^\text{56,57}\). Inhibition of neutrophil elastase and gelatinase activity in a pig model of ARDS reduced neutrophil accumulation and damage to the lungs \(^\text{54}\). The combined data support a substantial role for neutrophil proteases in the pathogenesis of ARDS.

**Lipid mediators**

Eicosanoids are other toxic products released by neutrophils as well as other cell types. They are all products of arachidonic acid (AA) and include prostaglandins, thromboxanes and leukotrienes \(^\text{58}\). These metabolites, especially leukotriene B4 (LTB4), are potent neutrophil stimuli providing a paracrine loop to stimulate other leukocytes. LTB4 is a potent stimulus for ROS production, elastase release,
increased adhesion and production of IL-1, TNF-α and other cytokines. LTB4 and thromboxane A2 are also potent neutrophil chemoattractants.

The characteristic findings of acute inflammation such as vasodilatation, edema and early neutrophil are caused by eicosanoids. They also increase pulmonary vascular permeability and resistance and have been implicated as early mediators of ARDS. Eicosanoid generation involves the production of ROS, which in return induce eicosanoid synthesis. Free radical antagonists inhibit both classes of agents. Not every lipid mediator has pro-inflammatory activity, PGE2 is an anti-inflammatory prostaglandin that inhibits cytokine production by negative regulation of NF-κB pathways. PGE2 also inhibits neutrophil chemotaxis and aggregation. PGE2 and IL-10, another anti-inflammatory factors, are detected 2-3 h after the precipitating stimulus and act in synergy with all other factors to modulate the neutrophil response.

**Neutrophil and apoptosis**

Delayed neutrophil apoptosis contributes to the perpetuation of the destructive inflammatory response. Unstimulated neutrophils have a life span of about 5 to 6 h that is usually terminated by apoptosis. Neutrophil programmed death is markedly delayed by β2 integrin-mediated migration and by pro-inflammatory mediators such as endotoxin, IL-1β, IL-8 and GM-CSF. There is substantial evidence supporting a pivotal role for delayed apoptosis in the pathogenesis of ARDS. Neutrophil apoptosis is delayed in both ARDS patients and experimental models of ALI. ARDS resolution is marked by the increase in alveolar macrophage/neutrophil ratio, consistent with the removal of neutrophils by macrophages. The anti-inflammatory cytokine IL-10 improves pulmonary inflammation by promoting the restoration of normal neutrophil apoptosis.
Table 2. Potential Mediators of Acute Respiratory Distress Syndrome

<table>
<thead>
<tr>
<th>Pro-inflammatory Mediators</th>
<th>Humoral Mediators:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular Mediators:</strong></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Complement</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Cytokines/chemokines</td>
</tr>
<tr>
<td>Platelets</td>
<td>Coagulation/fibrinolysis</td>
</tr>
<tr>
<td>Tissue macrophages and monocytes</td>
<td>Kinin system</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Soluble adhesion molecules</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Endorphins</td>
</tr>
<tr>
<td></td>
<td>Histamine and serotonin</td>
</tr>
<tr>
<td></td>
<td>Proteolytic enzymes</td>
</tr>
<tr>
<td></td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
</tr>
<tr>
<td></td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td></td>
<td>Eicosanoids and Vasoactive peptides</td>
</tr>
<tr>
<td></td>
<td>Monocyte chemoattractant protein-1 and -2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Anti-inflammatory Mediators</strong></td>
<td></td>
</tr>
<tr>
<td>IL-1 receptor antagonist</td>
<td></td>
</tr>
<tr>
<td>IL-4, IL-10, IL-13</td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor (\beta)</td>
<td></td>
</tr>
<tr>
<td>PDGF, IGF-1</td>
<td></td>
</tr>
<tr>
<td>Soluble TNF receptor</td>
<td></td>
</tr>
<tr>
<td>Leukotriene B4 receptor antagonist</td>
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</table>

**Other cellular mediators**

**Endothelial cells**

Endothelial injury is a hallmark of ARDS\(^{65,66}\) and endothelial activation is one of the critical factors in the pathogenesis of ARDS\(^{67}\). Endothelial activation is nearly synonymous with increased expression of adhesion and signaling molecules and include: selectins, immunoglobulins, IL-8, oncostatin M, the chemokine epithelial neutrophil activating peptide 78 and cyclooxygenase-2\(^{67-70}\). These molecules recognize different classes of leukocytes in an agonist- and time-dependent fashion, and thus regulate the microvascular accumulation of different inflammatory cells at different times\(^{67}\). Activated endothelium can even bind to non-activated neutrophils, accounting partially for the early CD18-independent lung sequestration of these cells in ALI\(^{71}\). Endothelial cells are activated by numerous factors including endotoxin, cytokines, PAF/thrombin, histamine and ROS\(^{5,67}\).
There is extensive literature on the participation of the endothelium in the pathogenesis of ARDS. Endothelial ICAM-1 participates in lung trafficking of neutrophils as shown in a murine model of hemorrhage and trauma ALI. Conversely, others demonstrated that ICAM-1 or P-selectin inhibition reduces both tissue injury and organ dysfunction. Nathens et al reported that the glutathione-depleting agent diethylmaleate ended the neutrophil-dependent ALI induced by LPS, specifically by preventing ICAM-1 upregulation in the lungs. Levels of soluble endothelial molecules are used as markers of injury. Soluble ICAM-1 levels during resuscitation correlate with the development of MODS in severely traumatized patients and other conditions. Likewise, subsets of major trauma patients, especially those later dying from infection or organ failure, had elevated levels of shed E- and P-selectin during resuscitation.

Intercellular adhesion molecule-1 (ICAM-1) is an inducible cell surface glycoprotein of the immunoglobulin supergene family, present on both leukocytes and endothelial cells at low levels. This inflammation-enhancing molecule is the most important ligand for integrins. ICAM-1 expression on endothelial surface is dramatically increased by inflammatory mediators such as LPS, IL-1 and TNF-α and inhibited by glucocorticoids. Induction of ICAM-1 requires de novo protein synthesis and increased surface expression 4 to 24 h to reach a maximum, remaining elevated for several days. ICAM-1, ICAM-2 and VCAM expression correlates more with influx of monocytes and lymphocytes to inflamed sites than neutrophils. ICAM-2 and ICAM-3 expression are not modified by inflammatory cytokines, increasing only in tumoral endothelium. ICAM-4 expression is restricted to erythrocytes and erythroid precursors while ICAM-5 or teleenchehalin, binds to CD11a/CD18 and is expressed in some parts of the brain. Endothelial VCAM is upregulated by LPS, IL1 and TNF but since it supports adhesion through α4β1, they do not bind to neutrophils, which do not express this integrin.

Platelets

Within less than 30 min of an insult, platelets colocalize with neutrophils in pulmonary and hepatic capillary networks. Platelets physically interact with neutrophils, and modulate their ability to adhere to the endothelium and migrate. Platelets directly activate the neutrophils by secreting diverse products such as PAF, IL-8, fibrinogen and fibronectin. Platelets also participate directly in the pathogenesis of ARDS. Increased platelet adhesiveness and aggregation give rise to microthrombi, which obstruct small vessels and cause ischemic injuries. Monocytes, alveolar macrophages and the coagulation cascade are also activated early, along with platelets.

Other humoral mediators

Complement

Complement activation occurs early after trauma or shock, and releases biologically active anaphylatoxins C3a, C5a and the cytolytic terminal complement complex (TCC). Fragments of activated complement contribute to SIRS and lung injury by promoting neutrophil sequestration in the
lungs. Complement fragments increase both neutrophil stiffness, facilitating intravascular retention and surface expression of adhesion molecules. Complement fragments also stimulate neutrophil chemotaxis, phagocytosis, increase vascular permeability, smooth muscle contraction and promote the release of histamine, arachidonic acid products, as well as TNF-α and IL-1 by macrophages and monocytes.

Even moderate trauma or hemorrhage causes significant activation of the complement that persists for days. Sustained complement activation and elevated elastase levels are the most significant parameters determining the development, severity and survival from ARDS after trauma. The important role of the complement in ARDS is further supported by the fact that the acute lung injury is reduced in complement-depleted animal models.

**Cytokines**

Cytokines are low-molecular-weight soluble proteins that mediate immune and inflammatory responses. Each cytokine binds to a specific receptor in the target cell and their mode of activity may be autocrine, paracrine and/or endocrine. Cytokines are produced in cascade, in which target cells such as neutrophils, fibroblasts and endothelial cells amplify the initial cytokine signals. Growing evidence suggests that an imbalance with predominance of pro-inflammatory cytokines is important in the post trauma and hemorrhage inflammatory response, especially TNF-α, IL-1 and IL-6. A comprehensive review of cytokines and lung disorders is beyond the scope of this dissertation and excellent reviews recently published on this topic are suggested.

TNF-α or cachectin is a pleiotrophic cytokine with many pro-inflammatory actions. Local release of TNF-α activates neutrophils, and therefore participates in the initiation of SIRS and ARDS. TNF-α is also involved in propagating, enhancing and perpetuating the inflammatory response by stimulating the production of many pro-inflammatory cytokines including IL-1β and IL-6, and chemokines such as human IL-8 and rat CINC and MIP. TNF-α has other deleterious effects such as diaphragmatic muscle dysfunction. TNF-α is mainly synthesized by blood monocytes and tissue macrophages, but also by neutrophils, lymphocytes and endothelial cells. TNF-α activates NF-κB, a critical transcription factor in the initiation of neutrophilic inflammation and ARDS. Many studies in trauma and hemorrhagic shock patients showed that while TNF-α increases in the BALF of patients with ARDS, its serum levels remain low, suggesting an important role in the trauma- and hemorrhage-induced lung dysfunction. Unfortunately, clinical trials with anti-TNF-α both antibodies and antagonists failed to reduce mortality in ARDS over the placebo groups.

IL-1β is an early phase pro-inflammatory cytokine with pivotal role in maintaining a persistent inflammatory state in the lungs of ARDS patients. It is the major pro-inflammatory bioactive substance in the BAL fluid of patients with ARDS. According to recent studies, IL-1β might be the major pro-
inflammatory cytokine in ARDS, with a more crucial role than TNF-α. IL-1β produces fever, anorexia and hypotension and stimulates the release of chemokines such as IL-8, MCP-1, MIP-1, consequently promoting neutrophils chemotaxis and organ damage. Circulating levels of IL-1β do not increase trauma/hemorrhage, but the use of IL-1β receptor antagonist increased post hemorrhage survival rate in an experimental model.

IL-6 is responsible for integrating signals produced early in the inflammatory response and stimulates acute-phase and procoagulants responses. It is not clear whether its physiological role is of a pro or anti-inflammatory cytokine. IL-6 enhances neutrophil phagocytosis, release of oxidant and PAF generation. Activated macrophages produce IL-6, partly by TNF-α and IL-1β stimulation. In contrast to TNF-α and IL-1β, IL-6 levels consistently increase in response to trauma and hemorrhage. IL-6 is very high in the BAL of patients at risk for ARDS and remains elevated throughout the course of established ARDS. IL-6 is a marker of the post trauma inflammatory state.

Chemokines

Chemotactic chemokines control the process of leukocyte attraction into tissues. To date, 40 chemokines have been identified and grouped into four families according to the relative position of their cysteine residues in their primary structure. Two families are well characterized. Alpha chemokines contain one amino acid separated by two cysteine residues (cysteine – X amino acid – cysteine or CXC), whereas the beta chemokines contain two cysteine residues adjacent to each other (CC). CXC chemokines include IL-8, GRO, ENA-78, granulocyte chemotactic protein 2, growth-related oncogene- α, -β and -γ, epithelial neutrophil-activating protein and epithelial neutrophil-activating protein-2. Beta chemokines in general do not attract neutrophils but monocytes and eosinophils. Chemokines are produced by many different cells under the stimulation of early pro-inflammatory cytokines TNF-α and IL-1β, bacterial and viral products. They are retained on matrix and cell-surface heparan sulphate proteoglycans establishing a concentration gradient surrounding the inflammatory stimulus. Chemokines stimulate specific G-protein-coupled cell surface receptors.

The IL-8 family of cytokines plays a major role in lung neutrophil chemotaxis and activation. IL-8 increased synthesis and release by alveolar macrophages occur as early as 30 minutes after an acute insult such as trauma, peaking in blood by 2-3 h. IL-8 concentration is elevated lung edema fluids of ARDS patients and correlate well with neutrophil accumulation in BAL fluid but not with severity of injury. In experimental models of ALI, both IL-8 and CINC, a rat cytokine with structural and functional homology to human IL-8, had a pivotal role in the enhanced accumulation of neutrophils in the lungs. Neutralizing concentrations of anti-IL-8 and anti-CINC antibody or anti-oxidant N-acetyl-cysteine, reduced the neutrophil sequestration in these models, from 50 to 66%, implicating IL-8 as a major mediator of neutrophil-mediated tissue injury.
Other important factors in the pathogenesis of ARDS

The role of the gut

The importance of the gut barrier to luminal microflora and toxic products has been increasingly recognized in the pathogenesis of SIRS\textsuperscript{106}. Diverse stresses such as trauma and hemorrhage, result in failure of the gut mucosal barrier resulting in increased intestinal permeability and translocation of bacteria/endotoxin into the systemic circulation and remote organs\textsuperscript{5,60}. Appearance of endotoxin in the plasma occur early after major blunt trauma or multiple injuries\textsuperscript{107} and in 30-90 min after hemorrhagic shock in animal models\textsuperscript{108}. Translocation of bacterial and/or endotoxin could directly lead to SIRS as well as function as a second stimulus triggering an exaggerated inflammatory response from a system primed by the initial injury. However, the clinical significance of translocation remains controversial, with no convincing reduction in morbidity and mortality in clinical trials of selective decontamination of the digestive tract\textsuperscript{5}.

Ischemia-reperfusion injury

Catabolism of ATP during ischemia leads to an increased concentration of purine metabolites, hypoxanthine and xanthine. Ischemia also mediates the conversion of xanthine dehydrogenase to xanthine oxidase. Oxygen, the only substrate lacking for superoxide generation is introduced suddenly and in excess with reperfusion and the xanthine oxidase-dependent oxidation of hypoxanthine and xanthine generates a burst of ROS that cause tissue injury. The free radicals generated by the endothelium cause local cell damage, which is enhanced by the accumulation and activation of neutrophils\textsuperscript{34,109}.

Clinical management of ARDS

The tremendous progress in understanding the basic cellular and molecular mechanisms involved in acute lung injury has unfortunately not been matched by similar advancement in therapeutic strategies. There are currently no specific measures, of proven benefit, for the treatment of ARDS. The clinical management of these patients consist of primary supportive measures including managing the precipitating condition, preventing complications, nutritional support, fluid balance and mechanical ventilatory support\textsuperscript{110}. A major breakthrough in therapeutic intervention was the April 1999 report from the on-going phase III ventilator trial, of the dramatic reduction in mortality (39 to 31\%) in patients ventilated with low tidal volume group\textsuperscript{111}.

No pharmacological strategies have a proven benefit for ARDS patients and clinical trials commonly report disappointing results. Preliminary data indicated that inhaled nitric oxide could improve arterial oxygenation without hypotension. However, prospective studies showed that arterial oxygenation was minimally improved, with no impact on either the length of positive pressure ventilation or mortality\textsuperscript{111}. Other therapies that failed clinical trials including n-acetylcysteine, prostaglandin E\textsubscript{1}, ketoconazole, lysofylline, methylprednisolone, extracorporeal membrane oxygenation,
extracorporeal carbon dioxide removal, prophylactic PEEP, high-frequency ventilation, partial liquid ventilation, aerosolized surfactant therapy (20, 21, 110, 112 and reviewed in 111). Regardless of these results, there are strong expectations of major discoveries in the near future. Most of the future clinical trials appear to be directed at targeting the elements responsible for the recruitment of neutrophils to the lungs such as anti-IL-8 therapy, or the administration of the anti-inflammatory cytokine IL-10 111.
Section II. Fluid Resuscitation and Hypertonic Saline

Objectives

The objective of this section is to review current and relevant literature on hemorrhagic shock resuscitation with hypertonic saline. The focus is on the established hemodynamic effects and beneficial clinical repercussions, as well as the recently suggested immunological effects. The following are the most relevant issues:

1. The primary goal in the management of hemorrhagic shock is to stop the bleeding and replace circulating volume;
2. The most common fluid resuscitation method is to infuse large volumes of isotonic crystalloid fluids, however large volumes might be deleterious;
3. An alternative method proposed is to resuscitate with small volumes of hypertonic saline/dextran (HSD) or NaCl 7.5% + 6% dextran;
4. HSD resuscitation has marked hemodynamic effects: it increases blood pressure, cardiac output and organ perfusion, and consequently HSD reduces post trauma mortality and morbidity;
5. Recent studies suggest that HSD also has immune and anti-inflammatory effects, which would have an enormous impact in reducing post trauma SIRS and ARDS;
6. HSD resuscitation is safe and free of significant toxic side effects.

Fluid resuscitation, therapeutic options

Large-volume resuscitation

The primary goals in the management of post trauma hemorrhagic shock are to terminate blood loss, frequently accomplished by surgery, and to replace both circulating volume and oxygen-carrying capacity. Early restoration of the circulating volume leading to adequate tissue perfusion prevents the aggravation of the initial injury. The concept of fluid resuscitation has evolved and become widely accepted since World War II. However, the debate over the best resuscitation method in trauma remains unresolved.

Today, most Emergency Rooms and Trauma Centers in North America accept the guidelines established by the American College of Surgeons in the Advanced Trauma Life Support (ATLS) manual as the standard for resuscitation. The most widely preferred solution for the treatment of hemorrhagic shock is the non-carbohydrate, non-protein isotonic crystalloid fluids such as Ringer's lactate and normal saline (0.9% NaCl) given together with packed red blood cells for the most severe cases. Resuscitation with colloidal fluids during the initial phases of shock has been abandoned by most. Colloid solutions increase intravascular colloidal pressure leading to intravascular influx of interstitial fluid thus reducing fluid requirements compared to crystalloid solutions. However, colloid solutions are more expensive, bind and decrease ionized fraction of serum calcium, decrease circulating levels of immunoglobulins, decrease endogenous production of albumin and might further compromise the extracellular fluid volume.
deficit rather than restore it. In fact, a meta-analysis of colloid versus crystalloid fluid resuscitation that included the most relevant human studies to date on this subject, concluded that crystalloid fluids are superior to a colloid-based regimen, with a 12% reduction in mortality rate when used to resuscitate hypovolemic shock patients.

According to the ATLS guidelines, crystalloid fluids are given liberally in order to restore the patients' hemodynamic parameters to normal, frequently resulting in massive infusions and edema. Extensive edema invariably accompanies major trauma or hemorrhage. Edema is the result of large shifts of volume into the extravascular space, or "third space", at the expense of an already inadequate circulatory volume. Interstitial and cellular edema impairs microcirculation and affects a multitude of cellular responses. The mechanisms underlying the shift of fluid are not well understood, but an important one is the increased capillary permeability that profoundly affects plasma volume and hemodynamic stability. Diverse inflammatory mediators, including neutrophils, are responsible for the systemic disruption of the endothelial barrier. An ideal resuscitation fluid should both replace circulating volume and prevent or attenuate the extravasation of plasma proteins through leaking capillaries. There is currently no resuscitation regimen, proven to limit capillary leak, but massive infusions of fluid certainly worsen it and resuscitation with large volumes of fluid has come into intense criticism.

No fluid resuscitation

The patient's final outcome results not only from tissue damage, blood loss and homeostatic responses but also from the iatrogenic interventions during resuscitation. Large-volume fluid resuscitation might worsen a patient's outcome by disrupting an early soft thrombus leading to rebleeding, diluting coagulation factors leading to coagulopathy, worsening systemic peripheral edema or causing heat losses and hypothermia. These arguments form the basis for questioning of the current ATLS guidelines and the no-fluid resuscitation proposition or hypotensive resuscitation. Instead of the aggressive resuscitation with large amounts of fluids, some authors have proposed avoiding pre-operative fluid resuscitation for traumatic hemorrhagic shock. They argue that the ensuing hypotension would facilitate hemostasis, which in turn, would allow compensatory mechanisms to maintain organ perfusion.

In diverse experimental animal models of uncontrolled hemorrhage, no fluid resuscitation had a better survival rate than those resuscitated with large volumes of fluids. Only one large and controlled study has tested this hypothesis in humans. This intensively debated clinical trial from the Ben Taub Hospital in Houston, Texas, concluded that injured patients in hypovolemic shock in whom fluid resuscitation was restrained until after achieving surgical control of the bleeding had a better chance to survive than comparable patients who underwent conventional large-volume resuscitation. Unfortunately this fascinating study is flawed in that the difference in outcome achieved statistical significance only after retrospective stratification of the patients, solely penetrating and not blunt injuries.
were included, the causes and timing of patients' death were not reported and all patients waited more than 44 minutes to reach the operating room from the emergency department, an inordinately long time considering their critical condition \(^{117}\). Considering the inconsistencies of this controlled trial and the lack of other studies most trauma experts agree for the time being, that the results of this study do not justify the recommendation to withhold all infusions until hemostasis \(^{120}\).

Another important consideration is that delaying or withholding fluid resuscitation in patients with profound blood losses or prolonged periods of hypotension might overwhelm the compensatory responses and result in either death or an exaggerated inflammatory response and multiple organ failure. In a rodent model of severe uncontrolled hemorrhage, mortality rates were the same for the no-resuscitation group and the group of animals resuscitated with large amounts of Ringer's lactate aiming to restore normal hemodynamic parameters \(^{121}\). This study suggests that large-volume resuscitation might be just as deleterious as avoiding all fluids.

**Small-volume resuscitation**

The debate over the large volume or no fluid resuscitation has become a dominant issue of discussion among trauma surgeons. Different concerns, some better founded than others, lead 2 decades ago to the proposition of small-volume resuscitation regimen with hypertonic saline. Few years later, concerns over a theoretical increased in blood loss following hypertonic saline resuscitation, lead to its complete abandonment. The novel verification that hypertonic saline might exert beneficial anti-inflammatory effects, compounded by the increased awareness of the inflammatory and immunological repercussions of trauma (more patients die of SIRS/MODS than directly from trauma) lead to a renewed interest in small-volume resuscitation.

**Historical overview**

There are sporadic reports of the administration of high-salt crystalloid solutions dating back to the beginning of the century. According to these reports, hypertonic solutions were used most often to correct electrolyte abnormalities, but also to induce peripheral vasodilatation or transient increases in blood pressure \(^{122,123}\). In 1980, de Felippe et al reported the first use of a hypertonic crystalloid solution, in this case 7.5% NaCl in water, to resuscitate trauma patients in hemorrhagic shock \(^{124}\). In this study, small volumes of hypertonic saline (HS) were administered to 12 patients *in extremis* of hemorrhagic shock with 11 showing a remarkable increase in blood pressure with a 90% drop in fluid requirement over 24 h. Nine patients left the hospital alive \(^{124}\). Since then, small-volume hypertonic saline resuscitation of trauma patients in hemorrhagic shock has been tested in over 1500 patients, mostly in North America \(^{123,125-132}\).

These clinical studies confirmed de Felippe's observation that small volumes of HS, usually 250 ml for an adult, normalized the crumbling hemodynamic parameters. In spite of these remarkable hemodynamic effects, HS resuscitation did not improve survival and the mortality rate was comparable
to standard large-volume resuscitation. Enthusiasm for this modality was later tempered by concerns over potential adverse effects of normalizing blood pressure prior to surgical hemostasis, raised mainly by the Ben Taub clinical trial mentioned earlier, and the routine use of hypertonic saline for resuscitation was abandoned almost completely. The interest in HS reappeared recently after studies suggested important immune and anti-inflammatory effects, heightened by concerns over large volume resuscitation. The following is a review of the current knowledge on hypertonic saline resuscitation and its potential application to the human condition of post hemorrhagic shock inflammation and cell-mediated organ damage.

**Hemodynamic effects**

In most clinical studies HS was administered in association with colloids such as dextran (HSD) or hydroxyl-ethyl starch. Colloids increase the duration and degree of volume expansion from 15 min to at least 2-3 h, without adding significant side effects. The hemodynamic effect of hypertonic saline is proportional to the sodium load and seem to plateau at 7.5% concentration. Thus, HSD containing 7.5% NaCl, 2400 mOsM/L and 6% dextran-70 is the standard hypertonic solution for clinical application. HSD is given as a rapid (4 to 10 minutes) single bolus of 4 ml/kg, in most studies adults received a bolus of 250 ml, through any available venous access. Alternatively, HSD may also be infused by intraosseous route. The small volume of infusion makes HSD regimen very attractive for use in the civilian pre-hospital and military setting.

Intravascular volume expansion by HSD is 8 to 10 times larger than that of a similar infusion of isotonic saline infusion. It is believed to result primarily from a rapid osmotically driven shift of water from the interstitial and intracellular spaces. Another fascinating possibility is that the anti-inflammatory effects of hypertonicity, especially on neutrophils, would reduce intravascular fluid and protein losses through highly permeable capillaries.

Neuroendocrine changes might also have a role, even though some of them seem to reflect the correction of hypotension and hypovolemia, being observed in animals fully resuscitated to their baseline hemodynamics with either HS or Ringer’s lactate. In HS resuscitated animals, the levels of epinephrine and nor-epinephrine decrease to approximately 25% of those resuscitated with isotonic saline. HSD also decreases circulating levels of ACTH, cortisol, aldosterone, vasopressin, renin and angiotensin compared to an equal volume of isotonic saline. Some studies suggest that some of the systemic effects of HS might be mediated centrally, differences in regional blood flow support the concept of peripherally mediated control. Venoconstriction might occur through a neural reflex, the afferent pathway of which would lie in the pulmonary vagal afferent with an efferent pathway via sympathetic venomotor fibers.

The increase in circulating volume improves blood pressure, cardiac output, organ perfusion and significantly reduce the volume of fluids required for resuscitation. The hemodynamic effects
become clinically detectable in 2 min of infusion, and last 2 to 3 h. Other effects also cooperate to the normalization of these parameters. HSD resuscitation increases cardiac preload by causing musculo-cutaneous venoconstriction, while reducing afterload and systemic vascular resistance by causing precapillary vasodilatation \(^{143, 149}\). Vasodilatation is due to an effect of HSD both on the vascular smooth muscles and on endothelial cells causing prostacyclin production, a paracrine mediator responsible for local vasodilatation and inhibition of platelet aggregation \(^{150}\). Another well-characterized attribute of HSD is a positive inotropic effect \(^{151, 152}\), but its clinical relevance questioned by some \(^{153, 154}\).

Interestingly, in post-ischemic hearts, HSD improves ventricular contractility mostly by reducing oxidative damage, indicating its potent anti-inflammatory effects \(^{155, 156}\).

The cells mostly affected by HSD resuscitation appear to be the endothelial, erythrocytes and leukocytes, probably due to their immediate contact with the hypertonic fluid. The remarkable reduction of endothelial and erythrocyte swelling accounts for the opening of shock-narrowed capillaries and improved organ perfusion \(^{157}\). Other diverse cell types are also exposed to hypertonicity and their function strongly affected by it. The effect of cellular volume changes on cell function is presented later in this dissertation.

A 250-ml bolus of HSD would raise plasma Na\(^+\) levels by 100 mM, however the body quickly corrects plasma Na\(^+\) levels to about 20 mM beyond isotonic levels, which remain elevated for as long as 4 to 6 hours \(^{158}\). In human and animal models of hemorrhagic shock, HSD causes a transient elevation of serum sodium ranging from 135 to 155 mEq/L, serum chloride ranging from 118 to 128 mEq/L and serum osmolarity ranging from 300 to 350 mOsm/L, while normalizing glucose, base excess deficit, pH and lactate values \(^{127, 143, 159}\). HSD causes a mild decrease in serum potassium and calcium and no changes in serum urea, bicarbonate, total plasma proteins, hematocrit and coagulation factor levels. All values are essentially normalized by 24 hours \(^{132}\).

**Immune and anti-inflammatory effects**

Most of the hemodynamic effects of HSD are known since the initial report by de Felippe in 1980 \(^{124}\). A revolution in this field started a few years ago with reports that HSD exerted immune and anti-inflammatory effects when administered to experimental models of trauma and hemorrhagic shock. In 1994, a group headed by Hoyt published the first of a series of studies focusing on the HS-mediated restoration of immuno competence after trauma and hemorrhage, potentially reducing the susceptibility to post-trauma septic complications \(^{160}\). These authors showed that hypertonicity enhanced in vitro T-cell proliferation and IL2 production \(^{160, 161}\) and prevent IL-4 and PGE2 inhibition of lymphocyte function \(^{134}\). Similar findings were reported in a rodent model of hemorrhagic shock and HS resuscitation \(^{134}\). Later, Coimbra and other members of this group showed that HS in fact reduces septic complications and increases survival in an experimental model of hemorrhage and cecal ligation and puncture \(^{135}\).
Our laboratory made significant contributions in this area that will be presented in the second part of this dissertation as our original data. We have focused on neutrophil function and some of our findings such as changes in neutrophil surface expression of adhesion molecules, have been corroborated by other investigators \textsuperscript{136, 162}. Still in the context of post trauma/hemorrhage inflammatory response, HS resuscitation protected organs from early shock-induced organ apoptosis, as observed in both small bowel and liver \textsuperscript{163} and increased neutrophil apoptosis, potentially reducing inflammation and organ damage \textsuperscript{141}. More recently our laboratory extended these observations to a model of liver ischemia/reperfusion wherein HS prevented neutrophil-dependent and -independent hepatocellular necrosis (Oreopoulos et al). In a separate model, Corso et al recently reported that HS reduced leukocyte in the liver accumulation after hemorrhagic shock \textsuperscript{133}.

The common point on all studies is the immense potential of HSD to modulate the inflammatory response to trauma and hemorrhage and consequently reduce trauma's most common cause of death, organ damage. Besides, a number of recently published biochemical and molecular studies agree that diverse cellular functions can be profoundly affected by a hypertonic environment, much in the same way as trauma and hemorrhage models do.

**Clinical benefits**

A large number of randomized clinical and experimental studies have compared HSD with traditional isotonic fluid resuscitation in the past \textsuperscript{123, 125-132}. Most of the clinical studies were completed before any suggestion of immune effects to HSD, and thus had the common goal of determining whether the hemodynamic effects of HSD would reduce in-hospital mortality rate. The following is a compilation of the most relevant clinical and experimental effects of HSD resuscitation according to these extensive studies.

**Decreases overall mortality rate**

Most of the studies consistently showed a trend toward decreased overall mortality rate for the HSD groups, but failed to yield any conclusive reduction. However, a recent meta-analysis by Wade et al of the most relevant clinical studies done to date showed a significant decrease in overall mortality rate by 5.1\% in favor of HSD compared to standard resuscitation \textsuperscript{132}. Since the early 1990’s, it is well establish that HSD significantly reduces trauma-related deaths in certain subgroups including severe head injury and patients trauma patients requiring operative intervention \textsuperscript{127, 128}. 
Decreases ICP and mortality rate in head injury

Head injury is the leading cause of death following trauma and remains a challenge with respect to fluid resuscitation since hypotension/hypoperfusion are as deleterious as cerebral edema caused by the fluid resuscitation. Several studies reported that HSD resuscitation of patients with severe head injury, resulted in decreased brain swelling and intracranial pressure (ICP) compared to standard resuscitation, plus the added benefit of increasing cerebral blood perfusion by increasing systemic blood pressure. Simma et al reported that children with severe head injury treated with HSD had a significantly lower ICP, required less medical and surgical interventions and had fewer complications. A comprehensive review of the experimental data on head injury by Anderson et al concurred to these results, showing that HSD decreased cerebral water content and ICP without delayed rebound effect.

Using the data from 12 large prospective studies, Wade et al found a better overall survival rate for severe head injury patients resuscitated with HSD instead of crystalloids, 37.9% versus 26.9%. Logistic regression analysis of these data revealed that head injury patients are twice as likely to survive when resuscitated with HS rather than conventional crystalloids.

Reduces the incidence of ARDS

The original study by de Felippe et al already reported significant improvements in pulmonary circulation and respiratory parameters. In a large multicenter study, Mattox et al reported that HSD resuscitation significantly reduced the incidence of post-trauma ARDS and pneumonia. Simma et al reported no cases of ARDS among children with severe head injury resuscitated with HSD versus 23% of those resuscitated with Ringer's lactate. Similar findings were reported by Holcroft et al, where HSD resuscitation resulted in less respiratory dysfunction, less need of mechanical ventilation and shorter ICU stay compared to Ringer's lactate. Animal models of post-hemorrhage lung injury also corroborate this protective effect, suggesting that the lungs might be one of the organs most likely to benefit from HSD resuscitation.

Reduces fluid requirement and improves pulmonary function after major surgery

Clinical trials in Austria, Germany, France and Brazil have shown that the use of HSD for fluid loading or fluid substitution during major vascular surgeries such as cardiac bypass and abdominal aortic aneurysm repair, reduced the positive perioperative fluid balance, improved hemodynamic stability and pulmonary function.

Improves spinal cord blood flow

HS resuscitation improves organ perfusion, causing a demonstrable increase in spinal cord blood flow after injury. Tuma et al reported that in three different animal models, HS resuscitation helped to preserve spinal cord function in the acute models and to recover faster in the chronic models of spinal cord injury. These authors concluded that the enhanced spinal cord blood flow was responsible for the beneficial effect of HS resuscitation.
Protects the heart and liver from ischemia-reperfusion injury

Resuscitation from hemorrhagic shock with HS prevented leukocyte stasis in hepatic sinusoids as well as leukocyte adherence to endothelial lining of post-sinusoidal venules and was associated with decreased shock-induced liver injury \textsuperscript{133, 135}. Other animal models have also consistently demonstrated the protective effect from ischemia-reperfusion injury of the heart compared to isotonic resuscitation \textsuperscript{170, 171}. In an animal model of burn injury, HSD resuscitation was reported to improve myocardial performance and exert significant cardio-protective effect after thermal injury, as measured by a significant reduction of troponin-I levels \textsuperscript{172}.

HSD side effects

HSD toxicity has been extensively tested revealing that up to five times (20 ml/kg) of the usual doses are free of toxic and collateral effects \textsuperscript{114, 173-176}. Diverse randomized double-blind prospective studies on HSD resuscitation showed that HS is safe and free from collateral, toxic and undesirable side effects \textsuperscript{123, 127-132, 177-179}. No clotting, renal, neural, cardiopulmonary or septic complications were noted.

The most feared complication is the increase in blood losses by HSD, a concept supported by some animal models of uncontrolled large vessel hemorrhage \textsuperscript{118, 119; 180; 181}. Interestingly this complication has been conspicuously absent from all clinical studies \textsuperscript{122, 132}. Blood loss measured during operation for trauma was not greater with HSD resuscitation and by all counts, blood requirements are significantly lower \textsuperscript{130}. According to Matsuoka et al, the disparity between animal models and human clinical data comes from the fact that most human traumatic bleeding originates in solid organs not large vessels \textsuperscript{182}. The concept of increased bleeding by HSD is well founded but appears to be of insignificant clinical relevance.

Two other potential complications of HSD administration include neurological disturbances due to acute hypernatremia and hyperosmolarity and coagulopathy by dextran. No human instances of HSD-related neurological disturbances have ever been described, even by an autopsy review of 64 deceased patients that had been treated with HS \textsuperscript{125, 127; 132, 183}. No cases of central pontine myelosis, demyelination syndrome, coma, seizures or hypernatremic subarachnoid hemorrhagic encephalopathy have so far been described in association with HSD infusion \textsuperscript{125, 132, 184}. Dextran solutions are associated with increased bleeding by decreasing platelet adhesion, depressing factor VIII and increasing fibrinolysis, anaphylactic reactions and impaired renal function. The small volume of dextran infused in association with HS has so far not been charged of causing any of these complication and in fact its oxygen-free scavenging abilities has been implicated in the improved myocardial performance after HS resuscitation \textsuperscript{127, 128, 130}.

The absence of side effects might be simply due to the difficulty in identifying and linking them to HSD, in patients who already have a multitude of disarrangements. In euvolemic healthy volunteers HSD administration caused no more than a transient unpleasant sensation of headache and heat, and transitory minimal hemodynamic changes \textsuperscript{185}. Patients in hemorrhagic shock do not report these
unpleasant sensations and in these patients the hemodynamic effects are remarkable. Present evidence points to the fact that the macrocirculatory changes that occur in humans are very similar to those observed in research animals following HS infusion.
Section III. Neutrophils

Objectives

The objective of this section is to review current and relevant literature on neutrophil physiology, focusing on those aspects of neutrophil function related to post trauma ARDS. The most relevant issues are:

1. neutrophils have an enormous oxidative and proteolytic potential to initiate and perpetuate an inflammatory state, which may result in SIRS and ARDS;
2. sequestration of activated neutrophils in the lungs precede any evidence of ARDS and is the result of a complex cascade of events and interactions of a multitude of mediators;
3. selectins are adhesion molecules that mediate the initial neutrophil-endothelial interactions, they are shed by unknown proteases and their role in ARDS varies under different conditions;
4. β2 integrins, predominantly CD11b/CD18, are essential for firm neutrophil adherence to the endothelium and emigration into the lungs, with a prominent role in the pathogenesis of ARDS;
5. most neutrophil inflammatory mediators are pre-synthesized and stored in cytoplasmic granules, the exocytic process remains incompletely understood.

Introduction

Neutrophils are the most abundant leukocyte in blood, with a mean concentration of 4.4 x 10⁶ cells/ml, accounting for 40 to 75% of all leukocytes. Normally, about 5% of all circulating neutrophils are immature or band cells. During infections or other conditions of stress, the bone marrow release large numbers of immature precursors into the circulation that respond differently than mature neutrophils to various stimuli. Neutrophils have nuclei with two to five lobes and abundant cytoplasmic granules. Neutrophils are short lived and in less than 12 hours after release into the blood stream, they migrate into extravascular tissues where they dye by apoptosis. Neutrophils are terminally differentiated cells that produce rapid responses independent of transcriptional or translational mechanisms. They have a limited repertoire of synthetic functions. Neutrophils derive almost all of their energy from the glycogen deposited in their cytoplasm, have extremely few mitochondria and use fermentation rather than oxidative phosphorylation to obtain energy. At any given time, there are 30 times more neutrophils in the bone marrow than in the blood stream. Approximately half of all neutrophils released from the bone marrow accumulate within vascular spaces and are called the “marginal pool”. Pulmonary capillaries contain a large pool of marginated neutrophils, with a concentration of neutrophils to red cells 60 to 100 times higher than that found in the systemic circulation.

Neutrophils are inflammatory cells with potent oxidative and proteolytic potential responsible for the destruction of invading pathogens by eliciting and maintaining an inflammatory state. The ability to exit the blood stream and migrate into extravascular sites is crucial in fulfilling this role. Most of the neutrophils' mediators of inflammation are presynthesized and stored in their granules, then released
upon stimulation. The neutrophil toxic potential however, can paradoxically be directed against host tissues and neutrophils have been implicated as perpetrators of many inflammatory processes, including ALI/ARDS.

**Granules**

Neutrophils, along with eosinophils and basophils, are described collectively as granulocytes due to the presence of granules in their cytoplasm. Neutrophils contain at least four different types of granules that store diverse membrane and bactericidal proteins. Different granules and contents are synthesized during different phases of myelopoiesis, and their corresponding genes turned off. Granule release radically affects the way neutrophils interact with the environment.

**Primary or azurophil granules**

Primary granules are spherical or football-shaped granules that appear in the promyelocytic phase and contain the neutrophil-specific protein myeloperoxidase and diverse hydrolytic enzymes. They are regarded as modified lysosomes due to their high content of acidic hydrolases, their binding to phagosomes and minimal or no extracellular release. Markers or proteins present exclusively in this granule include the lysosomal membrane protein of 53kDa CD63 or granulophysin, myeloperoxidase and defensins. The other three granules are referred to as peroxidase negative granules.

**Secondary or specific granules**

Secondary granules are smaller, less dense, staining heavily for glycoproteins. They appear in both myelocytic and metamyelocytic phases and contain lysozyme, collagenase, lactoferrin, B12-binding protein and cytochrome b558. A protein exclusively stored in this granule is CD66b or the nonspecific cross-reacting antigen-95. It is a member of the carcinoembryogenic antigen and its function is mostly unknown.

**Tertiary or gelatinase granules**

Tertiary granules were classified as a separated entity from the specific granules only after the advent of electron microscopy. They are morphologically similar to the specific granules although less dense and rich in gelatinase and appear during the myelocyte stage. Tertiary granules are the main store for gelatinase, but also for large amounts of CD11b/CD18 and cytochrome b558 an essential component of the NADPH oxidase. Neutrophils contain two matrix-degrading metalloproteinases, which are a family of zinc- and calcium-dependent enzymes. One 75kDa interstitial collagenase or matrix metalloproteinase 8 (MMP8) located in specific granules and another 92 kDa gelatinase (MMP9) also named gelatinase B or neutrophil gelatinase. Gelatinase B, commonly used as a marker for 3rd granules, denatures type IV, V and XI collagen and enhances the activity of MMP8. As in malignant cells, both gelatinases are essential in allowing neutrophils to invade solid tissues and extravasate from the circulatory system.
**Secretory vesicles**

Secretory vesicles are the most rapidly mobilizable intracellular structures, being mobilized by signals originating in the L-selectin-mediated rolling. They are of endocytic origin but are not re-formed once exocytosed. Secretory vesicles contain plasma and the complement receptor 1 or CD35, a membrane cofactor protein exclusively stored in these vesicles. CD35 binds to cleaved third (C3b) and/or fourth (C4b) components of the complement, promoting phagocytosis and secretion.

**Determinant factors for neutrophil sequestration in the lungs**

The very early events in ARDS include extensive sequestration of neutrophils within lung capillaries accompanied by a proportional reduction in circulating neutrophils. Fifteen minutes after many triggering stimuli, neutrophils already accumulate in the pulmonary microcirculation and this initial accumulation is followed by a decrease in PaO₂, increase in pulmonary artery pressure and protein-poor lymph flow.

Several studies suggest that changes in the biomechanical properties of neutrophils, particularly size, increased stiffness and decreased ability to deform are responsible for the early and initial sequestration within the lungs. Under normal conditions, circulating neutrophils are larger than most lung capillary segments, and have to contort and elongate in order to transit through the lungs. They move significantly slower than red blood cells, stopping at least once on the way. Small reductions in the cross section diameter of neutrophils result in shorter transit time and no measurable stops. In contrast, an increase in stiffness, as caused by increased content of polymerized actin, results in trapping of neutrophils within the capillaries.

Complement activation is believed to mediate the very early lung neutrophil sequestration and neutropenia. Fragments of activated complement induce actin polymerization and increase neutrophil stiffness. Infusion of complement fragments causes neutrophil lung sequestration and drastic neutropenia within 1/2 to 1 min. This effect is short-lived and independent of surface adhesion molecules. In fact, the early sequestration occurs independent of neutrophil adhesion molecules, including CD18.

Also participating in this process is the fact that after hemorrhagic shock there is a marked swelling of both endothelial and red blood cell, worsens neutrophil sequestration. There is also a massive release of immature and less flexible neutrophils by the bone marrow. Immature neutrophils are rapidly sequestered within the lungs, increasing the risk of neutrophil-mediated lung damage. Alternatively, neutrophil sequestration might follow an early activation of the endothelium by IL-8, TNF-α, IL-1 or IL-6 resulting from direct trauma to the lungs or their systemic release. In fact, P-selectin is able to initiate and maintain neutrophil-endothelial adhesion independent of neutrophil activation. When P-selectin was rapidly mobilized to the endothelial surface by thrombin and histamine in an experimental study, even formaldehyde fixed neutrophils adhered to the endothelium.
More recently Abraham et al proposed that subsets of neutrophils that accumulated in the lungs immediately after hemorrhage or endotoxemia, were responsible for the initiation of the inflammatory process. The rapidly sequestered neutrophils were the major source of lung cytokines and chemokines such as IL-1β, TNF-α and MIP-2, not the resident alveolar macrophages or vascular endothelium. Therefore, according to this study, neutrophils not only initiate but also enhance and perpetuate the inflammatory process in the lungs.

Maintenance of neutrophil sequestration requires adhesive interactions between cognate receptors on both neutrophil and endothelial cells. Both soluble or membrane-bound mediators such as IL-8 and PAF, or signals received through adhesion molecules cause the neutrophil to become activated. Activation leads to increased expression and avidity of β2 integrins resulting in firm adhesion and transendothelial migration. The role of the different adhesion molecules in neutrophil sequestration and transmigration in the lung capillaries is not fully known and is discussed next.

**Adhesion molecules: selectins**

Selectins constitute a group of three transmembrane glycoproteins, L-, E- and P-selectins. Their characteristic array of structural motifs include a calcium-dependent lectin domain at the amino terminal end, an epidermal growth factor (EGF)-like domain, a variable number of short consensus repeats (SCR), a transmembrane domain and a short cytoplasmic tail. Latin and EGF domains are the optimal recognition units for ligand interaction and SCR domains both stabilize the receptor structure and mediate signal transduction.

All selectins bind weakly to specific carbohydrate ligands: 1) oligosaccharides related to sialyl Lewis X (sLEX) and sialyl Lewis a, 2) phosphorylated mono and polysaccharides and 3) sulfated or phosphorylated polysaccharides and lipids. Specific high-affinity ligands for L-selectin include two sulfated glycoproteins, the 50 kDa glycosylation-dependent cell adhesion molecule-1 or GlyCAM-1 and the 90kDa Sgp90 or CD34. A third glycosylated mucin-like protein, the mucosal addressin cell adhesion molecule-1 or MadCAM-1 and a fourth sulfate-bearing high endothelial venule ligand for L-selectin have recently been described. Specific targeting of neutrophils to venules in inflamed tissue may involve L-selectin binding, via sLEX, to E- and P-selectin on endothelial cells as well as non sLEX-dependent interactions with other unknown receptors. Finally, other molecules also mediate leukocyte initial attachment and rolling under flow. The α4 integrins, were described as mediators of lymphocyte initial attachment and rolling on to VCAM-1 and Mad-CAM receptors present on high endothelial venules in Peyer’s patches.

The participation of the selectins in acute lung injury and ARDS is not fully understood. In the lungs, neutrophils emigrate from capillaries rather than postcapillary venules as in other organs, the capillaries are narrower than the neutrophils preventing rolling and the expression of endothelial ligands to selectins varies in different parts of the vascular bed. There is however, abundant proof...
indicating that the selectins have a crucial role in mediating accumulation of neutrophils in the lungs and subsequent damage. Soluble L-selectin levels are associated with the development and mortality of ARDS. The studies on the role of selectins in ALI/ARDS are summarized in Table 3 (page 38).

**L-selectin**

Leukocyte selectin is constitutively present and functional at high levels on neutrophils, most lymphocytes, monocytes, eosinophils, basophils and immature thymocytes. L-selectin is a highly glycosylated protein of 95-105 kDa on neutrophils and 74 kDa on lymphocytes. This difference is solely due to post-translational modifications since only a single L-selectin mRNA species has been identified. Kahn et al described a precursor form of L-selectin suggesting the possibility that at least some L-selectin can be replenished by de novo synthesis.

L-selectin is responsible for the initial attachment and rolling of leukocytes on to the endothelium and to each other, under conditions of flow. The result of these interactions is the localization and accumulation of neutrophil in inflamed or injured sites and lymphocyte migration into lymphoid tissues. Leukocyte adhesion via L-selectin depends on the association between cytoplasmic tail of L-selectin and actin cytoskeleton. Partial deletion of the cytoplasmic domain or treatment with cytochalasin B or D inhibited all adhesion via L-selectin. L-selectin is linked to the cytoskeleton through the actin-binding protein α-actinin, which in turn forms a complex with vinculin and possibly talin. The cytoplasmic domain is required neither for proper localization of L-selectin on the neutrophil surface nor ligand recognition.

L-selectin is rapidly shed by proteolytic cleavage following cell activation or rolling along vascular endothelium, as a prelude to migration into peripheral tissues. Endoproteolytic cleavage occurs between the last SCR domain and the membrane spanning region. Soluble L-selectin retains functional activity and regulates leukocyte attachment to cytokine-activated endothelium in a dose-dependent manner. Plasma levels of soluble L-selectin were significantly lower in patients that progressed to ARDS after multiple trauma, pancreatitis or perforated bowel than in normal volunteers, and considered to be of prognostic value. Other studies on L-selectin level after trauma report contradictory results. Among trauma patients progressing to SIRS, Ahmed & Christou reported elevated soluble and decreased surface expression of L-selectin. Maekawa et al found that both soluble and surface expression of L-selectin were elevated after major trauma (ISS>16) while Cocks et al reported that neutrophil L-selectin surface expression was elevated.

In lymphoblastoid transformed cell lines and Jurkat T cells, the cytoplasmic domain of L-selectin was phosphorylated by many different agents on serine residues or by cross linking on tyrosine residues. These reports suggest the possibility of L-selectin activity being regulated by phosphorylation. Phosphorylation enhanced L-selectin binding activity, but neither basal levels of adhesion nor proteolytic release of L-selectin was affected by it. The role of phosphorylation
remains mostly undetermined.

**E-selectin**

Endothelial leukocyte adhesion molecule (ELAM-1) is neither stored nor constitutively expressed but synthesized by vascular endothelium following stimulation by various cytokines including TNF-α, IL-1β and lipopolysaccharide. Surface expression on endothelial cells occurs 1 hour after stimulation with maximal expression between 4 to 8 hours, diminishing by 24 to 48 hours. It has a short half-life, is internalized in a temperature-dependent fashion and is rapidly degraded by lysosomes. A soluble form of E-selectin exists in the serum and is the consequence of both proteolytic cleavage and/or activation-related shedding. It is increased in various inflammatory conditions.

**P-selectin**

Platelet selectin is not constitutively expressed but is stored in Weibel-Palade bodies of both endothelial cells and platelets. It is rapidly mobilized to the cells surface minutes after stimulation with histamine, hydrogen peroxide and thrombin and has an important role in the early leukocyte adhesion to the endothelium at sites of inflammation. Its expression on endothelial cells is usually transient and downregulated by endocytosis. A functional and active soluble form of P-selectin is also found on the serum. P-selectin participates in both inflammation and thrombogenesis following tissue injury. Soluble levels of these receptors can be used as marker of the extent of endothelial injury. In a study of severely traumatized patients, levels of soluble E- and P-selectin were elevated during resuscitation especially among those in shock or later dying from infection or organ dysfunction.

**Experimental studies on the role of selectins**

Presently, most of the scientific evidence indicate that selectins mediate the initial attachment of leukocytes to endothelium, a necessary pre-requisite for subsequent integrin-dependent adhesion and transendothelial migration. However, the precise role of each selectin varies considerably in different models of inflammation or injury, in different organs and even in different vascular segments (i.e. venules and capillaries). The following is a compilation of the most relevant experimental studies published on the topic, with emphasis on neutrophil L-selectin. The largely discrepant results reported in these studies reflect the limited knowledge of the physiological role of the selectins.

The neutralization of L-selectin prior to physiological shedding that occur during interaction with vascular endothelium, by diverse manipulations significantly reduces leukocyte influx to site of acute inflammation or injury. Loss of L-selectin by FMLP or inhibition with anti-L-selectin antibody DREG56 lead to a 50-60% reduction of neutrophil adhesion to endothelial cells in vitro. In mice lacking L-selectin there was a reduction of neutrophil (56-62%), lymphocyte (70-75%) and monocyte (70-72%) migration into inflamed peritoneum up to 4 h and returning to baseline at a later time, reduced rolling and capture of neutrophils after trauma and TNF-α treatment, slower rejection of allogeneic skin grafts and resistance to death resulting from LPS-induced toxic shock. In male
Baboons inflicted with hemorrhagic shock and complement activation by cobra venom factor, a single infusion of anti-L-selectin antibody DREG55 reduced overall mortality, decreased organ damage, improved hemodynamic parameters and peripheral perfusion while decreasing fluid requirements. Challenging these results, L-selectin inactivation with sulfo Lewis C resulted in worsening of LPS-induced lung injury in pigs, with higher degree of hypoxemia and increased lung accumulation of neutrophils. L-selectin deficient mice studies also established that L-selectin mediates neutrophil sequestration caused by prolonged (i.e. over 5 min) injection of complement fragments, *Escherichia coli* and *Streptococcus pneumoniae* pneumonia.

All three selectins have overlapping and unique functions. After trauma, leukocyte rolling over post capillary venules is initially, up to 40-60 min, P-selectin dependent, and only then, L-selectin dependent. In this scenario however, L-selectin was the determinant factor in leukocyte localization at sites of inflammation while P- and E-selectins played complementary roles. A series of studies on the role of selectins in leukocyte recruitment were performed using null mutated mice at L-, E- and P-selectin locus and summarized by Jung et al. Importantly, E/P selectin double mutant mice had marked neutrophilia, high levels of circulating cytokines, severely impaired acute neutrophil recruitment and increased neutrophil margination in the lungs. These animals, as well as those with simultaneous inhibition of L- and P-selectin by fucoidin, showed increased neutrophil lung emigration, lung edema and histological evidence of lung injury following intra-tracheal instillation of *Streptococcus pneumoniae*. Rolling was drastically reduced in both single and double mutant mice, but only triple mutant mice showed no tissue recruitment under all conditions, suggesting that any selectin can support neutrophil recruitment. In humans, the lack of functional selectins was described in two unrelated human disorders of the fucose metabolism. In both conditions, the neutrophils do not express sLEx and do not adhere or roll on venules. These patients suffer recurrent bacterial infections in addition to being short in stature and mentally retarded.

Many anti-inflammatory drugs function by blocking or inducing shedding of L-selectin, and consequently reducing neutrophil recruitment into inflamed tissues *in vivo*. Some of these drugs include diclofenate, aceclofenac, indomethacin, aspirin and leumedics but not dexamethasone and piroxicam.
Table 3. Role of selectins in diverse experimental models of ALI

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<tr>
<th>Neutrophil-dependent</th>
<th>Dependent</th>
<th>Independent</th>
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<tbody>
<tr>
<td>IV <em>Pseudomonas aeruginosa</em></td>
<td>E-selectin</td>
<td>E-selectin</td>
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<td></td>
<td>L-selectin</td>
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<tr>
<td>Complement activation with cobra venom factor</td>
<td>P-selectin</td>
<td>E-selectin</td>
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<td></td>
<td>L-selectin (&gt;5min)</td>
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<tr>
<td>IT IgG immune complexes</td>
<td>E-selectin</td>
<td>P-selectin</td>
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<td></td>
<td>L-selectin</td>
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<tr>
<td>Hind limb ischemia-reperfusion</td>
<td>E-selectin</td>
<td>P-selectin</td>
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<tr>
<td>IT <em>Streptococcus pneumoniae</em></td>
<td>L-selectin</td>
<td></td>
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<tr>
<td>IV LPS</td>
<td>L-selectin</td>
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**Neutrophil-independent**

| IT IgA immune complexes | L-, P- and E-selectin |

IV = intravenous, IT = intratracheal

**L-selectin shedding**

L-selectin, like FcγRIIIb, CD43, CD44 is rapidly cleaved from the cell membrane by an unknown metalloprotease(s) \(231^{242}\). This distinct metalloprotease(s), also referred to as sheddase, appears to be membrane bound, constitutively active and regulated by tertiary conformational changes of L-selectin \(243^{245}\). The sheddase cleaves L-selectin between the second SCR and the transmembrane domain, between Lys\(^{283}\)-Ser\(^{284}\). The length of this putative region, but not its amino acid sequence, is critical for the sheddase activity \(224\). The sheddase activity is resistant to cysteine/serine protease inhibitors and to a wide range of metalloprotease inhibitors including gelatinases A and B inhibitors \(224^{244}\). Two hydroxamic acid-based inhibitors of zinc-dependent metalloprotease KD-IX-73-4 (N\(_L\)-(2-(hydroxyamino-carbonyl) methyl)-4-methylpentanoyl) \(\Psi\)-3-(2'-naphthyl)-alanyl\(_L\)-alanine amide) \(231\) and Ro 31-9790 (N-2-((2S)-(hydroxycarbamoyl)methyl))-4-methylvaleryl]-N-1,3-dimethyl \(\Psi\)-valinamide were recently reported as potent inhibitors of L-selectin shedding \(219^{244}\). Auto proteolysis was also suggested, but since L-selectin lacks any obvious homology to known proteases, but this is an unlikely possibility. Soluble L-selectin might have a role in preventing leukocyte binding to endothelial cells and
downregulate the leukocyte-mediated inflammatory response.

L-selectin is shed following receptor occupancy, cross linking or cell activation. Neutrophils also shed L-selectin as they age in the circulation, and conversely, L-selectin shedding makes neutrophils more susceptible to apoptosis. The signaling pathways underlying the shedding of L-selectin remain mostly undetermined. One of the few studies on this topic reported that in lymphocytes, Leu-13 surface receptor occupancy or L-selectin crosslinking caused shedding in a tyrosine kinase-dependent and PKC-independent way. In contrast, PMA or CD3-mediated shedding were tyrosine kinase-independent and PKC-dependent indicating that shedding is a complex process triggered by multiple intracellular signaling pathways. In a 1999 study, Middlehoven et al reported that in neutrophils, jasplakinolide-induced actin polymerization resulted in pronounced shedding of L-selectin, implying the involvement of the cytoskeleton in this process. Interestingly, neither cytoskeletal disassembly with cytochalasin B nor PTK inhibition with herbimycin A or PP1 prevented the jasplakinolide-induced shedding of L-selectin.

Most of the available literature support the concept that the physiological role of L-selectin shedding is in regulating the rolling velocity of leukocytes and limiting leukocyte aggregation and accumulation at sites of inflammation. This argument however, is not widely accepted. Allport et al reported that inhibition of L-selectin shedding resulted in no significant change in the rate of neutrophil attachment, rolling velocity, stable adhesion and transmigration, both under flow and static conditions. Thus, a complete understanding of the process of shedding as well as its physiological significance remains to be determined.

**Adhesion molecules: integrins**

Integrins constitute a family of heterodimeric transmembrane receptors composed of two gene products, α and β chains, linked within the endoplasmic reticulum in a very stable but noncovalent structure. Both chains are required for normal receptor expression and ligand binding. This is a growing family with at least 14 α and 8 β known chains. β chains may combine with more than one α chain, resulting in a variety of heterodimers with many different functions able to recognize a multitude of ligands. A common classification separates this superfamily according to the β chain. Therefore, heterodimers containing β1 chains are known as VLA or very late activation antigen family, β2 chains are LeuCAMs or leukocyte cell adhesion molecules, which are exclusively expressed on leukocytes and β3 chains are known as cytoadhesins.

Neutrophils express VLA and cytoadhesion integrins at a very low level and their importance in neutrophil activation and inflammatory remains equivocal. The predominant integrins in all leukocytes are those from the β2 family. There are four β2 integrins: lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18 or αLβ2), MAC-1 (CD11b/CD18 or αMβ2) and glycoprotein (gp) 150,95 (CD11c/CD18 or αxβ2) and the novel CD11d/CD18 (αDβ2) constitutively expressed with CD11b/CD18
in synovial macrophages. CD11a/CD18 is primarily expressed on lymphoid cells, but to some extent in all leukocytes, whereas CD11c/CD18 and CD11d/CD18 are most strongly expressed on monocytes and macrophages. They may partially overlap in function and no human disorder has been described with defects in individual α chains.

The most abundant of the β2 integrins in neutrophils is CD11b/CD18, also referred to as complement receptor 3 (CR3). CD11b/CD18 is essential for adherence to endothelium, chemotaxis, diapedesis and phagocytosis of complement-opsonized particles. It binds to ICAM-1, -2 and -3 expressed on many different cells. CD11c/CD18 binds to iC3b and probably to ICAM1, it is also called complement receptor 4 (CR). The leukocyte adhesion deficit syndrome (LAD) is a congenital disorder that occurs in patients with β2 integrins deficit due to mutations in the common β2 chains. It is clinically characterized by recurrent infections, elevated levels of circulating neutrophils, failure to involute the umbilical stump and death often at an early age. Neutrophils isolated from these patients do not respond normally to chemoattractant and are unable to emigration into skin windows, skin chambers, peritoneal, laryngeal, esophageal, periodontal, gingival, dermal or umbilical tissues.

Leukocyte integrins, constitutively present on the cell surface, are not constitutively active and require activation in order to become adhesive. Mechanisms of integrin activation are still poorly understood, but are believed to be the result of three distinct modes. First insertion of pre-formed integrins stored in cytoplasmic granules, onto the cell surface by exocytosis. Secondly, by rapid change in individual integrin ligand affinity, which involves conformational changes in their extracellular domain. Thirdly by increased avidity, accomplished by clustering integrins thus allowing more efficient multivalent interaction and increased binding.

It is well established that the β2 integrins are responsible for immobilizing the neutrophils on the endothelial surface and the subsequent emigration from the postcapillary venules into inflamed or injured tissues. However in the lungs, neutrophils leave the circulation through capillaries and may or may not require the involvement of these integrins. Doerschuk et al have extensively investigated the role of β2 integrins in lung inflammation, by using blocking antibodies and mutant mice deficient in CD18 (CD18). According to their and other authors' work, neutrophil emigration is CD11/CD18-dependent when inflammation is caused by Escherichia coli, Pseudomonas aeruginosa, E. coli lipopolysaccharide, IgG immune complexes, IL-1β and PMA. It is CD11/CD18-independent when is caused by Streptococcus pneumoniae, group B streptococcus, Staphylococcus aureus, hydrochloric acid or hyperoxia. Possible explanation for the discrepancies includes the differential ICAM-1 endothelial expression. ICAM-1 expression increases after E. coli or P. aeruginosa challenge but not after S. pneumoniae, possibly determining whether a CD18 dependent or independent pathway should be utilized. Another important concept is that there is always a component of CD11/CD18-independent emigration, estimated to be of about 15 to 30% of the total. In trauma patients and after major elective
surgery, both CD11b/CD18 and ICAM-1 expression are consistently reported to increase. The concept of reducing neutrophil-mediated organ damage by interventions that block CD11b is presently being tested in North America in a large multicenter study. Patients suffering severe trauma are treated with anti-CD11b antibody. The results of this study might establish the clinical utility of directly inhibiting adhesion molecules in reducing organ damage that follows hemorrhage and severe injury.

**Sequence of events in neutrophil extravasation**

Having presented the individual participants, we will next present the classical description of the complex cascade of events and interactions involved in the process of leukocyte extravasation into inflamed or injured tissues. P-selectin is quickly released from vesicles to endothelial surface seconds after an inflammatory stimulus. Passing neutrophils adhere weakly to the endothelium, they are slowed but not stopped, seeming to roll along the endothelial surface in a process involving L-selectin. Neutrophil β2 integrin molecules are activated by PAF released by activated endothelial cells and inflammatory mediators such as IL8, C5a, thrombin, TNF-α and LTB4 as well as by L-selectin-mediated signals and other unknown mechanisms. Activated β2 integrins then bind to constitutively expressed ICAM-1, ICAM-2 and fibrinogen, forming a tight adhesive bond between neutrophil and endothelium, completely stopping the cell that then spreads over the endothelium and move between adjacent endothelial cells into the underlying tissues.

In ARDS, 30 minutes after the initial injury, neutrophils are already migrating into the interstitium and interstitial edema develops. One hour after most triggering injuries there is clear evidence of endothelial and alveolar damage and from 2 hours onwards, increasing progressive disruption of the endothelial layer results in increasing edema and perfusion/ventilation mismatch manifested by worsening hypoxemia, hypercapnia and lung compliance.

Adhered neutrophils respond to inflammatory mediators by releasing ROS and granule constituents, which play a central role in inflammatory damage caused by neutrophils. These responses are tightly coupled with cytoskeletal rearrangement that dictates spreading over the adhesive surface, involving mostly β2 integrins and diverse non receptor PTK, which are discussed later in Chapter 4.

**Exocytosis of granules in neutrophils**

Neutrophils have unique characteristics in the conditions required for exocytosis of different granule populations. First, granules are released according to a strict hierarchy both in vivo and in vitro indicating that differences in mobilization of subsets are due to quantitative differences and not to qualitative differences in the machinery controlling exocytosis. Secretory vesicles are always the first to be released, followed by gelatinase whereas specific granule release occurs considerably slower. The intensity of the stimulus required for exocytosis also follows this hierarchical sequence. Small increases in \([Ca^{2+}]\), by 40 to 50 nM, result in release of proteins from secretory vesicles but only higher values will induce gelatinase granules exocytosis. Specific granules require \([Ca^{2+}]\), of approximately 1
μM whereas the release of primary granules require extreme values 258-260.

Second, different stimuli mobilize different amounts of the same type of granules. FMLP causes 100% release of secretory granules but only 15 to 25% of gelatinase and less than 3% of 257 granules while PMA causes almost 100% release from all three granules 258. Considering that an unstimulated neutrophils, 5% of all CD11b/CD18 is in the plasma membrane, 75% is within 257 and gelatinase granules and 20% within secretory vesicles 261, PMA stimulation results in a 2 to 3 times higher increase in surface membrane content than FMLP 191.

Third, intracellular calcium release is not an essential signal initiating exocytosis in neutrophils and other mechanisms do exist, increasing the cell flexibility to respond to different stimuli 188. No exocytosis occurs in neutrophils suspended in Ca\(^{2+}\)-free medium and stripped of calcium by millimolar EGTA treatment and loaded with large doses of Ca\(^{2+}\) chelators. However, a prominent exocytosis occur following stimulation by these same agonists even when they are unable to alter [Ca\(^{2+}\)]\(_i\) 262. Therefore, [Ca\(^{2+}\)]\(_i\), transient rise are necessary but not sufficient for degranulation or ROS generation by FMLP, PAF and LTB4 262. FMLP, PMA, LPS and activation of GTP-binding proteins by intracellular perfusion with GTP\_S all are able to induce exocytosis independent of [Ca\(^{2+}\)]\(_i\) 263.

Transduction signaling pathways underlying exocytosis

The pathways signaling between the various membrane receptors to the exocytic machine are presently unknown. The role of the small GTP-binding proteins and the cytoskeleton are discussed in details in another part of this dissertation. Recent data suggests that PTK participate in the organization of the exocytic response. In neutrophils, PTK are activated by various receptor agonists and broad-spectrum inhibition of PTK results in inhibition of primary and secondary granule exocytosis 264. However, such broad spectrum inhibition of PTK failed to suppress PMA-induced degranulation 265.

Specific neutrophil PTK implicated in exocytosis include MAPK, Src-family kinases and Syk with has distinct role in FceRI-mediated secretion in basophils and mast cells 257, 266-268. Chemotactic-induced exocytosis was not prevented by MEK1/Erk 264, 269, and only partially by p38 inhibition 260, 269. In Fgr "". Hck "" double knockout mice and PPI-treated neutrophils, exocytosis induced by spreading was defective, however induction by phorbol ester or ionomycin were not 257, 260. These results identify a role for Fgr and Hck as well as PKC, in signaling to granule-plasma membrane fusion 257, 260. However, selective depletion of the predominant isoform β-PKC in HL-60 cells or inhibition of PKC by sphingosine abolished the PMA but not the FMLP- or A23187-induced primary and specific granule exocytosis 270, 271. The combination of all the data presented in this section indicate the participation of PTK and PKC in neutrophil degranulation, however, neither PTK nor PKC are part of the final common pathway of regulated exocytosis.

The SNAP-SNARE theory

A widely conserved, universal molecular mechanism of exocytosis has been proposed the
SNARE hypothesis \textsuperscript{272}. Integral membrane proteins both in the vesicular (SNAP-receptors) and target membranes (v- and t-SNAREs) are able to interact with each other allowing the correct docking of the vesicle to be fused \textsuperscript{272}. Different sets of v- and t-SNARE proteins function at various fusion steps. Additional proteins, such as Rab family of small GTP-binding proteins and Sec1 family, are responsible for supervising the specificity of docking \textsuperscript{273}. Other ubiquitous cytosolic proteins also participate in efficient fusion: NSF or N-ethylmaleimide-sensitive fusion protein and the soluble NSF-attachment proteins: \(\alpha\), \(\beta\) and \(\gamma\) or SNAPs. According to this hypothesis, fusion is mediated by the combined action of NSF and the attachment protein for NSF, the SNAPS in further combination with \(Ca^{2+}\), synaptotagmin and other membrane proteins. NSF is an ATPase that binds firmly to SNAPs attached to SNARE proteins but not to soluble SNAPs. ATP hydrolysis is required for the dissociation of the fusion complex and the preparation of SNARE proteins for the next round of membrane fusion \textsuperscript{260}. It is currently believed that membrane fusion occurs by the same mechanism from yeast to man and from the endoplasmic reticulum to the plasma membrane. Ligeti \& Mocsai have recently questioned this concept in a review of the considerable differences in exocytic mechanisms between neural secretory cells and granulocytes \textsuperscript{260}.

The arguments of these authors are supported by Brumell et al that recently demonstrated that the neural-type SNARE proteins syntaxin 1, VAMP-1 and SNAP-25 are not detected in circulating neutrophils \textsuperscript{274}. Even though more recent studies have identified homologs of the neural SNARE proteins in neutrophils, no data is available on the existence of other core proteins such as NSF, \(\alpha\)-, \(\beta\)- or \(\gamma\)-SNAP, the neural \(Ca^{2+}\)-sensor synaptogmin or the participating Rab proteins. Presently, neither neutrophils nor other hematopoietic cells have a whole set of proteins that according to the SNARE hypothesis are required for granule release as described for neuroendocrine tissues \textsuperscript{260}. The determination of this process as responsible for exocytosis in neutrophils remains to be proven.

\textbf{Respiratory burst}

Oxygen has two unpaired electrons with parallel spins, it is a biradical. To overcome spin restriction, oxygen prefers to accept electrons, one at a time, and the sequential addition of electrons lead to the formation of highly reactive oxygen species (ROS) including superoxide anion (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), hydroxyl radical (OH) and products of myeloperoxidase \textsuperscript{275}. ARDS patients have increased production of oxidants in the lungs as indicated by increased concentration of \(H_2O_2\) in expired air, a deficiency in alveolar epithelial lining fluid glutathione with a greater percentage in the oxidize form and potentially toxic levels of peroxynitrate (reviewed in \textsuperscript{275}). In addition, F2 isoprostanes, markers of nonenzymatic lipid oxidation, are notably elevated in ARDS patients \textsuperscript{275}.

ROS cause lung dysfunction by diverse mechanisms. ROS interact with many cellular components, oxidizing proteins, lipids, DNA bases, enzymes for intermediate metabolism and extracellular matrix components including collagen and hyaluronic acid. Depending on the extent of
oxidant stress, cells exposed to ROS will undergo apoptosis or necrosis. In endothelial and epithelial cells, oxidant injury may also impair macromolecular barrier function leading to pulmonary edema. Other possibilities include ROS changing the redox state of ABP and actin itself thus inducing actin polymerization, and ROS functioning as second messengers within cells and initiating production of potent chemotaxins such as IL8, adhesion to the endothelium via activation of NF-κB mediated transcription of integrin genes. Thus, localized production of ROS may initiate a cascade that culminates in a fulminating inflammatory response, tissue destruction and organ malfunction. ROS can be generated by non-phagocytic sources within the lungs like the molybdenum hydroxylases such as xanthine dehydrogenase oxidase and aldehyde oxidase, the reduced form of NADPH oxidoreductases, mitochondrial electron transport chain and arachidonic acid metabolizing enzymes such as cyclooxygenase. The best studied source for ROS in the lung and the focus of this discussion is NADPH oxidase of phagocytic cells such as neutrophils.

The NADPH oxidase of phagocytes is a multisubunit complex that generates \( \text{O}_2^\cdot \) in one-electron reduction of \( \text{O}_2 \) using electrons supplied by NADPH. The oxidase consists of two membrane proteins gp91 and p22 that together form a unique cytochrome and several cytosolic components, including p47 and p67 which are essential, the small GTP-binding protein Rac2 (in humans) and p40phox. The oxidase is regulated by inflammatory cytokines such as TNF-α, IL-1β and LPS. Assembly of the cytosolic proteins with membrane components activates the oxidase. The active enzyme complex is membrane-associated and interacts with NADPH on one side of the membrane and \( \text{O}_2 \) on the other.

The NADPH oxidase is regulated by a large number of different factors. The actin cytoskeleton closely regulates this metabolic machinery, even though dissociation of these two processes is also known as in patients with neutrophil actin dysfunction syndrome that have normal oxidant generation. In unstimulated neutrophils, both p67 and p40 are associated to the triton-insoluble cytoskeleton whereas p47 is mainly found on the soluble fraction. Disassembly of the cytoskeleton results in the well-known enhancement of the respiratory burst triggered by chemoattractants. Furthermore, ROS generation is enhanced by adhesion and is defective when adhesion is prevented. Exocytosis is also an important regulator of this process since most proteins of the NADPH oxidase are stored in intracellular granules. Finally, heritable defects of gp91, p22, p47 or p67 are the basis for chronic granulomatous disease, a disorder of white blood cell function characterized by recurrent, severe bacterial and fungal infections.
Section IV. Signal Transduction Pathways in Neutrophils

Objectives

The objective of this section is to review current and relevant literature on the complex and rapidly evolving field of cell signaling transduction pathways. We will focus on the signaling pathways mediated by two major superfamily of kinases, MAPK and small Rho GTP-binding proteins, due to their importance in mediating neutrophil functions affected by osmotic stress. The following are the most relevant issues:

1. Cells have four different types of surface receptors, G protein coupled receptors are of major importance in neutrophil inflammatory response;
2. MAPK pathway is present in all eukaryotic cells, and is composed of at least 3 families with dissimilar functions, Erk and the stress-activated kinases p38 and JNK;
3. MAPK p38 is activated by inflammatory and stress signals, intermediates almost all TNF-α-, FMLP-, LPS- and TGF-β1-induced cellular responses and is important in neutrophil respiratory burst, chemotaxis, adhesion and apoptosis;
4. Small Rho GTP-binding proteins intermediate a myriad of cell functions and are the main regulators of actin cytoskeleton organization in eukaryotic cells;
5. Rac and Cdc42 mediate diverse stress responses and appear to be upstream activators of p38/JNK;
6. L-selectin binding triggers diverse biochemical changes resulting in respiratory burst, increased actin polymerization, exocytosis and activity of β2 integrins CD11b/CD18;
7. Integrin occupancy triggers diverse signaling pathways, resulting in neutrophil activation and migration into tissues;
8. A plethora of signaling intermediates have been recently described, including the non-receptor protein tyrosine kinases, which appear to be of major importance for neutrophil inflammatory response.

Introduction

It is important to review some basic aspects of signal transduction in order to understand the behavior of the neutrophils in conditions such as ARDS or the changes caused by exposure to hypertonicity. Many cellular responses to a particular extracellular stimulus result from the binding of a signaling molecule or ligand to a specific receptor protein on the cell surface. This binding leads to a conformational change of the receptor that initiates a sequence of biochemical reactions, collectively referred to as signal transduction, resulting in cellular changes. Other molecules are able to start this biochemical cascade directly, without the intermediation of surface receptors.

This chapter has been divided into 5 segments, a brief review of surface receptors, the MAPK pathway, the small GTP-binding proteins, the signaling events generated by surface adhesion molecules,
and signaling intermediates and protein kinase inhibitors of relevance to the studies presented in the second part of this dissertation.

Receptors

There are basically 4 types of cell surface receptors: ion channel receptors, G protein-linked receptors, receptors lacking intrinsic catalytic activity and receptors with intrinsic catalytic activity. The G protein-linked receptors are by far the most important and well-studied receptors in neutrophil chemotaxis, phagocytosis and oxidative metabolism.

G protein-linked receptors or chemokine receptors

Neutrophils respond to a variety of chemoattractants including formyl peptides, C5a, PAF, LTB4 and IL-8. Receptors for chemoattractants have been cloned and identified as having seven transmembrane spanning domains and being coupled to heterotrimeric G-proteins. Receptor occupancy results in dissociation of the G-protein subunits that in turn activates adenylate cyclase, PLC and other downstream effectors. PLC hydrolyzes PIP2 to DAG and PIP3. G proteins are constituted by three subunits designated α, β and γ. When no ligand is bound to the receptor, the α subunit (or Gαs) is bound to GDP and complexed with β and γ subunits (or Gβγ). Agonist coupling induces receptor conformational changes, causing it to bind to trimeric G protein and GTP replaces GDP. The α-GTP complex then dissociates from the β and γ subunits and subsequently binds and activates a second messenger-generating systems. Once GTP is hydrolyzed, the complex resumes its initial inactive form. There is another separate class of G proteins that is also involved in chemoattractant signaling, the monomeric or small G proteins, which will be reviewed later in this chapter.

Receptors directly linked to tyrosine kinases but with no intrinsic enzymatic activity

Ligand occupancy of these receptors, which have no intrinsic catalytic activity, causes receptor monomers to dimerize triggering the binding and activation of one or more cytosolic protein tyrosine kinases. Neutrophil recognizes and binds to opsonized invading microorganisms through its Fc receptors. Neutrophils express two receptors for IgG: FcγRIIa (CD32) and FcγRIIIb (CD16). These receptors are involved in signal transduction through the involvement of several families of non-receptor tyrosine kinases, leading to remodeling of the actin cytoskeleton, phagocytosis and exocytosis. FcγRIIIb also associates with Mac-1 (CD11b/CD18) on the neutrophil surface, thus cooperating in the generation of respiratory burst and phagocytosis. Fc receptors are shed from the neutrophil surface by proteolytic cleavage, following stimulation, apoptosis, chemotaxis or enhanced actin cytoskeleton polymerization.

Receptors with intrinsic enzymatic activity or receptor tyrosine kinase (RTK)

The binding of ligands to these receptors causes most of these receptors to dimerize, the protein kinase of each receptor then phosphorylates a distinct set of tyrosine residues in the cytosolic domain of its dimer partner, a process called autophosphorylation. Activated RTK subsequently stimulates signal
transduction cascades affecting a wide spectrum of functions including regulation of cell proliferation and differentiation and adjustments in cell metabolism. Receptors for insulin and many growth factors are examples of RTK.256,282

From chemoattractant receptors to MAPK

The major pathway leading to tyrosine kinase activation in neutrophils is triggered by occupancy of the heterotrimeric G protein-linked receptors that then activate non-receptor protein tyrosine kinases (PTK) through not yet completely elucidated pathways. The level of second messengers cAMP, PIP3, DAG, Ca^{2+}, phosphatidic acid and arachidonic acid increase dramatically in response to chemoattractant-receptor binding. The role of these second messengers in modulating neutrophil function is presented throughout this dissertation.

The non-receptor PTK bind to different adapter proteins that couple these kinases with signaling molecules but have no intrinsic signaling properties. Adapter proteins such as Grb2, bind to different phosphotyrosine residues via conserved polypeptide domains called Src homology 2 (SH2) and Src homology 3 (SH3) domains. These domains are homologous to the prototypical cytosolic tyrosine kinase domain encoded by Src. Grb2 has one SH2 domain and two SH3 domains which are present in a large number of signaling molecules. While the SH2 domains of Grb2 binds to tyrosine-phosphorylated residues of either non-receptor PTK or RTK, its SH3 domains bind to cytosolic Sos, a protein with guanine nucleotide-exchange activity (GEF activity). Sos binds to Ras-GDP leading to the release of GDP, and binding of GTP generating active Ras-GTP form. Activated Ras then binds to Raf (or MEKK), a serine/threonine kinase, which in turn binds and phosphorylates MEK1 and MEK2, dual-specificity protein kinases that phosphorylate on both tyrosine and threonine residues. MEKs phosphorylate and activate MAPK Erk 1 (p42) and Erk2 (p44), kinases that phosphorylate many different proteins including transcription factors that regulate many specific neutrophil function.282,283
Mitogen-activated protein kinase pathway

In all eukaryotic cells, extracellular signals activate a highly conserved cascade of three families of mitogen-activated protein kinases (MAPK). The extracellular signal-regulated kinases Erk 1 and 2, and the stress-activated kinases JNK/SAPK and p38. These MAPK families share high sequence homology, similar structures and conformations, all appear to be activated by PTK cascades initiated by small GTP-binding proteins in the Ras superfamily and all require dual phosphorylation in threonine and tyrosine residues. In contrast, each family is activated by different stimuli, has different substrates and their activation leads to different cellular responses, Erks to growth and differentiation, while in certain cells JNK and p38 inhibit cell growth and promote apoptosis.

MAPK Erk

The extracellular signal-regulated kinases (Erk) are activated in response to growth factors and the best-characterized cascade of activation is the one regulated by Ras, as described above. However, many cell surface receptors utilize the MAPK Erk cascade in their repertoire of signal transduction mechanism. This fact can be verified in neutrophils where a variety of soluble agonists, including FMLP, PAF and phorbol myristate acetate or PMA, activate Erk. These molecules are capable of activating Erk.
pathway by PKC, both in a Ras-dependent and –independent manner. One important and specific MAPK Erk pathway inhibitor has been recently described, 2'-amino-3'methoxyflavone or PD98059. In fact PD98059 is a specific inhibitor for MEK1, which is upstream to Erk1 and 2. In neutrophils, PD98059 inhibits FMLP-induced oxidative burst, reduces phagocytosis, reverts the GM-CSF-induced delay in apoptosis but has minimal effects on adhesion, exocytosis or activation of phospholipase A2. These results have helped to clarify the role of Erk in many neutrophil functions.

**MAPK p38**

Efforts to uncover signaling mechanisms activated by inflammatory and environmental stress lead to the identification of MAPK p38 in *Saccharomyces cerevisiae*. The first description was that p38 became phosphorylated in response to endotoxin and hyperosmolarity, and was termed HOG1 or high osmolarity glycerol response. Subsequently, p38 homologs were described in a wide variety of tissues, including human, suggesting that this family of kinases have an important function in cellular response. TNF-α, IL-1, endotoxin, arachidonic acid and diverse environmental stresses including hyperosmolarity and UV radiation activate p38 in many cell types. Recent studies suggest that p38 might also be activated by the small GTPases CDC42 or Rac, a possibility discussed in details in the following section.

At least three dual specific kinases MEK3, MEK4 and MEK6 are able to phosphorylate p38, and currently 4 isoforms have been identified, namely: p38α (the original described human homolog to HOG1), p38β which is 74% identical to p38α; p38γ (or Mxi2, Erk6) which is 63% identical to p38α and p38δ (SAPK) which is 60% identical. So far, only p38α (or HOG1) and p38δ were detected in neutrophils. These isoforms phosphorylate transcription factors including MAPKAP-K2, MAPKAP-K3 and heat shock protein 27 (HSP27). The isoform p38α specifically activates ATF-2 (activated transcription factor-2), serum response factor accessory protein-1 and myocyte enhancer factor 2C. It is not clear whether these p38 homologs are regulated like p38 itself.

**Functional consequences of p38 inhibition**

The pyridinil imidazoles SB203580 and SK&F86002 have recently been described as highly specific inhibitors of p38 activity. They exert negligible inhibitory effects on other important mammalian kinases and phosphatases, including Erks or JNK, even though extreme high concentrations might affect some isoforms. Many recent studies used these inhibitors to elucidate the functional effects of p38, which has long been implicated in the regulation of apoptosis, growth arrest and activation of immune and reticuloendothelial cells.

Environmental stress activates both JNK and p38, however, the anti-inflammatory effects of SB203580 and SK&F86002 indicate that p38 and not JNK, has primary importance in mediating inflammatory signals. In human neutrophils, the pyridinil imidazole inhibitors significantly impair the generation of reactive oxygen species (this inhibition varies according to different stimuli), chemotaxis,
adhesion and stress-induced apoptosis. In monocytes they inhibit the synthesis of IL-1, IL-8 and TNF-α, as well as platelet aggregation and secretion, and in L929 cells the TNF α-induced IL-6 mRNA expression. Studies with SB203580 on neutrophils have strongly implicated p38 as a common denominator in the TNF α-, LPS- and many FMLP-mediated cellular functions, but not in those functions induced by PMA or ionomycin. Recently, Hannigan et al used SB203580 to inhibit p38 activation by the chemoattractant TGF-β1. They reported that preventing p38 activation resulted in inhibition of the neutrophil chemotactic response and actin polymerization, suggesting that p38 might also have a role in chemoattractant-induced remodeling of the neutrophil cytoskeleton.

**MAPK JNK/SAPK**

The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is a family of MAPK formed by 3 subfamilies and at least 10 isoforms, 4 isoforms of JNK1, 4 isoforms of JNK2 and 2 isoforms of JNK3, all expressed as either 46 kDa or 55 kDa proteins. They are activated by environmental stresses such as anisoosmolarity, cytokines, UV radiation, hypoxia-reoxygenation, reactive oxygen species and DNA-damaging agents. Calcium activates JNK through Pyk2 and calmodulin-dependent kinases while hyperosmolarity and UV radiation by cell surface receptor clustering, a hypothesis still not proven. JNK regulates a wide range of dissimilar functions including cell proliferation, apoptosis, growth arrest and tissue morphogenesis. JNK is also required for the induction of E-selectin in endothelial cells activated by inflammatory cytokines such as TNF-α. This important step for leukocyte infiltration at sites of inflammation requires concomitant c-Jun activation by JNK and ATF2 activation by p38.

JNK is activated by two MAP kinase kinases, MEK 4 and 7, and inactivated by serine/threonine and tyrosine phosphatases. MEK 4 is also known as the SAPK/ERK kinase 1 or SEK1, which also activates p38. MEKK for JNK pathway have been recently described and include MEKK1, 2, 3, 4, 5, MUK/DLK, MLK3/SPRK, MST/MLK2, TAK1 and Tpl-1/2. Other kinases such as p21-activated kinase (PAK) and germinal center kinase (GCK) activate JNK and are discussed next. JNK phosphorylates the transcriptional factors ATF2 and ATF3, c-Jun and Jun D, Elk-1 and Sap-1.

**From the small GTPases to p38/JNK**

Recent studies have reported that the Rho GTPases Rac1 and Cdc42 could activate both p38 and JNK, suggesting an analogous role to that played by Ras in the MAPK Erk cascade. It was shown that Rac1 and Cdc42 activated JNK and p38 upon cotransfection. However, the exact role of the Rho GTPases in the activation of these kinases is far from clear.

The first element implicated in linking Rac1/Cdc42 to p38/JNK is the p21-activated kinase or PAK. PAKs are 60 to 70 kDa proteins whose C-terminal catalytic domain is 60-70% identical to the Saccharomyces cerevisiae Ste20p. Three isoforms have been described, PAK1 or α, PAK2 or γ and
PAK3 or β that are directly activated upon interaction with the GTP-bound form of Rac/Cdc42. In neutrophils, phorbol esters (PMA), chemoattractant receptors, genistein-sensitive tyrosine kinases and PIP3K activate PAK. Recently, Clerk & Sugden reported that hyperosmotic shock rapidly activated PAK1 in ventricular myocytes, suggesting the possibility that the osmotic activation of p38 might be mediated by PAK.

There are many studies implicating PAK as a potential mediator of the Rac/Cdc42-induced p38/JNK activation. It has been reported that over expression of wild type PAK1 activates p38 kinase activity and over expression of a dominant negative PAK1 mutant as well as a dominant negative Cdc42 mutant protein inhibited the IL-1dependent activation of p38 in HeLa cells. Inactive PAK fragments inhibit the EGF activation of JNK and IL-1β activation of p38. In COS cells, constitutively activate PAK3 and PAK1 led to a modest to substantial induction of JNK in vitro kinase activity. PAK transfection into human kidney fibroblasts also led to the activation of JNK and p38. However, it is still not resolved whether PAK is a physiological regulator of this pathway and the identity of the PAK substrate linking these kinases to JNK/p38 is not known. Loop mutations of Cdc42 and Rac have questioned the role of both PAK and another potential intermediate, the mixed-lineage kinases (MLK) as immediate downstream targets for these GTPases since Rac and Cdc42 mutants unable to bind PAK or MLK, still effectively stimulate JNK.

A novel Rac target POSH, or plenty of SH3 domains, might be the link, but it lacks a kinase domain and the mechanism by which it stimulates JNK remains unknown. Another possibility is MEKK4, which both binds to Rac and Cdc42 and enhances signals toward JNK, but probably not p38. However, a dominant negative kinase defective MEKK4 did not affect the PAK-dependent induction of JNK activity in COS cells, suggesting that PAKs and MEKKs can function in separate of the Cdc42-dependent signaling pathways leading to JNK activation.

**The small GTP-binding proteins**

There are two separate classes of G proteins, the heterotrimeric mentioned earlier, and the monomeric, referred to as small G proteins or the Ras superfamily. Small GTPases are monomeric guanine nucleotide-binding proteins of 20-25 kDa molecular mass that play major roles in the regulation of growth, morphogenesis, cell motility, axonal guidance, cytokinesis and trafficking through the Golgi, nucleus and endosomes. The first small GTPase discovered was Ras, and now the members of this superfamily are grouped in five families: Ras, Rho, ADP-rybosylation factor (ARF), Rab and Ran.

They bind and hydrolyze guanosine nucleotides. The exchange between hydrolyzed GDP for GTP results in conformational changes unmasking structural domains by which they bind to target proteins. The exchange is regulated by three main classes of proteins. Those that facilitate the exchange of hydrolyzed GDP for GTP and thus promote activation, called guanine nucleotide exchange factor (GEF). Those that speed up the hydrolysis activity of the GTPases and thus promote inactivation, the
GTPase-activating proteins (GAP). Those that inhibit the dissociation of the GDP from the GTPases, the GDP dissociation inhibitors (GDI) that seem to stabilize the inactive GDP-bound form\textsuperscript{305, 313}.

The small GTPases of the Rho subfamily have been identified as the master regulators of actin organization in all eukaryotic cells. They act as molecular switches that coordinate multiple pathways by assembling multimolecular complexes, which are held together by scaffold proteins\textsuperscript{310}. Their vast array of functions include membrane trafficking, cell transformation, apoptosis, transcription and cell cycle control, oxidant generation, axonal guidance, gene expression and morphogenetic processes involving changes in cell shape and polarity\textsuperscript{277, 305, 310}. The Rho family is currently formed of 14 distinct members: Rho (A-E, G, H), Rac (1-3), Cdc42 (Cdc42Hs and G25K), TC10 and Rnd (1 and 2)\textsuperscript{305, 313}. The additional GEFs and GAPs increase considerably their number and add to the current complexity of the signaling pathways that involve Rho GTPases.

**Rho family GTPases**

Regulation of the actin cytoskeleton by the Rho family of small GTPases was described and mostly studied in fibroblasts. Classically, Rac1 promotes \textit{de novo} actin polymerization at the cell periphery to form lamelipodial extensions and membrane ruffling\textsuperscript{313, 314}. Activation of Cdc42 elicits extension of hairlike filopodia or microspikes\textsuperscript{310} but cross talk between Cdc42 and Rac results that filopodial extensions are usually seen with lamellipodial protrusions. Activation of RhoA triggers clustering of integrins and associated proteins into focal adhesion complexes and bundling of actin filaments into stress fibers.

In neutrophils, studies with C3 enzyme, a clostridial toxin that ADP-ribosylates and inactivates Rho, showed that basal actin polymerization was reduced, whereas chemoattractant-induced F-actin formation was unaffected or even enhanced\textsuperscript{315}. Functional inhibition of Rho with C3 transferase exoenzyme also reduced \(\beta_2\) integrin-dependent adhesion in intact neutrophils, neutrophil migration (as reviewed in\textsuperscript{315}), Fc receptor-mediated phagocytosis and in lymphocytes inhibited the LFA1/ICAM-1-dependent aggregation\textsuperscript{316}. Evidence suggests that Rho is more likely involved in leukocyte adhesion rather than locomotion\textsuperscript{305, 310, 314}.

Cdc42 induces actin polymerization in neutrophil extracts\textsuperscript{317} whereas Rho, Rac and Cdc42 were implicated in migratory responses to chemoattractants, phagocytosis\textsuperscript{277} and a variety of signaling pathways including phospholipase D. In macrophage chemotaxis in response to colony-stimulating factor 1, Rho and Rac were required for the ability of cells to migrate while Cdc42 was required for giving the locomotion a direction\textsuperscript{318}. Rac is an essential component of the NADPH oxidase enzyme complex in phagocytes and consequently a regulator of ROS generation\textsuperscript{319}.

**Rho and the cytoskeleton**

About 8 putative downstream targets for Rho have been identified. All substrates for these kinases are components of the cytoskeleton, most notably they phosphorylate the myosin light chain
phosphatase and myosin light chain itself resulting in increased actinomyosin contractility, stress fiber formation and recruitment of integrins to focal adhesion structures\textsuperscript{320,321}. Rho also appears to regulate the Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity probably through ROCK\textsuperscript{322}, which is also involved in the integrin-dependent activation of Erk upon cell adhesion. Another target is p140mDia that can interact with profilin and have a direct role linking Rho to the actin cytoskeleton. Overexpression of p140mDia in COS cells resulted in pronounced accumulation of F-actin in these cells\textsuperscript{305}.

**Rac/Cdc42 and the cytoskeleton**

Rac and Cdc42 are mediators of a wide variety of cellular stresses. They have each around 8 known targets, some are common to both such as PAK. The effect of PAK on p38/JNK activation has been presented earlier in this chapter. PAK also regulate a complexity of signals. PAK1 is able to induce lamellipodia and filopodia-like protrusions when microinjected in fibroblasts\textsuperscript{310}, phosphorylate p67\textsuperscript{phox} and p47\textsuperscript{phox} subunits of NADPH oxidase in neutrophils\textsuperscript{305}, are involved with cell transformation, morphological changes during apoptosis\textsuperscript{323} and can be activated by pathways independent of Rac/Cdc42, e.g. by sphingosine\textsuperscript{324}. Rac also activates LIM kinases that phosphorylate the actin depolymerizing protein cofilin that once non functional leads to accumulation of F-actin in the periphery of cells expressing active LIM kinases\textsuperscript{325}.

Important Cdc42 targets linking to the actin cytoskeleton include the Wiskott-Aldrich syndrome protein (WASP) involved in filopodia formation and IQGAP1\textsuperscript{315} that binds directly to F-actin and cross-links them\textsuperscript{305}. Novel Cdc42-specific class of serine/threonine protein kinases include MRCK (myotonic dystrophy kinase-related Cdc42 binding protein kinase) and its *Drosophila* homologue Genghis Khan (Gek) are important in mediating the Cdc42 effect on polymerized actin. Gek mutations resulted in abnormal accumulation of F-actin\textsuperscript{305}.

**Small GTPases and membrane phosphoinositides**

An important target for Rac, and Rho, is phosphatidylinositol 4-phosphate 5-kinase one of the three isoenzymes that converts PIP into PIP2, which affects actin polymerization and filament assembly\textsuperscript{305}. In permeabilized platelets both Rac and Cdc42 were found to be essential for the release of capping proteins from the barbed end of actin filaments, a prerequisite for rapid actin polymerization in response to thrombin\textsuperscript{326}. In neutrophils, Arcaro found that cytosolic Rac activation by FMLP receptors was responsible for the dissociation of gelsolin from the barbed ends of F-actin and subsequent actin polymerization, independent of PIP2 or PIP3 activity\textsuperscript{327}. Arcaro concluded that neither PIP2 or PIP3 was required in neutrophils for this process\textsuperscript{327}. This author also suggested that Cdc42 acted simultaneously upstream to Rac in increasing barbed end availability, and possibly via PAK to "de-sequester" monomeric actin\textsuperscript{327}. This observation corroborates other studies demonstrating that in contrast to Rho\textsuperscript{315} and Rac, Cdc42 does not affect phosphatidylinositol 4-phosphate 5-kinase, and in fact, Cdc42 can stimulate actin polymerization in a cell-free system independent of PIP2\textsuperscript{317}. 

Intracellular signaling pathways triggered by adhesion receptors

The function of selectins and integrins extends beyond simply attachment, they also participate in neutrophil activation. In this regard, neutrophils do not cause damage while suspended in the bloodstream but rather release toxic compounds only while adhered to the endothelium, epithelium or extracellular matrix. Furthermore, adhesion greatly enhances the effect of soluble mediators on both respiratory burst and release of proteases. Abundant literature indicates that most of the enhanced neutrophil activation comes from signals generated by integrins, and antibodies against CD11b/CD18 inhibit these signals.

Neither integrins nor selectins possess enzymatic activity, therefore studies have focused on protein interactions occurring at the cytoplasmic domain of these receptors. The short intracellular domain of L-selectin is not coupled to any signaling molecule but is attached to the cytoskeleton, which provides a link to intracellular signaling pathways. The cytoplasmic domain of β2 integrins also interacts directly with cytoskeletal elements such as α-actinin and talin, but also with signaling molecules such as integrin associated protein (CD47), focal adhesion kinases, cytohesin-1, and the integrin-linked kinase (ILK). These molecules are speculated to regulate the affinity state of the integrin adhesive function (inside-out signaling) or the signals emanating from these receptors (outside-in signals). Other molecules involved in the integrin-mediated signaling include: calreticulin that binds to the α-chains and regulates the calcium-dependent events during adhesion, Fc receptors, and the transmembrane-4 super family proteins.

L-selectin

L-selectin ligation increases both the number and avidity of CD11b/CD18 molecules. In addition, L-selectin colocalizes with these β2 integrins resulting in enhanced microvascular retention of circulating neutrophils, firm adhesion to endothelial surface, enhanced aggregation and transendothelial migration. Cross-linking of L-selectin significantly enhanced the FMLP- and TNF-α-induced respiratory burst, and this effect required transient increases of [Ca^{2+}].

Calcium changes, oxidative burst, Erk activation and increased TNF-α and IL-8 mRNA expression induced by sulfatides or cross-linking of L-selectin are dependent on PTK and were almost completely inhibited by genistein, herbimycin A or PTK deficient Jurkat cells. Neither pertussis toxin nor PTK inhibitors inhibited the cytoskeletal changes induced by L-selectin while...
cytoskeletal disruption with cytochalasin B did not prevent tyrosine phosphorylation. These results suggest that both L-selectin-mediated processes occur independently of each other.

Antibodies to different epitopes of L-selectin have different potency in inhibiting the L-selectin-mediated intracellular signals and neutrophil responses. Antibodies recognizing the SCR domains (Mab LAM1-14) are more potent than those against the lectin domains (DREG-200 or -56)\textsuperscript{211}, suggesting that specific domains have different roles in the L-selectin-mediated biological responses\textsuperscript{222}.

\textit{β2} integrins

In different models of neutrophil activation, β\textsubscript{2} integrins occupancy or cross-linking initiates a cascade of biochemical events resulting in reorganization of the cytoskeleton, cell spreading over adhesive surfaces, release of ROS and degranulation\textsuperscript{268}. Cytoskeleton reorganization and activation of protein tyrosine phosphorylation are central in signal transduction by integrins in neutrophils. Integrin ligation leads to the activation of MAP kinases, Ras, paxillin, FAK, the non-receptor PTK Fgr, Lyn, Syk, phospholipase C-γ2 and the proto-oncogene Vav\textsuperscript{340}. Integrin ligation also causes changes in the lipid membrane resulting in increased substrate for phospholipase C, consequently increased PIP\textsubscript{3}, \([Ca^{2+}]\), DAG and finally PKC\textsuperscript{248}. These pathways are linked to functional responses such as granule exocytosis, enhanced phagocytosis, activation of the respiratory burst and cell migration. The integrin-generated signals can be modified by endogenously produced ROS, which also modulates the activity of tyrosine kinases redistributed to sites of integrin clustering\textsuperscript{341}.

Src-family kinases play a pivotal role in signaling and cytoskeletal reorganization dictated by integrins in human neutrophils\textsuperscript{342}. Neutrophil spreading is accompanied by activation of Fgr and Lyn that are distributed to the actin-based cytoskeleton. Neutrophils from Fgr and Hck double deficient mice do not spread on fibrinogen, ICAM-1 or anti-integrin Ab-coated surfaces\textsuperscript{267}. Syk is an important signaling molecule activated by integrins as previously demonstrated in platelets and monocytes\textsuperscript{268}. In neutrophils, Syk associates with Fgr or Lyn to form protein complexes that are necessary for cytoskeleton rearrangement and neutrophil spreading\textsuperscript{268,340}. Activation of Syk by integrin signals result in partial translocation of this molecule to the triton-insoluble fraction suggesting that it might have a role the cytoskeleton remodeling\textsuperscript{268}.

Another important signaling molecules activated by integrin signals are the focal adhesion family of kinases (FAK), functionally linked to focal adhesion complexes. FAK transfers the integrin signals into diverse signaling pathways, including those of PIP\textsubscript{3}K. In human neutrophil, a cell lacking focal adhesion complexes, p125 FAK itself seems to have a less certain role. Another member of the FAK family Pyk2 or calcium dependent tyrosine kinase (CADTK) has been identified in neutrophils. Pyk2, like FAK itself, couples to several signaling pathways and regulate many different functions. It is activated by diverse stimuli including UV radiation, hypertonicity, cytokines, chemoattractants and PMA\textsuperscript{298,298,340}. Pyk2 is a stress-sensitive upstream mediator of the JNK signaling pathway\textsuperscript{298}, which can also
be activated in Pyk2-independent ways. Pyk2 appears to be downstream to Lyn, Syk and PKC and function in signaling events associated with adhesion and cytoskeletal rearrangements. Finally, integrin-mediated transduction signaling involves the Rho family of small GTPases. In lymphocytes, integrin adhesion to fibronectin triggers filopodia and membrane ruffling, hallmarks of Cdc42 and Rac while dominant negative Cdc42 and Rac inhibit these effects. Rho A is also activated by integrin-mediated adherence.

**Signaling intermediates**

**Non receptor PTK**

There are nine known families of non-receptor PTK, and they are: Src, FAK, Jak, Syk, Csk, Fes, Tec, Ab1 and Ack. They are involved in the regulation of a multitude of cellular responses in neutrophils including adhesion, chemotaxis, priming. Src is the largest family, comprising 10 enzymes with common Src homology domains such as SH2 and SH3, which are involved in regulation and binding to proteins and substrate recognition, whereas SH1 is catalytic. All Src family PTK, except Yrk and Fyk, are involved in signal transduction processes. Hck is expressed mainly in neutrophils, where it is predominantly associated with azurophilic granules, but also to non-granular membranes and cytosol. Hck translocates to the phagosomes during phagocytosis, suggesting it might be a regulator of degranulation or late stages of phagocytosis. Fgr is partially associated with secretory vesicles and specific granules and translocates to the plasma membrane during cell activation suggesting that both Hck and Fgr might regulate degranulation of their respective granule population. Both Fgr and Hck are involved in immune complex-linked neutrophil activation and are physically and functionally associated with FcγRII and FcγRIIIb respectively. Presently, the understanding of the function of Src, Ab1 and Fes in neutrophils is sparse. PP1 or 4-amino-5-(4-methylphenyl)-7(t-butyl)pyrazolo[3,4-d]pyrimidine is a potent and selective Src family tyrosine kinase inhibitor. PP1 inhibits Src-family kinases at nanomolar concentration without interfering with the phosphorylation of other kinases such as Syk/Zap70.

Syk is a 72-kDa protein that contains two SH2 domains and one catalytic domain. It is almost immediately activated after engagement of many types of antigen receptors including IgE receptors, B-cell receptors and IgG receptors (i.e. FcγRI and -II) in neutrophils. Osmotic stress, ionizing radiation, oxidative stress or genotoxic agents all activate Syk. Syk phosphorylation is a combination of both autophosphorylation and the activity of additional tyrosine kinases. Autophosphorylation accounts for its rapid and robust activation. Syk is expressed in a wide variety of hematopoetic cells, including neutrophils, T cells, B cells, mast cells, macrophages, platelets and thymocytes. Piceatannol or 3,4,3',5'-tetrahydroxy-trans-stilbene, preferentially inhibits Syk in a dose-dependent fashion by competing for the tyrosine-containing substrate binding, and not by interfering with antigen receptors or Src family kinase activation, at doses up to 50 μg/ml.
Calcium

Localized increases in cytoplasmic free Ca$^{2+}$ controls a vast array of neutrophil functions, from short-term responses such as granule exocytosis and shape changes to long-term regulation of cell growth, proliferation and even cell death by either necrosis or apoptosis. Most intracellular calcium ions are sequestered in the mitochondria and endoplasmic reticulum or other vesicles, thus allowing localized calcium release. ATPases pump calcium across cell membrane to the cell exterior, or into the lumen of vesicles for storage keeping its cytosolic concentration below 0.2 µM.

Many stimuli increase cytosolic calcium, even in the absence of calcium in the surrounding medium, by mobilization of cytoplasmic stores. The generation of receptor-induced cytosolic Ca$^{2+}$ signals involve two interdependent components, one rapid transient release from stores followed by slowly developing extracellular Ca$^{2+}$ entry. Occupation of a seven spanning G protein-linked receptor or RTK causes activation of phospholipase C (PLC) which in turn hydrolysis phosphatidylinositol 4,5 biphosphate (PIP2) found on the cytosolic leaflet of the plasma membrane. This hydrolysis yields two important products: 1,2-diacylglycerol (DAG) that remains in the membrane and inositol 1,4,5-triphosphate (PIP3), which is water-soluble. PIP3 diffuses to the ER surface where it binds to a calcium channel protein causing it to open allowing calcium ions to exit ER and enter the cytosol. PIP3 is rapidly hydrolyzed and thus the release of calcium from ER terminated. Depletion of calcium stores triggers a message leading to the opening of cell membrane “store-operated” calcium channels and extracellular calcium entrance. This second phase mediates long term cytosolic Ca$^{2+}$ elevations and replenishes intracellular stores.

Diacylglycerol and PKC

DAG remains associated to the cell membrane and activates a family of plasma membrane proteins called protein kinase C (PKC), important for cell growth, differentiation and tumor promotion. PKC is a family of enzymes, composed of at least 10 different isoforms (α,β,δ) that differ in responsiveness to phospholipids and Ca$^{2+}$. PKC mediates signals for multiple neutrophil functions including ROS generation, degranulation, adherence and actin filament assembly. They are divided in three groups, the classical PKC (cPKC) isoforms α, βI, βII and γ that are DAG- and Ca$^{2+}$-dependent. The Ca$^{2+}$-independent but DAG-dependent novel PKC (nPKC) isoenzymes δ, ε, η, μ and θ, and finally the Ca$^{2+}$- and DAG-independent atypical (aPKC) isoforms ζ, λ and τ. The DAG surrogate phorbol 12-myristate 13-acetate or PMA, also activates cPKC and nPKC.

Human neutrophils contain cPKC isoforms α and predominantly βI and βII, nPKC isoenzyme δ and atypical ζ-PKC. Diverse PKC inhibitors are available, from the traditional but unspecific staurosporin and H7 to the newly developed and isoform specific bisindolylmaleimide Gö 6850 and indolocarbazole Gö 6976. Other targets for phorbol esters and DAG include protein kinase D (PKD) or η-chimerin, which mediate certain effects of these components, either partially or exclusively.
**Phosphatases**

Phosphotyrosine accumulation, which is crucial for many neutrophil functions, results from the balance between protein tyrosine kinases (PTK) and phosphatases (PTP). Not much is known about the role of PTP in regulating neutrophil function, but appear that most of their effects are primarily as negative regulators. PTP are divided into receptor-like transmembrane PTP (e.g. CD45 and PTPβ) or cytosolic soluble PTP (e.g. SHP-1 and Meg 2).

CD45 is believed to contribute to the desensitization of neutrophils following repeated stimulation with FMLP and its inhibition contribute to the oxidant-induced activation of MAPK. SHP-1 or SH2-containing tyrosine phosphatase 1 or PTP1C, is a phosphatase expressed primarily in hematopoetic cells and detected in large quantities in neutrophil cytosol. SHP-1 is also a potential regulator of growth promoter receptors in hematopoetic cells. Mutations in SHP-1 are responsible for serious autoimmune and immunodeficiency defects in Moth-eaten mice. Neutrophils from these animals show abnormal myeloid maturation, increased tyrosine phosphorylation, delayed apoptosis, hyperadherence due to increased expression and activation of CD11b/CD18 and defective chemotaxis (Downey G. personal communication). Very preliminary results on Meg-2, a 68kDa cytosolic soluble PTP recently found to be present in neutrophils, appear to localize exclusively in the primary granules and the triton X 100-insoluble fraction suggesting a potential involvement in membrane fusion (Downey G. personal communication).

Hypertonic stress activates SHP2 in COS cells. This PTP acts as an adapter molecule linking the Jak/STAT pathway to MAPK pathway in a p38-dependent manner. Another phosphatase activated by hypertonicity is MKP-1 in H411E hepatoma cells. MKP-1 is also induced by growth factors, oxidative stress and heat shock and contributes to the deactivation of MAPK.

**Lipopolysaccharide**

The component of the outer membrane of most Gram-negative bacteria, referred as endotoxin or LPS, is a highly potent inflammatory agent. As a single stimulus however, LPS is a weak stimulator of neutrophil chemotaxis and exocytosis, but primes and/or enhances ROS generation and adherence. LPS also increases F-actin assembly making the neutrophil stiffer and more resistant to deformation and activates NF-κB stimulating the synthesis of TNF-α, IL-1, cyclooxygenase-2, prostaglandin E2 and thromboxane B2.

LPS treated neutrophils undergo significant shedding of L-selectin and concomitant increased in CD11b/CD18 expression in vitro. While this sequence of events is critical for transmigration, the “untimely” mobilization of these adhesion molecules results in significant impairment of neutrophil migration. Clinical endotoxemia mimics other clinical conditions such as trauma, burn injury and major surgery in that migratorially depressed yet oxidatively hyperactive neutrophils become incapable of leaving the vasculature promoting injure to the endothelial cells and...
increasing vascular permeability. Inability to migrate into inflammatory sites can increase the risk of infections. In fact, emigration of neutrophils toward infected areas of the lungs can be blocked by systemic administration of LPS. Neutrophil responses to LPS are independent of PKC, do not use the Ras/Raf/MAPK Erk pathway or cause changes in \([\text{Ca}^{2+}]_i\), but activates p38 via MEK3 by pathways undetermined in humans.

CD14 is the most important LPS receptor on monocytes and neutrophils, at physiological concentrations of LPS. CD14 is a 55-kDa glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that binds LPS exclusively in the presence of LPS binding protein. CD14 has no intracellular domain and requires another membrane-spanning protein for signaling such as the toll-like receptors (TLR) described in Drosophila and mice. CD14 shedding is both constitutive and inducible, resulting in a soluble molecule normally present in human serum. Neutrophil activation by internalization of LPS and sCD14 seems more important in rats than humans since rats do not express CD14 and are not as sensitive to LPS as humans and other animals. CD14 is stored in both secretory vesicles and \(1^{50}\) granules in neutrophils, and its surface expression increased by stimulation with LPS, FMLP and other cytokines but not PMA or A23187. Mac1, CD11c/CD18 and L-selectin have all recently been shown bind LPS and activate cells. Different responses to LPS may be due to the fact that the specific activity of commercially available LPS is rarely supplied by vendors and rarely reported in publications and their Limulus amebocyte activity can vary by more than 10-fold in between lots.

**Specificity of selected protein kinase inhibitors**

Pharmacological inhibitors that can target individual kinases are powerful tools for use in signal transduction research. Most compounds however, affect more than one individual target due to the enormous number of protein kinases and the highly conserved nature of their catalytic domain. Recently, novel, potent and more specific agents such as PP1 and SB203580 have been reported, which gained rapid and wide acceptance. The following table shows schematically the selectivity of the pharmacological agents used for the studies presented in this dissertation.
### Table 4: Specificity of some pharmacological PTK inhibitors

<table>
<thead>
<tr>
<th>KINASES</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>INDUCED BY</th>
<th>OTHER EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SB203580</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly selective p38 inhibitor</td>
<td>p38 **, **</td>
<td>600 nM</td>
<td>IL-1, stress, LPS</td>
</tr>
<tr>
<td></td>
<td>p38 ***</td>
<td>0.3-0.5 μM</td>
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<tr>
<td></td>
<td>JNK-1 and 2 **</td>
<td>&gt;10 μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDK-1, PDK-2 PKB (T- Bcells)</td>
<td>3-5 μM</td>
<td></td>
</tr>
<tr>
<td><strong>PPI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly selective Src-family inhibitor.</td>
<td>p56lck</td>
<td>5 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p59fyn</td>
<td>6 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p60Src</td>
<td>170 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hck</td>
<td>20 nM</td>
<td></td>
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<tr>
<td></td>
<td>EGFR</td>
<td>250 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JAK2</td>
<td>50 μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Syk/ZAP-70</td>
<td>&gt;100 μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c-Abl</td>
<td>&gt;1 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p38</td>
<td>&gt;1 mM</td>
<td></td>
</tr>
<tr>
<td><strong>Genistein</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Broad-spectrum PTK inhibitor.</td>
<td>Inhibits tyrosine phosphorylation of isolated enzymes, receptors and whole cells</td>
<td></td>
<td>Platelets, leukocytes and diverse cultured tissue</td>
</tr>
<tr>
<td></td>
<td>EGFR</td>
<td>2.6 μM</td>
<td>Auto-phosphorylation Histone 2B</td>
</tr>
<tr>
<td></td>
<td>EGFR</td>
<td>22 μM</td>
<td>Ang. II</td>
</tr>
<tr>
<td></td>
<td>EGFR</td>
<td>1 μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>v-Src</td>
<td>26 μM</td>
<td>Casein Poly-GT v&lt;sup&gt;2&lt;/sup&gt;-Ang. II</td>
</tr>
<tr>
<td></td>
<td>c-Src</td>
<td>&gt;50 μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>v-Abl</td>
<td>39 μM</td>
<td></td>
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<tr>
<td></td>
<td>PKA</td>
<td>&gt;100 μM</td>
<td>Histone 2A</td>
</tr>
<tr>
<td></td>
<td>PKC</td>
<td>15 μM</td>
<td>Histone H1</td>
</tr>
<tr>
<td></td>
<td>PKC</td>
<td>&gt;100 μM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PKC</td>
<td></td>
<td>anti-CD3</td>
</tr>
<tr>
<td>Herbimycin A</td>
<td>Erbstatin Analog</td>
<td>Staurosporin</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Reacts with thiol groups</td>
<td>Competitive with substrate; noncompetitive with ATP</td>
<td>Interacts with ATP binding site</td>
<td></td>
</tr>
<tr>
<td>p60Ssrc</td>
<td>Antagonist to most PTK EGFR kinase v-Abl</td>
<td>PKA PKG MLCK PKC Ca**2+/calmodulin kinase II Other PTK</td>
<td></td>
</tr>
<tr>
<td>PDGF Thrombin anti-CD3</td>
<td>780 nM</td>
<td>7 nM 8.5 nM 1.3 nM 0.7 nM 0.5 nM 70 nM</td>
<td></td>
</tr>
<tr>
<td>900 nM 14 μM</td>
<td>780 nM</td>
<td>Produces cell cycle arrest and apoptosis in Jurkat T cells; Inhibits topoisomerase II.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Thymocyte apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induces thymocyte apoptosis; Inhibits G2/M cell cycle</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activates bcl-2 apoptosis</td>
<td></td>
</tr>
</tbody>
</table>

PDK = 3-phosphoinositide-dependent protein kinase, PKB = protein kinase B, HSP = heat shock protein, EGFR = epidermal growth factor receptor, PK = protein kinase, PTK = protein tyrosine kinase, PL = phospholipase

* = Cuenda et al (reference 291),
** = Clerk A., Sugden P.H. FEBS lett 1998; 426:93-96,
Section V. **Volume and Neutrophil Function**

**Objectives**

The objective of this section is to review current and relevant literature on the functional consequences of volume changes, focusing on the sparsely known outcomes of neutrophil shrinkage. The following are the most relevant issues:

1. cell volume changes are by themselves, an important signal induced by and necessary for the action of many hormones and mediators, regulating numerous cellular functions;
2. cells detect volume changes probably by the combination of mechanisms including macromolecular crowding, integrin receptors, ligand-independent receptor activation and membrane deformation;
3. volume changes trigger diverse signaling pathways, in neutrophils shrinkage causes extensive tyrosine phosphorylation and p38 activation, in other cells Src-family kinases, Syk, Pyk2, STAT, SHP2 and inositol phospholipids are also activated by shrinkage;
4. cellular consequences of shrinkage in neutrophils are mostly depressive effects and include inhibition of motility, respiratory burst, phagocytosis and bacterial killing as well as induction of apoptosis;
5. in other cells shrinkage profoundly affects cell metabolism, commonly promotes catabolism, inhibits protein synthesis and stimulates apoptosis; it also modifies gene expression and inhibits secretory functions.

**Introduction**

Every cell is separated from its environment by a plasma membrane, which allows the maintenance of distinct intracellular and extracellular settings. The uneven distribution of ions and other molecules on either side of the membrane is the basis of a multitude of vital physiological phenomena. Any intracellular or extracellular change in osmolarity results in transmembrane movement of water and consequently a change in cell volume. A fact only recently appreciated is that even minute changes in cell volume affect a wide variety of cellular functions (reviewed in 365-367). While some of these changes serve the sole purpose of restoring near-normal volume to avoid extreme size variations and lysis, other changes modify cellular metabolism and gene expression 368-371. In fact, cell volume modification is both regulated by and required for the action of hormones and other mediators 365; 372; 373. Overall, volume change is an essential mechanism of cellular adaptation to an ever-changing environment.

This chapter is divided in three parts, how the volume change is detected (osmosensing), transduced (osmosignaling) and finally how it impacts on diverse cell functions. Not much is presently known about each of these steps and how they are linked to each other.

**Sensing volume changes**

The mechanisms of sensing osmotic and volumetric changes are far from being understood. Distinct osmosensing membrane proteins have not been identified in mammalian cells, like the ones described in yeast 374. In yeast the histidine kinase Sho1p is activated by osmotic shrinkage and activates
Hog-1, the homologous to the mammalian p38MAPK. Subsequently, Hog-1 triggers the synthesis of glycerol that acts as osmolyte in yeast. In a classic 1993 paper, Parker argues that mechanical events like bending or stretching of the membranes are unlikely to be important sensors of volume changes. He postulated that the critical signaling depends on cytoplasmic protein concentration, in the so-called macromolecular crowding theory. According to this author, cellular responses to shrinkage and swelling strive not to restore volume but to preserve an optimum degree of cytoplasmic protein concentration.

Recent evidence however suggest that other mechanisms may have relevant roles in sensing osmotic changes, that one or more mechanisms might be simultaneously involved and the relevance varies according to the cell type. Potential candidates, besides macromolecular crowding, include: the integrin system, cytoskeleton, ligand-independent activation of membrane receptors and membrane deformation leading to generation of phospholipids and opening of ion stretch channels.

**Integrins**

Two lines of evidence suggest that integrins are important mechanical transducers, converting membrane stresses into chemical signs. Stretching of the cell membrane by either external mechanical overload, cell swelling or drugs activate signal transduction pathways and gene expression events. Membrane stretching affects integrin interaction with extracellular matrix at focal adhesion sites resulting in autophosphorylation of the focal adhesion kinase (p125FAK). FAK tyrosine phosphorylation promotes the binding of Src-family protein tyrosine kinases to FAK and subsequently Grb2, leading to the activation of ERK. Alternatively, under mechanical stress, integrins can activate the Ras-ERK pathway by recruiting the adapter protein Shc, another SH2-domain containing protein, to a subset of β1 integrins linking various tyrosine-phosphorylated signal transducers to Ras. Further evidence comes from the tensigriity models using magnetic twisting cytometry that apply direct mechanical stress to cell surface integrins. Pulling of the integrins transmits mechanical signs to the cytoskeleton that undergo reorientation and redistributed along the axis of the applied tension inducing a force-dependent stiffening response. This provides a molecular connection between integrins, cytoskeletal filaments and nuclear scaffolds, which by unknown ways intersects with the chemical signaling events in the cell and its membrane. Considering however that in fibroblasts shrinkage do not result in integrin-mediated FAK phosphorylation, it seems that integrins are important sensors and transducers of mechanical stress/tension, but their role in sensing volume changes awaits confirmation.

**Cytoskeleton**

Volume change due to exposure to anisoosmotic environment results in both cytoskeletal rearrangement and activation of diverse intracellular signaling pathways, but the relationship between them is an unexplored domain. It has been shown that drugs that alter the cytoskeleton also affect agonist-induced activation of the stress-activated protein kinases. On the other hand, activated
kinases are important regulators of the cytoskeleton organization. The bulk of current evidence suggests that cytoskeletal remodeling is more likely to be regulated by rather than the regulator of the osmosensitive signaling pathways.

**Ligand-independent receptor activation**

One form of macromolecular crowding is the aggregation of cell surface receptors. Rosette et al demonstrated that hyperosmolarity causes multiple growth factor and cytokine receptors to cluster and internalize in HeLa cells, thus initiating a cascade leading to a ligand-independent activation of JNK. This fascinating hypothesis however, has not yet been conclusively proven. It is supported by a recent study by Qin et al in which pre treatment of B cells with suramin, an inhibitor of cell surface aggregation, inhibited the osmotic stress-induced activation of Syk. Schliess et al suggested that clustering of EGF receptors by mechanical forces on the plasma membrane due to hyperosmotic shrinkage, resulted in their activation in H41IE rat hepatoma cells, and many other authors also speculated that receptor clustering triggered different biochemical signals, unfortunately without conclusive evidence. Other questions about this hypothesis are whether swelling also triggers such ligand-independent activation of receptors or whether it occurs under physiological conditions.

**Membrane deformation and generation of phospholipids**

Membrane deformation by volume changes might also lead to the hydrolysis of one or several inositol phospholipids found in the cytosolic leaflet of the plasma membrane and thus initiate several signaling pathways. This hypothesis was recently proposed by Dove et al that reported that in yeast, hyperosmotic shock induced PIP2 synthesis, by a process involving the activation of a PtdIns3P 5-OH kinase. While PIP2 generation may be an acute osmotic adaptation response, the authors reported similar findings in plants and mammalian cells, and suggest that this process may be a prevalent regulatory pathway that most certainly should be explored.

**Membrane deformation and stretch ion channels**

Finally, a variety of ion channels are activated by cell membrane stretch or increased tension of the cell membrane. Stretch increases the open probability of the channels without affecting single channel conductance or selectivity of the channels. These most non-selective channels allow passage of K⁺, Na⁺ and Ca²⁺, thus favoring cell swelling. Mechanisms linking membrane stretch and channel activation have not been clearly defined. It may be mediated by activation of Src family kinases such as p56lck. Membrane stretch channels probably function more as last line of defense rather than fine-tuning of cell volume.

Other possible sensors of osmotic and volume changes include the small Rho-family of GTPases that under different stresses, coordinate the regulation and translocation of diverse kinases from the cytosol to the plasma membrane. In conclusion, it appears that multiple mechanisms besides macromolecular crowding are important for sensing the state of cell hydration in mammals.
Volume-sensitive signal transduction pathways

Volume changes initiate many different signal transduction pathways, involving both protein kinases and phosphatases. In 1997, two groups demonstrated in a variety of cells, including neutrophils, that a reduction in cell volume (shrinkage) resulted in extensive tyrosine phosphorylation of many intracellular proteins. Detailed investigation into the identity of the tyrosine-phosphorylated proteins demonstrated in neutrophils the shrinkage-induced activation of members of the Src-family p59fgr and p56\(s/c\)ck. Later, Kapus et al demonstrated in CHO cells, the volume-regulated activation of p59fyn and inhibition of p60Src. The selective pyrazolo pyrimidine Src-family inhibitor PP1 prevented the hypertonicity-triggered tyrosine phosphorylation of almost all bands, suggesting that most osmosensitive tyrosine kinases are regulated by the Src-family kinases. Furthermore, this study also revealed the existence of Src-independent pathways, one of which involving MAPK p38.

Other studies have investigated the effect of hypertonic shrinkage and MAPK activation in neutrophils. All studies agree that hypertonic stress do not activate Erk 1 and Erk 2, while JNK is activated only by extremes of hypertonicity such as 900 mOsM. They however disagree concerning the shrinkage-induced activation of p38. One study reported that hypertonicity did not activate MAPK p38 in neutrophils. In contrast, Junger et al reported p38 activation as early as 1 min after hypertonic shrinkage, followed by a progressive increase over time reaching 10-fold above baseline by 2 h. In this manuscript, Junger et al were also the first to report that hypertonicity paradoxically activated p38 and inhibited the FMLP-induced p38 and Erk 1/2 activation. The authors speculated that this paradoxical effect could result from the uncoupling of the signals originating in the FMLP receptor. This argument was based on the known fact that Na\(^+\) ions, and not increased ionic strength, efficiently reduces G-protein activity and might constitute the physiological signal by which the activity of the FMLP receptor is decreased. While this argument could account for the termination of the FMLP signal, it does not fully explain the fact that hypertonicity does not prevent other FMLP-mediated signals such as transient increase in [Ca\(^{2+}\)]. The authors also reported that pharmacological inhibition of p38 with SB203580 prevented the effect of hypertonicity on both PMA- and FMLP-induced exocytosis, leading to the hypothesis that the hypertonic effects on neutrophils could be mostly due to p38 activation. Our own investigation supports a shrinkage-induced activation of p38. However, our findings on the role of p38 in exocytosis and other neutrophil functions challenge the conclusions of Junger’s study and are presented later in this dissertation. Considering that only two other groups, besides ourselves, have investigated the possible shrinkage-mediated activation of p38MAPK in neutrophils, it remains a matter open for discussion.

In most other cells hypertonic exposure activates both p38 and JNK, also known as stress-activated protein kinases, with variable effect on Erk 1/2 activation. All three MAPK, Erk 1/2, p38 and JNK are activated by hypertonicity in vascular endothelial cells, MDCK, fibroblasts, rat
pheochromocytoma PC-12, rat myocytes, Chinese hamster ovary (CHO), H4IIE hepatoma cells and hepatocytes. Shrinkage-mediated activation of the stress-activated kinases but not of Erks occurs in mouse macrophages and Kupffer cells. In hematopoietic cells, p38 is activated by hypertonic stimulation in peripheral mononuclear cells, Jurkat cells and promyelocytic leukemia cells U937. In the latter, activation of the NHE antiporter and the resulting cytosolic alkalinization mimicked the shrinkage-induced activation of JNK and p38, raising the possibility that cytosolic alkalinization mediates the hyperosmotic activation of these kinases. Interestingly however, hypertonicity was still able to activate both JNK and p38 even when the NHE was inhibited, thus establishing that activation of this antiporter is not necessary for the activation of the stress kinases upon hypertonic challenge.

In MDCK epithelial cells, hypertonicity sequentially activated the MAPK cascade starting with PIP3 + DAG then PKC, raf-1, MEK, Erk 1/2 and 90 kDa ribosomal S6 kinase (p90rsk). In other cells, osmotic stress activated MEK 4 then p38 and JNK, which were downstream to Pyk-2 via PIP3-kinase and the small G proteins Ras and Rac. Other tyrosine kinases known to be activated by hypertonicity include Erk 5 (or Big MAPK), Syk (activated by both autophosphorylation and Src-family kinases), Pyk-2, PKC, the Jak/Stat pathway and the tyrosine phosphatase SHP2 and MKP-1. The exact cascade of biochemical events and their functional consequence remain mostly undetermined.

**Functional responses to volume changes: ionic compensatory mechanisms**

The content of osmotically active solutes and the osmolarity of the extracellular fluid determine cell volume. In a hypotonic or hypertonic environment, cells initially swell or shrink almost like perfect osmometers. Many cell types are able to initiate volume regulatory responses such as regulatory volume decrease (RVD) or regulatory volume increase (RVI). Primary RVI occurs when cells are exposed to a hypertonic environment, and secondary RVI occurs when cells undergo initial hypoosmotic swelling and are subsequently returned to isotonic medium, which is now hypertonic relative to these cells. This distinction is important because cells like neutrophils, lymphocytes and the human promyelocytic leukemic cell (HL-60), show little or no secondary RVI, whereas almost all cells show significant secondary RVI.

Neutrophils have an average volume of 280 ± 30 μm³ under isotonic conditions, or 6-8 μm in diameter, and these measurements vary considerably according to different reports. Under slow hypoosmotic swelling, neutrophils reach a maximum volume of approximately 1,200μm³ or 2.6 times the initial area, just before lysis, while hyperosmotic shrinkage reduces their volume roughly by a third.

Ions contribute to the bulk of both intracellular (mainly K⁺) and extracellular (mainly Na⁺) osmolarity. Regulatory volume adjustments are efficiently and rapidly accomplished by transmembrane ion transport. RVD by extrusion of K⁺ and Cl⁻ ions, and RVI by accumulating ions through simultaneous activation of the Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers. The Na⁺/H⁺ exchanger (NHE) and Cl⁻/HCO₃⁻ exchanger are responsible not only for the regulation of cell volume but intracellular pH as well. Among the different members of the Na⁺/H⁺ exchanger family, neutrophils express solely NHE-1, which is
activated by cell shrinkage. Another characteristic of the NHE isoforms 1, 2 and 3 is their dependence on ATP.

Volume adjustments by ion transport may compromise cell functions. Some cell types adjust their volume by breakdown or uptake organic substances or “osmolytes”, molecules specifically designed to create osmolarity. They are especially important in the volume regulation of the renal medulla cells where osmolarity may reach extreme levels and in the brain where volume changes are limited by the rigid skull. Osmolyte gathering may take hours to days, and neutrophils do not accumulate osmolytes.

**Functional responses mediated by volume-sensitive signaling pathways**

We are far from understanding the functional consequences initiated by changes in cell volume and most of the current knowledge refers to swelling-induced functional changes. In general, cell shrinkage supports a catabolic situation in most cell types, whereas swelling exerts insulin-like effects. Presently, very few cellular functions have been convincingly linked to the hyperosmotic activation of specific signaling pathways.

**Activation of the NHE**

The Na⁺/H⁺ exchanger is activated by cell shrinkage. Recently, Krump et al suggested that in neutrophils, the Na⁺/H⁺ exchanger (NHE-1) is activated by osmosensitive tyrosine kinases since the inhibition of the shrinkage-induced tyrosine phosphorylation with genistein prevented its activation. Later, Kapus et al demonstrated in CHO cells that the activation of both NHE-1 and -3 was not mediated by the volume-regulated tyrosine kinases of the Src family. Similarly, Src-family kinase inhibition with PP1 did not prevent hypertonic NHE-1 activation in neutrophils.

**Phosphorylation of cortactin**

Another functional consequence of shrinkage-induced activation of Src-family kinases was also reported in the latter study. The osmotic activation of Src kinases caused phosphorylation of cortactin, a cortical actin-binding protein. Cortactin is involved in the organization of the cortical actin cytoskeleton and its phosphorylation in vitro by Src resulted in decreased actin crosslinking activity and increased cell migration. Considering that osmotic stress in yeast induces rapid disassembly of filamentous actin and subsequent reassembly in the cortical area, it is plausible that cortactin phosphorylation might participate in the hypertonic reorganization of the cytoskeleton.

**Apoptosis**

Hyperosmotic shock and other environmental stresses can elicit apoptosis, and cell volume loss is one of the apoptosis hallmarks. The role of volume in apoptotic cell death is still ill defined and remains mostly a matter of speculation. In human neutrophils, Frasch et al demonstrated that hyperosmotic stress induced apoptosis in a p38-dependent manner. Curiously however, in many cell types both p38 and JNK have been implicated in pathways leading to two distinct outcomes, either
proliferation or apoptosis. To explain this discrepancy, Chen et al proposed that the kinetics of activation determine the fate of the cell, such that rapid and transient activation is associated with proliferation and persistent and sustained elevation with apoptosis. This hypothesis is corroborated by the observation that in neutrophils transient and rapid p38 activation by FMLP promoted survival while UV radiation caused a delayed and sustained activation for over 1-2 hours resulting in increased apoptotic rate. This concept may also prove correct for hypertonicity-induced apoptosis since p38 osmotic activation is similarly sustained.

Another signaling molecule apparently involved in hyperosmotically-induced apoptosis is the p21-activated kinase or PAK. Chan et al reported that hyperosmotic shock caused cleavage and activation of PAK2 and thus caused apoptosis in mouse Balb/c 3T3 fibroblasts, human hepatoma hep 3B and human epidermal carcinoma A431 cells. They also report that caspase 3 was activated by hyperosmotic shock, and its inhibition or the use of antioxidants, prevented the hyperosmotic activation of PAK2 and subsequently apoptosis. Recently Rudel and Bokoch also implicated PAK2 in Fas- and TNF-α-induced apoptosis of Jurkat T cells. These findings suggest that PAK2 might be a common signaling mechanism involved in apoptosis of diverse cell types triggered by environmental stress including hyperosmolarity. In B cells, hyperosmotic shock induction of apoptosis appears to be modulated by the presence of functionally active Syk, which protects the cells from osmotic stress-induced apoptosis.

Other functional responses

Recently shrinkage-induced activation of p38 in MDCK cells was suggested to participate in RVI of these cells, which was almost completely prevented by p38 inhibition with SB 203580. Arbabi et al reported that hyperosmotic activation of another MAPK, Erk 1/2 in human endothelial cells, results in prostacyclin (PGE2) synthesis, which might be involved in the hypertonic saline-induced vasodilatation in humans. The hyperosmotic activation of Erk 1/2 and p38 was responsible for gene activation resulting in synthesis of both betaine in MDCK cells and sorbitol in HepG2 cells. The p38 inhibitor SB203580 and the MEK1 inhibitor PD098059 inhibited the generation of both osmolytes.

Other functional responses to cell volume changes

At this time, it is important to present some considerations. First, the entire cell changes volume following exposure to anisoosmotic conditions, including the volume of subcellular compartments such as mitochondria, endoplasmic reticulum and peroxisomes. Second, the intracellular ionic strength is altered along with changes in volume, even when extracellular ionic strength is kept constant. Therefore, the sequelae of osmotic alterations of cell volume are not necessarily identical to the consequences of isotonic alterations of cell volume. Third, different degrees of hypo or hypertonicity besides the length of exposure trigger different responses. Fourth, many of the pharmacological agents used in scientific studies directly or indirectly affect the cell volume or its regulation. This fact might make it difficult to
determine whether cellular responses are the consequence of volume change or rather other effects. Some of these agents include PKC inhibitors, NDGA, PLA2 inhibitors, calmodulin antagonists, toxins that inhibit trimeric G proteins, dibutyl cAMP and forskolin. Fifth, changes in several proteins might result not from gene transcription but from relocation between the cytosol and the cytoskeleton and vice-versa during volume changes.

**Actin cytoskeleton**

Apparently both cell swelling and shrinking affect the polymerization state of the microfilamentous cytoskeleton. While it is long known that cell swelling causes actin filaments to depolymerize in many different cells, including neutrophils, solely on HL60 cells there has been any suggestion that cell shrinkage induces actin polymerization. This finding however, is disputed by two early studies by Sha'afi & Molski and Yassin et al in that hypertonicity not only did not increase the amount of polymerized actin but also inhibited the FMLP- and leukotriene B4-induced actin polymerization. Both studies were done in rabbit neutrophils.

Hallows et al demonstrated in HL 60 cells that the amount of F-actin during regulatory volume response, is inversely proportional to the cell volume shift. Treatment with cytochalasin B lowered levels of F-actin but did not prevent volume regulatory response, suggesting no causal relationship between these two events. The authors also speculate that volume-dependent changes in actin assembly could result from signaling events (phosphoinositide turnover, and phosphorylation/dephosphorylation activity), changes in intracellular concentration of Mg\(^{2+}\) or Ca\(^{2+}\), or dilution/concentration of proteins (macromolecular crowding). In another study, these authors describe different pools of F-actin that respond differently to volume changes. Triton-soluble F-actin pool is very much influenced by the physicochemical effects of volume changes while the triton-insoluble remains virtually unchanged.

It is known that in purely *in vitro* studies using isolated actin, in the absence of nucleotides and divalent ions, actin polymerization can be enhanced by osmolytes such as sucrose. Few months ago, Fuller & Rand offered a theoretical basis to explain this phenomenon. Under osmotic stress, solutes excluded water from the aqueous compartment around actin (or actin associated water). This lead to a significant reduction in the critical concentration of monomeric Ca\(^{2+}\)-ATP actin, and favored polymerization since fewer water molecules were associated with F-actin. The magnitude of the polymerizing effect was proportional to the osmotic pressure exerted by the different neutral osmolytes tested. These results suggest that water is part of the G- to F-actin polymerization and osmotic dehydration drives the G- to F-actin equilibrium to the more dehydrated state. Extrapolated to a cellular level, it could mean that the hydration state of the cell is also an important parameter in determining its state of polymerization.

Intact microtubule and microfilament networks are required for RVD, which is inhibited in several tissues by drugs such as colchicine, nocodazole and cytochalasins B and D. While...
the same is true for Jurkart cells, in other leukocytes such as neutrophils and HL-60 cells, RVD was dependent solely on microtubules and unaffected by microfilament disruption. Some authors proposed that the swelling-induced actin disassembly is the consequence of changes in actin-binding proteins such as gelsolin, due to increased intracellular Ca²⁺ concentration, or profilin, due to degradation of phosphatidylinositol 4,5-biphosphate.

**Granule exocytosis**

Changes in cell volume influence the secretory function of many cell types. It has been postulated that cellular swelling increases vesicle membrane tension, triggering the fusion with the plasma membrane and exocytosis of their content. It is known that cell volume changes alter intraluminal pH of secretory granules, swelling causes alkalization and shrinkage acidification of these compartments and the pH changes determine insulin release from pancreatic β-cells. In the same way, the uptake and release of neurotransmitters such as catecholamines, glutamate, GABA and acetylcholine is impaired by osmotic shrinkage. Hypertonicity also inhibits secretion in a number of other tissues including adrenal chromaffin cells, parathyroid gland, urchin eggs and platelets but induces vasopressin and histamine release from basophils.

In neutrophils, many studies suggest that hypertonicity significantly impairs granule exocytosis. No single study has ever investigated the osmotic effect on exocytosis of all four granules. Unrelated studies reported decreased release of MPO, lysozyme, lactate dehydrogenase, β-glucuronidase, lactoferrin, gelatinase, elastase under hypertonic conditions, following stimulation with FMLP, PMA, LB4, arachidonic acid, PAF or A23187, with or without pre-treatment with cytochalasin B. Kazilek et al observed by electron microscopy, the appearance of an empty layer of cytoplasm between the plasma membrane and neutrophil granules following hyperosmotic shrinkage. The authors reported no evidence of fusion or specialized contacts between granules and plasma membrane. Nonetheless, the authors reason that changes in intracellular Ca²⁺ might be responsible for the osmotic inhibition of exocytosis, even though hypertonicity also inhibited exocytosis elicited by calcium ionophores, which caused the expected changes in intracellular Ca²⁺. Yassin and Junger et al proposed another mechanism for the inhibitory effect of hypertonicity on exocytosis, receptor uncoupling. According to these authors, hypertonicity would dissociate the receptor from generating second messengers. This argument however, fails to explain the osmotic inhibition of exocytosis triggered by non-receptor-mediated agonists such as the direct PKC stimulation by PMA or calcium ionophores. Another proposed, but not investigated mechanism, was an increase in intracellular cell viscosity.

Osmotic swelling induces and/or enhances the release of the following hormones: insulin, prolactin, gonadotropin-releasing hormone, luteinizing hormone, thyrotropin, aldosterone and renin.
hormone releasing effect of cell swelling has been correlated to increase \([\text{Ca}^{2+}]\), and intact actin cytoskeleton \(^{367}\).

**Intracellular traffic of vesicles**

Lee et al reported a few months ago that osmotically induced cell volume changes blocked the ER-to-Golgi traffic \(^{416}\). The hypertonic-stress response was linked to the inhibition of COPI, a protein complex that forms transport vesicle coats at the ER Golgi intermediate compartment. The ER-to-Golgi vesicle traffic reemerged after hours and this process was independent of new protein synthesis but required PKC. This data suggests that cell volume regulates constitutive traffic and impacts in organelle structure since in parallel they described that the Golgi collapsed into the ER. This osmotic effect may be the basic mechanism underlying many of the responses elicited by osmotic stress \(^{416}\). Furthermore, hyperosmotic stress also inhibits the formation of coated pits and vesicles and thus prevents the endocytic internalization of both low-density lipoproteins and transferrin receptors \(^{442}\). This effect however, was linked to increased intracellular ionic strength and not to cell volume changes \(^{442}\).

**Cellular migration**

Neutrophils and other motile cells undergo major shape and volume changes during migration. Volume change is required for cell migration \(^{443,444}\) and involves localized RVD/RVI and asymmetrical distribution of channels and carriers. In order to migrate, neutrophils have to swell \(^{443}\). This concept is supported by measurements of neutrophil size during migration \(^{443,444}\), as well as the observation that swelling increases their ability to migrate \(^{445}\) while shrinkage suppress any movement \(^{158,444,446}\). While RVD/RVI suppression stands as an important mechanism for the hyperosmotic inhibition of migration, other mechanisms might also have a role.

**Respiratory burst**

Extensive literature has consistently demonstrated that hyperosmotic shrinkage inhibits the formation of ROS in neutrophils, while swelling stimulates it \(^{158,438,441,446-453}\). The mechanisms underlying this effect however, have not been determined. It has been proposed that shrinkage might inhibit the flux through the pentose phosphate pathway or prevent the assembly of NADPH oxidase system \(^{452}\). Iyer et al demonstrated that NADPH oxidase system assembly requires en bloc translocation of the macromolecular complex p47-phox/p67-phox, which *in vitro* was dissociated by high osmolarity and thus rendered non-functional \(^{454}\).

The inhibition of neutrophil respiratory burst might also account for the inhibitory effect of hypertonicity on bacterial killing \(^{448}\). Neutrophil phagocytosis and bacterial killing are reduced but not absent, in a hypertonic environment \(^{439,446,450,453,455,456}\).

**Myosin**

Not all of conventional myosin is organized into contractile bundles. In non-muscle cells, myosin II is also part of the cortical cytoskeleton with a pivotal role in providing resistance against high
external osmolarity by increasing cortical stiffness and mediating the movement of cell-surface proteins. Dictyostelium cells lacking myosin II fail to increase cortical tension and change to a round shape, even when a "rigor" contraction was induced by ATP depletion. These mutants also failed to cluster surface cross-linked proteins on binding to concanavalin A, suggesting that myosin II provides the force to aggregate these membrane proteins. As discussed in section V, clustering of surface receptor has been linked to hypertonically-induced triggering of transduction signals, suggesting that myosin might have a role in the osmotic cellular effects.

Myosin light chain kinase (MLCK) is the key regulator of cell motility. In addition to its kinase activity, MLCK binds and bundles F-actin in vitro, and act as an actin cytoskeleton stabilizer. In C6 glioma and glomerular mesangial cells, shrinkage causes phosphorylation of MLCK, which in turn also activates the Na+/H+ exchanger in the C6 glioma cells.

In Dictyostelium, Zischka et al demonstrated that during osmotic shock the protein pattern remained unaltered, but the amount of regulatory myosin light chain increased substantially in the cytoskeletal fraction. They also reported that both actin and myosin II became phosphorylated under hypertonic conditions. Kuwayama et al proposed that the osmotically-induced myosin II phosphorylation enhances the disassembly of myosin II filaments, which then translocates to the cortex, co-localizing with (and probably participating in the generation of) the highly cross-linked cortical cytoskeleton. Finally, while testing the hypothesis that cortical F-actin might act as a physical barrier to secretion, Becker et al reported that both F-actin and myosin II content were reduced at putative sites of secretion in zebrafish eggs.

**Cytosolic pH**

Swelling causes cytosolic acidification by the exit of HCO₃⁻ through anion channels, by the release of H⁺ from acidic intracellular compartments and by enhancement of Cl⁻ and HCO₃⁻ exchange due to decreasing cellular Cl⁻ activity. Cell shrinkage causes alkalization due to the activation of the Na⁺/H⁺ exchanger.

**Chloride**

Intracellular Cl⁻ concentration in resting human neutrophils is unusually high, being four times higher than that predicted by Nernst equation. A large Cl⁻ ion efflux is an early and common event preceding neutrophil activation induced by most agonists. The Cl⁻ content falls by over 50%, and with the volume increase, intracellular [Cl⁻] decrease by more than two folds. Grinstein et al reported that the reduction of [Cl⁻] resulted in spontaneous activation of electroporated or streptolysin O-treated neutrophils suspended in a Cl⁻-free medium. These findings were corroborated by studies demonstrating that a reduction of [Cl⁻] precedes granule exocytosis (specific but not primary granules), activation of proadhesive molecules, reorganization of the cytoskeleton network and assembly of the NADPH oxidase. Alternatively, inhibition of the Cl⁻ efflux inhibited all these neutrophil
functions. Thus, Cl⁻ efflux is a common phenomenon during neutrophil activation and has an important role regulating neutrophil responses, irrespective of the type of agonist.

On the contrary, cell shrinkage would increase [Cl⁻], which could prevent neutrophil activation. This argument is supported by the observation that cells that are unable to regulate their volume in a hypertonic environment do accomplish RVI when intracellular Cl⁻ is lowered by prior RVD. Thus, some authors have suspected that increased [Cl⁻] might be a mechanism underlying some of the inhibitory effect of hypertonicity.

**Metabolism**

Cellular hydration state is an important determinant of cell function and hormones, oxidative stress and nutrients exert their effects on metabolism and gene expression in part by modifying cell volume. Persistent changes in cell metabolism occur within minutes after volume changes and shrinkage and swelling frequently exert opposite effects. Cell swelling favors synthesis and inhibits degradation of proteins, glycogen and lipids, while shrinkage supports mostly a catabolic situation. In hepatocytes, insulin induces a 20% increase in cell volume by activation of both Na⁺/H⁺ exchanger and Na⁺-K⁺-2Cl⁻ cotransporter whereas glucagon leads to an approximately 20% decrease by activation of ion channels. The opposing effects of insulin and glucagon on proteolysis are fully accounted for by their action on hepatocyte volume and the induction of the same degree of volume change can mimic their effect. Similarly the antiproteolytic effect of glutamine and glycine is prevented by inhibition of Na⁺/H⁺ exchanger and Na⁺-K⁺-2Cl⁻ cotransporter thereby preventing swelling of the hepatocytes. In fact, cell swelling correlates with cell proliferation in fibroblasts, mesangial cells, lymphocytes, HL-60 cells, GAP A3 hybridoma cells, smooth muscle cells and HeLa cells, while shrinkage, as discussed, correlates with apoptosis.

**Gene expression**

Cell volume changes influence cell metabolism on a long-term time scale by modifying a wide variety of genes. This involves the expression of genes coding for osmoregulatory proteins but also proteins not necessarily linked to cell volume regulation. Examples of the latter include P-glycoprotein, ClC-K1, cyclooxygenase-2, the immediate early gene transcription factors Egr1-1 and c-Fos, the GTPase activating protein for Rac α-1chimerin, vasopressin, matrix metalloproteinase 9, diverse matrix proteinases and the expression of heat shock proteins probably to counteract the detrimental effects of increased salt concentrations. Osmoresponsive promoters, well understood in bacteria and yeast, have also been identified in mammalian cells. Toncitivity-responsive enhancer (TonE) putative consensus sequence regulates genes for sodium/myoinositol cotransporter, the sodium/chloride/betaine cotransporter and aldose reductase in response to hypertonicity.
Cell swelling also stimulates gene expression of a wide variety of proteins including: β-actin, tubulin, cyclooxygenase-2, the transcription factors c-Jun and c-Fos, tissue plasminogen activators and many others 367.

ATP

Matsumoto et al suggested that the hypertonic inhibition on neutrophils was due to exhaustion of energy stores 469. The same group later demonstrated that the ATP precursor phosphoenol pyruvic acid would revert some of the suppressive effects of hypertonicity 446. This hypothesis has not been further investigated however current evidence does not support this premise. Exposure to a hypertonic environment causes activation of the NHE, which is remarkably sensitive to ATP levels 407, 408. Extensive tyrosine phosphorylation and activation of diverse signaling pathways also contradict this hypothesis. Furthermore, in a recent study, the ratio of ATP to protein in cells extracts at various times following exposure to hypotonic stress failed to demonstrate any significant differences 416.

Inotropic and vasoactive effects

Shrinkage exerts a positive inotropic effect in the heart, which was linked to the effect of increased ionic strength on the actinomyosin complex 470. In the microcirculation, osmotic swelling appears to favor vasoconstriction. Swelling activates anion channels with exit of Cl⁻ and depolarization of the cell membrane. The subsequent activation of voltage-sensitive Ca²⁺ channels stimulates Ca²⁺ entry and contraction of smooth muscle cells. Shrinkage has an opposite effect and leads to vasodilatation 471. This effect compounded by the release of prostacyclin induced by hypertonicity 150, results in increased organ perfusion, one of the most beneficial effects of hypertonic saline resuscitation in trauma patients.
Section VI. Neutrophil Actin Cytoskeleton

Objectives

The objective of this section is to review current and relevant literature on cellular actin cytoskeleton. The focus the consequences of cytoskeleton rearrangements in neutrophils motility and granule exocytosis. The following are the most relevant issues:

1. dynamic reorganization of the cytoskeleton affects diverse cellular functions and involves calcium, ionic forces, pH, temperature and actin-binding proteins (ABP);
2. ABP are in vivo regulators of actin cytoskeleton dynamics and some are also involved signal transduction;
3. neutrophils move by vigorous ameboid movements, and different from slow-moving cells like fibroblasts, they do not form stress fibers, focal adhesion complexes, lamellipodia and ruffles;
4. actin cytoskeleton may function as a physical barrier that prevents exocytosis in many neuroendocrine cells;
5. actin cytoskeleton is an important regulator of surface receptors such as integrins and FMLP and of many neutrophil functions.

Introduction

Actin filaments provide a lattice that supports the plasma membrane and organizes the cytosol while actin polymerization is required to generate some forms of cellular motility. Cell motility encompasses cell migration as well as the intracellular movement of vesicles. It requires fuel or ATP, proteins that transform ATP into motion (myosin) and a cytoplasmic system of fibers called cytoskeleton. The cytoskeleton of mammalian cells consists of 3 different types of filaments: 7 to 9 nm-diameter microfilaments, 10 nm-diameter intermediate filaments and 24 nm-diameter microtubules. Even though certain aspects of neutrophil motility requires synchronized action of microtubules and intermediate filaments with the actin cytoskeleton, the actin network is the prime component of the cell cytoskeleton involved in shape changes and neutrophil motility. In fact, Keller & Bessis demonstrated that heat-induced anucleated fragments of leukocytes are able to migrate even when lacking centrioles and microtubules 472.

The actin microfilamentous cytoskeleton is involved in every type of motility, from cell migration to cytosol transport and will be the sole focus of this review. This chapter is divided in four parts, one discussing the special properties of the proteins that comprise the cytoskeleton, another on cytoskeleton and cell motility, another on the functions modified by the cytoskeleton and finally a brief overview on myosins and drugs used for the study of the cytoskeleton.

Actin

Cytoskeletal fibers are built from small protein subunits actin, a 43kDa globular protein (G-actin), held together by noncovalent bonds. There are at least 6 different actins, three exclusively found
in muscle cells (α-actins), two present in virtually all non muscle cells (β- and γ-actins) and another γ-actin exclusively found in intestinal smooth muscle cells. Globular actin aggregates into two-stranded helical polymer filaments (F-actin) consisting of up to thousands of monomers. Polymerization is regulated by many actin-binding proteins, divalent cations and intrinsic ATPase activity. Actin is the most abundant intracellular protein in eukariotic cells, comprising 5% (non muscle cells) to 10% (muscle cells) of all cell protein.

The most important property of actin is the ability of G-actin to polymerize into F-actin, which lies at the core of motility. Since the discovery of actin by Straub in 1942, it has been well established that at a neutral pH, actin can exist in monomeric or filamentous form depending on the presence of metal ions such as Mg^{2+}, K^+ or Na^+. Assembly of F-actin may be caused in vitro solely by increasing the medium ionic strength or disassembly induced by diluting the medium. Intracellular ionic conditions favor polymerization and consequently nearly all intracellular actin should be in a filamentous form. However only ½ of all actin remains unpolymerized due to sequestering proteins such as thymosin β4 or profilin. Polymerization in vivo is accompanied by hydrolysis of ATP into ADP. F-actin is a long and flexible filament where the subunits are organized as a helix pointing toward the same end. Consequently, one end of the ATP-binding cleft of an actin subunit is exposed to surrounding solution. This end is called “barbed end” and has a 30 times higher affinity for G-actin and more rapid monomer incorporation than the other end, the “pointed end”. This difference leads to a phenomenon called “treadmilling” where subunits dissociated from one end are added to the other, thus generating work.

*Actin assembly*

Polymerization proceeds in 3 phases: nucleation, elongation and annealing. Nucleation is the formation of small unstable actin oligomers or nucleus (2 to 3 actin monomers), which act as growing points for filament elongation. This step is slow and accounts for the lag prior to the viscosity increase. Addition of actin filament nuclei to pure monomers reduces this lag time in a concentration-dependent fashion. Elongation occurs by addition of monomers to the both ends of nuclei or filaments, but the barbed end is strongly favored. Elongation causes viscosity to increase rapidly. The third step is the end-to-end annealing of short filaments to form longer filaments. This process occurs simultaneously with elongation and may be responsible for the increase in viscosity during polymerization. Eventually a steady state phase is reached when there is no net change in the mass of the filaments. Filamentous actin (F-actin) exists in two distinct pools in resting neutrophils: triton insoluble (cross-linked meshwork of F-actin located in pseudopodia and submembranous locations, not depolymerized by dilution and rich in α-actinin and filamin) and triton soluble (shorter oligomers of F-actin, diffusely distributed and rich in gelsolin). Certain drugs are known to affect the polymerization process and are discussed later in this chapter.
The role of calcium in the assembly of microfilaments has been extensively investigated. Downey et al conclusively showed in neutrophils that actin assembly is not mediated by an increase in \([\text{Ca}^{2+}]\), which instead, facilitates actin disassembly, possibly by \(\text{Ca}^{2+}\)-sensitive actin filament-severing proteins such as gelsolin. In the absence of calcium, FMLP, leukotriene B4, PAF, arachidonic acid, A23187, phorbol esters, IL-8 and C5a are capable of inducing actin polymerization and neutrophil polarization. FMLP probably via non-conventional calcium-independent PKC isoforms since inhibition of conventional PKC does not alter polymerization. Tyrosine protein kinases do not seem responsible for actin polymerization since PTK inhibition do not affect cytoskeletal characteristics elicited by chemoattractants such as IL-8, C5a and FMLP.

**Filamentous actin structures**

Intracellular filamentous actin (F-actin) is mostly arranged into two structures, bundles and network. Both structures provide the framework that supports the cell membrane and determine cell shape. Bundles are closely parallel packs of F-actin whereas in a network F-actin crisscross often at right angles and are loosely packed. Cells have two types of actin network, one associated with the plasma membrane, or cortical network, and another within the cells that confers gel-like properties to the cytosol. Actin bundles and network are connected to the cell membrane by anchoring proteins that tack the sheet of membrane to the underlying cytoskeleton framework. In neutrophils, \(\alpha\)-actinin is the one of the most important membrane anchoring protein.

The richest area of actin filaments in a cell lies in the cortex, a narrow area just beneath the plasma membrane. In this zone, the actin network excludes most of the cytoplasmic organelles and confers stiffness to the membrane. The complex formed by integral membrane proteins, anchoring proteins and the cytoskeleton forms an important link between the extracellular with the intracellular environment.

**Actin-binding proteins**

While in a test tube actin polymerization can be started by simply adding salts to G-actin, in a living cell, this process requires the participation of regulatory proteins. Several regulatory actin-binding proteins or ABP have been characterized, and much new information has emerged on the structure, function and regulation of these ABP. ABPs are classified according to their function in: a) severing, b) filament-capping, c) bundling, d) cross-linking and e) monomer-sequestering proteins. Most ABP however, have complex roles in actin cytoskeletal dynamics and exert more than one single function.

**Severing ABP**

Severing ABPs are able to fragment filaments by mechanisms that do not require ATP hydrolysis and are responsible for the gel-to-sol transformation of the cytosol. Gelsolin and ADF/cofilin are the two major groups of severing ABP. Gelsolin comprises 1% of total cell protein in neutrophils and is regulated by calcium, pH and PIP2. Gelsolin is capable of rapidly severing all actin filaments in a
network by rupturing the noncovalent bonds among actin subunits, remaining bound to the barbed end thus promoting depolymerization and preventing annealing or growth by addition of actin monomers.\(^{479, 480}\) Gelsolin capping and severing functions are inhibited by PIP2 as well as low concentrations of calcium.\(^{480}\) Repetitive waves of high Ca\(^{2+}\)/low PIP2 alternating with low Ca\(^{2+}\)/high PIP2, gelsolin can disassemble an existing filament through its severing activity and then lead to a site-directed reformation of an alternative structure contributing to the rapid motile changes in cells.\(^{480}\) Gelsolin expression increases during myeloid cell differentiation as the cells become more motile, and increased expression by stable transfection increases the rate of fibroblast movement in tissue culture.\(^{473, 479}\) Transgenic gelsolin-null mice have delayed neutrophil and fibroblast migration due to impaired actin depolymerization and excessive stress fiber formation.\(^{314, 479}\) Gelsolin might also be an important effector for the Rac-mediated actin dynamics.\(^{314}\)

ADF and coflin are two widely and highly expressed filament-severing ABP, with a weak severing activity of less than 0.1% that of gelsolin.\(^{479}\) ADF/cofilin have a role in phagocytosis and in chemoattractant-induced remodeling of the actin cytoskeleton. They also increase the rate at which actin monomers leave the filament from the pointed or slow-growing end.\(^{481}\)

**Capping ABP**

Filament capping proteins stabilize F-actin by binding mostly to the barbed end and preventing monomer addition or loss. The most important and well-known members of this group are the heterodimeric calcium-insensitive but PIP2-sensitive Cap Z (capping protein \(\beta_2\), Cap32/34 or CP) and macrophage capping protein (MCP). Cap Z binds to the fast-growing end of actin filaments in regions where the ends of the filaments are anchored to the membrane, such as the outer edge of the lamellipodia. Capping is needed in places where the organization of the cytoskeleton is unchanging, as in muscle sarcomere or at erythrocyte membrane. Capping proteins are also able to function as nucleating proteins thus facilitating filament formation.

**Bundling ABP**

Bundling proteins bind actin at a site exposed on the filament then self associate forming bundles of actin. Bundles might alternatively be formed from a single protein with two suitable actin-binding domains. One of the most abundant is the calcium-insensitive but PIP2-sensitive \(\alpha\)-actinin, which forms bundles and also connect the cytoskeleton to the plasma membrane. Bundling is regulated by the concentration of bundling ABP, of actin and the presence of other ABPs resulting in different structures, polarity and rigidity of the bundles. Bundling affects the physical properties of actin, therefore where bundling becomes prevalent viscosity diminishes as actin is drawn into isolate bundles. In contrast, when bundling and severing proteins combine to form a meshwork of interconnecting bundles, these bundles become very rigid and viscosity increases. Depolymerization occurs at a much lower rate in bundles than in isolated filaments.
Cross-linking ABP

Filamin cross-links F-actin in a perpendicular manner, resulting in very rigid gels, and cells lacking this protein have very unstable cortices. Filamin and its isoforms have an important role in regulating osmotically sensitive ion channels and may form a self-regulating stretch receptor. Some melanoma cell lines completely lack filamin and are unable to regulate their volume. Another family of cross-linking ABP is the ERM (ezrin, radixin and moesin) family, which behave as adjustable scaffold proteins that anchor actin filaments to the membrane. ERM proteins interact with integral plasma membrane and cell adhesion molecules such as CD44, ICAM-1 and ICAM-2 and are involved in signal transduction. ERM proteins are essential pre-requisites for Rho and Rac to induce stress fibers and lamellipodia respectively, and to regulate actin cytoskeleton organization, specially the cortical network.

Talin is localized in the tip of actin bundles close to the leading edge of moving fibroblasts where it mediates actin assembly as well as coupling to the membrane in focal adhesion areas. Talin, which is a true nucleating ABP, drives polymerization, cross-links F-actin into bundles and network, and also anchors the newly formed filaments into the lipid bilayer. It is rapid and reversibly controlled by ionic conditions/pH and temperature. Talin also binds the cytoplasmic tail of β integrins and L-selectin to the actin cytoskeleton, with an important role in transmitting signals generated by integrins.

Of all ABP, talin, vinculin and α-actinin have the major role of establishing the connection between microfilaments and membrane leaflets in specialized adhesion zones known as focal contact areas.

Monomer-sequestering ABP

While intracellular ionic concentration heavily favors polymerization, about 40% of all actin in a cell is unpolymerized due to ABP that sequester G-actin and prevent their polymerization. Thymosin β4 and profilin are the most important monomer-sequestering ABP and together account for all G-actin sequestration in neutrophils. Profilin binds in a 1:1 complex to actin monomers and has several interesting properties. Monomeric G-actin, while bound to profilin, is allowed to exchange ADP for ATP thus replenishing the pool of ATP-actin. Another property is the inhibition of profilin binding activity by phosphoinositol 4,5 biphosphate (PIP2), what may result in significant actin polymerization and enhancement of actin-based locomotion. In fact, 10 seconds after chemoattractant stimulation, profilin-actin complexes reduce drastically accompanied by appearance of barbed-end nucleating activity and an increase in total F-actin content in neutrophils. It has been suggested that profilin regulates the pool of unpolymerized actin by drawing actin from thymosin β4 and releasing it to actin filament thus having an important role in the complex membrane-based actin assembly system. Rho is another important regulator of profilin activity and is discussed latter in this dissertation.
Finally there is a group of ABPs responsible for the interaction between microfilaments and myosins, the most widely studied is tropomyosin, present in both muscle (large form) and non-muscle cells (smaller form). They regulate actomyosin interactions in a calcium-sensitive manner.

**Actin cytoskeleton and neutrophil motility**

The movement of neutrophils toward an inflammatory site may occur by random migration (unstimulated motility), chemokinetic migration (stimulated speed but no directionality) and chemotactic migration that are directed movement elicited by chemoattractants and the sole focus of this section.

Neutrophil is a fast-moving cell that crawls forward by vigorous and repetitive amoeboid movements with a clear display of polarity, that is, certain structures are always formed at the front of the cell, while other structures are found at the rear. The major features, as observed by videomicroscopy, are characterized by pseudopodia extension and cytoplasmic streaming. Chemoattractant-stimulated neutrophils undergo rapid morphological changes, from rounded, relatively smooth cells to elongate, ruffled cells with a single pseudopodium. Pseudopodium is a large, broad membrane protrusion that arches forward from the cell. A contractile uropod is formed posteriorly resulting in polarized cell morphology, which is required for efficient directed migration. As the pseudopodium attaches to the substratum, it quickly fills with cytosol that is flowing forward through the cell. The last step in movement is when the rear of the cell retracts toward the body, breaking its attachment to the substratum. Slow-moving cells such as fibroblasts, emit slender projections of membrane called lamellopodia from the leading edge toward the increasing chemoattractant concentration gradient. Some areas of the cell project upward as a thin veil or ruffle, that moves as an undulating ridge back along the dorsal cell surface toward the cell body.

During migration, both fibroblasts and neutrophils adhere to the substratum via rapidly assembled special structures on the ventral surface of the cells. Different from fibroblasts, neutrophils lack: stress fibers, focal adhesion sites (large integrin aggregates at the end of stress fibers), and focal complexes (smaller integrin clusters at the tips of filopodia or lamellipodia). Neutrophil's adhesion sites or podosomes are much more limited. Details of the molecular differences between podosomes and focal adhesions are unknown but both contain α-actinin, vinculin and talin and concentrate tyrosine kinases, PKC and a variety of adapter proteins like paxillin, p130cas Grb2 and Sos. The less organized cytoskeleton in podosomes is directly responsible for the ability of neutrophils to migrate more rapidly than fibroblasts.

**The osmotic pressure theory of neutrophil motility**

Neutrophil movement is accompanied by changes in the viscosity of the cytosol, which cycles between sol and gel. The fluid or sol cytoplasm flows rapidly toward the front of the cell filling the pseudopodium. Here it is converted into gel, which forms the cortex just beneath the plasma membrane. As the cell crawls forward, the gel is converted back to sol when it reaches the tail end and the cycle
repeats itself for as long as the cells are migrating. This observation forms the basis of the osmotic pressure theory for pseudopod formation proposed by Coates et al. These authors described that 30 seconds after neutrophil stimulation with chemoattractant, two waves of actin polymerization from the cell edge result in diffuse F-actin distribution. F-actin in the center of the cell then depolymerizes, yielding a round cell with a cortical ring of F-actin. Central actin depolymerization produces a large number of osmotically active particles resulting in an inflow of water. The cortical ring of F-actin then dissolves at a single locale causing an outpouching of F-actin-poor membrane area. Subsequent actin polymerization at the site of membrane protrusion results in a dominant pseudopod while the actin in the rear disassembles. The result is the formation of a pseudopod with asymmetrical distribution of F-actin.

The transformation between sol and gel states results from the disassembly and reassembly of microfilament network by several ABP. In the front of the cell, profilin promotes actin polymerization and α-actinin and filamin form gel like actin network. Other proteins like gelsolin, sever actin filaments to form more fluid endoplasm. Furthermore, isoforms of myosin II at the rear of the neutrophil cause cortical contraction, squeezing the tail and pushing the cytosol forward. Meanwhile other myosins at the front of the cell cross-link the cytoskeleton to the membrane in the pseudopodium.

Other hypothesis of cell motility

Other hypothesis have been proposed to explain the propelling of the membrane forward. specially in slow-moving cells like fibroblasts or neuron extending its growth cone. One hypothesis is that the membrane is pushed forward by polymerizing actin filaments. Another hypothesis suggests that myosin I with its cargo of plasma membrane crawls forward along actin cytoskeleton at the leading edge. And finally, that the protein-rich cytoskeleton in the lamellipodium swells by an osmotic mechanism, causing the membrane to balloon forward.

A large body of evidence supports the view that the machinery that powers cell movement is built from actin cytoskeleton. According to the first hypothesis, unidirectional polymerization of actin is utilized for vectorial force production and forward movement of the leading edge of the lamellipodia. Polymerization proceeds by linear condensation reactions at the ends of the filaments, which have distinct kinetics and structures. Filaments turn over a treadmilling mechanism, whereby the steady growth of barbed ends is fed by the subunits depolymerizing from the pointed ends, pushing the membrane forward. The observed rates of actin-based movement fall in the range 1-20 μm/min corresponding to the treadmilling rates of 7-30 subunits/second/filament.

It is known that cross-linking proteins stabilize actin filaments into bundles or networks while myosin I is thought to link F-actin to the leading edge of the plasma membrane. According to the second hypothesis, extension and retraction of F-actin is powered by movements of myosin I attached to the plasma membrane while the space between the barbed ends of the filament would is then filled by newly
polymerized actin. The third hypothesis is similar to that proposed for neutrophils.

**Cellular functions modified by the cytoskeleton**

Most of the neutrophil inflammatory responses are fully or partially affected by remodeling of the actin cytoskeleton, including exocytosis, chemotaxis, rolling, adhesion, transmigration, regulatory volume changes, assembly of the NADPH oxidase and regulation of the Na+/H+ exchanger and diverse signaling pathways. Most of these effects are discussed in other parts of this dissertation, in this section we will restrict the discussion to the effect on the bi-directional transmembrane traffic (exocytosis and ion influx), chemoattractant and integrin receptors and ion transport.

**Cytoskeleton as a barrier to exocytosis**

In many cell systems, the cortical cytoskeleton is known to modulate the trafficking, docking and fusion of granules to the plasma membrane. In fact, inhibition of cortical actin polymerization with cytochalasin not only enhances stimulus-induced exocytosis, but under certain conditions, secretion itself will not occur without cytoskeletal disassembly.

In contrast to the abundant evidence of enhanced exocytosis with cortical cytoskeleton disassembly, few studies have proposed the opposite, that polymerized actin might act as a physical barrier to exocytosis. The hypothesis of a cytoskeletal barrier to exocytosis was first suggested by Poste in 1973, and demonstrated years later in neuroendocrine cells by both Burgoyne et al and Aunis et al. These authors showed that disassembly of the cortical cytoskeleton of adrenal chromaffin cells was a necessary step for exocytosis. At resting conditions, a thick cortical ring of F-actin fills the submembranous area and exocytosis is necessarily preceded by disassembly of this network, with a measurable decrease of total F-actin and a parallel increase of G-actin. More recent publications continue to support the notion that in areas where F-actin is disassembled there is a reduced viscosity and increased trafficking of granules and exocytosis. F-actin disassembly by injection of the actin-severing proteins scinderin and gelsolin reduced the F-actin cortical network and enhanced exocytosis. In other studies microinjections of thymosin β4 or gelsolin into permeabilized pancreatic acinar cells, resulted in disassembly of the cortical network and “rapid and robust” exocytosis without any [Ca2+]i changes, and independent of any agonist stimulation. The latter findings make evident the presence of constitutive active fusion machinery, which is negatively clamped by the actin network. Similar actin clamp is not present in neutrophils and other neuroendocrine cells cytoskeletal disassembly alone is not sufficient to trigger exocytosis.

This hypothesis of actin as a physical barrier has been challenged by reports that rapid increases in F-actin are part of the exocytic process in diverse cells, including neutrophils. Furthermore, efforts to reproduce the scenario of an actin barrier in many cell types have been tempered by technical limitations, i.e. the difficulty to microinject neutrophils with phalloidin and induce polymerization. Therefore, the role of actin as an obstacle to exocytosis remains unproven in most cells.
Few recent studies have made important advances in this area. In three separate experiments, treatment with phalloidin resulted in assembly of a compact and stable actin network underneath the plasma membrane and inhibition of exocytosis in loach eggs, pancreatic acinar cells and zebrafish eggs. The novel polymerizing drug jasplakinolide (JK) has also been tested in diverse cell types. In two separate studies DDT1MF-2, A7r5 and MDCK cells were treated with jasplakinolide. In all three cell types, JK caused cortical bunching of actin and excluded cytoplasmic organelles from the vicinity of the plasma membrane. If the proposed theory is correct, such physical separation between vesicles and plasma membrane should prevent membrane fusion and inhibit exocytosis. In another study, JK-induced polymerization of the cortical cytoskeleton was implicated in the inhibition of prolactin secretion from pituitary cells. Sheik et al used jasplakinolide to induce actin polymerization in human neutrophils. The authors reported that a complete block of the FMLP-induced upregulation of CD11b membrane expression accompanied the marked increase in the amount of F-actin. Considering that surface expression of CD11b requires exocytosis, the study hints that the extensive actin polymerization caused by jasplakinolide could be involved in blocking CD11b exocytosis. Confirmation that the cortical F-actin might acts as a physical barrier to exocytosis in neutrophils waits for further proof.

Cytoskeleton as a barrier to ion influx

The theory of a cortical cytoskeletal barrier could be applied to the transmembrane traffic both leaving and entering the cells. There is currently only one study on the role of the actin barrier in incoming cellular traffic. The study suggests that a cortical ring of F-actin abrogated the store-operated calcium influx by preventing the physical coupling between the ER and plasma membrane, thus preventing the activation of the Ca\(^{2+}\) channels. The actin ring was caused by either jasplakinolide or the serine/threonine phosphatase inhibitors calyculin and okadaic acid. These agents caused a similar redistribution of F-actin that accumulated into a tight condensed ring underneath the plasma membrane. Cortical actin disassembly with cytochalasin D allowed reestablishment of coupling and activation of the store-operated Ca\(^{2+}\) entry channels.

Other studies have also investigated the role of a disassembled cortical actin cytoskeleton in the regulation of incoming traffic other than Ca\(^{2+}\). A recent study pointed that disassembly of the cytoskeleton with dihydrocytochalasin D enhanced the stimulated entry of multiple cations, including Ca\(^{2+}\), Na\(^{+}\), Mn\(^{2+}\), Ba\(^{2+}\) and Sr\(^{2+}\), and suggested that this entry of cations play a role in the activation of neutrophil respiratory burst and exocytosis. The scarce amount of data suggests that the polymerized state of the cortical actin cytoskeleton might affect the bi-directional transmembrane transport.

Cytoskeleton and surface receptors

The observation that cytoskeletal disruption by cytochalasin B enhances the chemoattractant responses, lead to the concept that the cytoskeleton might regulate the affinity changes of the chemoattractant receptors. During neutrophil activation, there is a rapid interconversion between...
chemoattractant receptor states, from rapid to slow dissociating forms, and different receptor states can
be isolated from different plasma membrane fractions. Desensitized receptors are found in the fraction
rich in F-actin while the active receptor is found in the fraction containing mostly monomeric actin. In
fact, actin microfilaments physically separate these receptors from the G proteins, turning off their
response, while disassembly of these filaments enhance the second messenger generation and/or
exocytosis.

In leukocytes, the cytoplasmic domain of the β2 integrins is connected to the actin cytoskeleton
via the protein talin. Activation causes proteolysis of talin, thus releasing the β2 integrins from the
cytoskeleton and allowing lateral movement and clustering. FMLP stimulation in the presence of
cytchalasin B increased CD11b/CD18 motility and diffusion constant by 24 and 74% respectively.
Following clustering, integrins reengage the cytoskeleton by binding to the actin cross-linking protein α-
actinin. The extensive actin polymerization caused by jasplakinolide prevented the TNF-α activation
of CD11b/CD18, an effect theorized result from the inhibition of lateral motility by the cytoskeletal-
bound integrin. Therefore, integrin-mediated adhesion both in resting and activated leukocytes are
regulated by the cytoskeleton which in turn is regulated by the small GTP-binding proteins Rho, Rac and
Cdc42.

Myosin

In many types of movement, actin does not function alone and depend on interactions with the
motor protein myosin, an ATPase that moves along actin filaments. Myosin moves by coupling the
hydrolysis of ATP to conformational changes, therefore it is a mechanochemical enzyme. Myosin is
considered the motor, actin filaments are the tracks along which myosin moves, and ATP is the fuel that
powers motility. However, their specific roles in non-muscle cell migration remain not completely
understood.

Myosins are present in all eukaryotic cells, this superfamily is presently represented by fifteen
distinct classes of molecules. Myosin I, II and V proteins are the most abundant and most thoroughly
studied. Myosin II powers muscle cell contraction, and in non-muscle cells, cytokinesis (or cell division),
cell motility, chemotaxis and cell morphology. Myosin I and V are involved in the cytoskeleton-
membrane interactions such as transport of membrane vesicles. Myosins are organized into three
structurally and functionally different domains: the head region that contains actin- and ATP-binding
sites and is responsible for generating force, the neck region that regulates the head domain by binding
either calmodulin or calmodulin-like regulatory light chain subunits and the tail that binds either to
membranes (plasma or intracellular organelle membranes, i.e. myosin I and V) or to other tails (i.e.
myosin II).

Myosin II, the conventional two-headed myosin, is composed of a pair of heavy chains, a pair of
essential light chains and a pair of regulatory light chains (RLC). Each heavy chain has a globular head
domain, an intermediate domain that forms a α-helical coiled coil (or rod) responsible for the heavy chain dimerization and a carboxy-terminal nonhelical tailpiece. The essential and RLC, which are structurally related to calmodulin, bind distal to the motor domain. Cytokinesis and polarized cell locomotion require the correct temporal and spatial regulation of myosin II and associated cytoskeletal proteins. There are at least two heavy chain isoforms, myosin IIA and IIB in non-muscle cells, with additional 3 splice variants of myosin IIB. In endothelial cells migrating into a wound and growth cone migration, myosin IIA concentrates in the extending cell edges while myosin IIB is retained in retracting regions suggesting that these isoforms are differently regulated and have distinct roles. Early immunofluorescence micrograph studies revealed that in migrating amebas, myosin I was localized at the leading edge and myosin II at the rear, suggesting that myosin I is in a position to guide and myosin II in a position to push the cell. Genetic deletion of myosin I had no effect on cell movement, while those lacking myosin II do not form pseudopodium and become non-motile. These findings support the concept that different myosin isoforms participate in coordinating cell movement as opposite parts of a cell, however, their particular role remain incompletely defined. Myosin IIA has a maximal ATPase activity that is 2.6-fold greater than myosin IIB and moves actin filaments at velocities 3.3-fold faster.

Myosin II motor activity and assembly into filaments are regulated by phosphorylation on the conserved sites on the RLC. These phosphorylation events are mediated by diverse kinases, suggesting that myosin II is subjected to regulation by multiple signal transduction pathways. Three kinases have presently been shown to phosphorylate RLC on Ser19: Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK), Rho kinase and p21-activated kinase (PAK). MLCK is a key regulatory molecule in signaling pathways mediating cell motility, division and morphology and RLC is the only known substrate to MLCK. In vertebrates, alternative splicing produces two MLCK isoforms, MLCK-108 and MLCK-210, the latter being the predominant isoform expressed in cultured nonmuscle cell lines. In fibroblasts, inhibition of MLCK by microinjection of polyclonal antibodies induces the disassembly of stress fibers and in eosinophils inhibits the extension of lamellipodia and cell locomotion (reviewed in §12). MLCK has a significantly higher specificity for the RLC than does Rho kinase suggesting an in vivo more relevant role in regulating myosin II activity. Rho-induced stress fiber assembly requires phosphorylation of the RLC and phosphorylation and inactivation of the myosin II phosphatase have led to the proposal that Rho kinase regulates the reorganization of the cytoskeleton by regulating the phosphorylation state of myosin II. Rho kinase apparently phosphorylates RLC directly while inhibiting MLC phosphatase, this mechanism however remains to be clarified. PKC stimulates phosphorylation of RLC on serine 1 and 2, inhibiting the rate of MLCK phosphorylation and the ATPase activity of myosin II. Myosin is also regulated in vivo through noncovalent interactions with other proteins including actin. Assembly, transport and disassembly of clusters of myosin II filaments during
cell locomotion and cytokinesis, suggest that molecules involved in stabilizing, targeting and disassembling myosin II filaments also play an important role in regulating its function.  

**Pharmacological manipulation of the actin cytoskeleton**

Few different toxins are known to disrupt the monomer-polymer equilibrium. This section will focus on toxins frequently used for the study of the actin cytoskeleton, the polymerizing jasplakinolide and phallotoxins, and the depolymerizing latrunculin and cytochalasin.

**Jasplakinolide**

Jasplakinolide is a cell permeant cyclic peptide isolated from the marine sponge *Jaspis johnstoni*. It causes extensive actin polymerization, and in virtually all cell types studied, JK is described to convert all G-actin to F-actin. The polymerized actin accumulates almost exclusively at the cell periphery with the development of a dense ring of actin underneath the plasma membrane. Jasplakinolide has an even higher affinity for F-actin than phalloidin (K<sub>D</sub> =15 nM), and competitively inhibits phalloidin binding to F-actin, thus precluding their combined use.

**Phallotoxins**

Phallotoxins prevent actin filaments from depolymerizing. Isolated from *Amanita phalloides* or the angel of death mushroom, phallotoxins bind at the interface between subunits of F-actin where they lock the subunits together. Even when actin is diluted below its critical concentration, phalloidin-stabilized filaments will not depolymerize. Phallotoxins cause both elongation of existing filaments and induction of new filaments predominantly in the cortical area. They do not permeate cell membranes and its use requires either permeabilization or microinjection. Phallotoxins bind exclusively to F-actin and its most common use is as a specific F-actin stain. The attachment of a variety of fluorescent groups to the phallotoxins does not change its properties. Certain fluorescent phallotoxins such as rhodamine phalloidin, increase 10 to 20 times their fluorescence upon binding to F-actin. Under suitable conditions, i.e. excess of phallotoxins, the increase in fluorescence is linearly proportional to the amount of F-actin and has been explored to measure micromolar F-actin concentrations. Phalloidin and phallacidin are different derivatives of phallotoxins with very similar properties.

**Latrunculin**

In 1983, Spector et al described two toxins, latrunculin A and B, purified from the Red Sea sponge *Latrunculia magnifica*. These sponges grow exposed, but are not damaged or eaten by fishes and when squeezed manually, they exude a reddish fluid that causes fish to retreat or die. Both toxins contain a new class of 16- (latrunculin A) and 14-membered (latrunculin B) marine macrolides attached to a rare 2-thiazolidinone moiety. Submicromolar concentrations of latrunculin cause rapid (2-5 min) morphological changes due to a complete disassembly of actin filaments and bundles. The effect of these toxins is fully reversible, is about 100 times more potent than cytochalasins and does not affect the...
microtubular network. It has been proposed that latrunculin exert its effect exclusively by binding and sequestering actin monomers.

**Cytochalasin**

Cytochalasins, which mean cell relaxation due to the gel-like consistency of the cytosol after exposure to these toxins, are a widely utilized class of membrane-permeant fungal alkaloids. They exert diverse effects, including inhibition of actin polymerization, phagocytosis, spreading and neutrophil chemotaxis. Treatment with cytochalasin does not prevent cell volume regulation under anisosmotic conditions (RVD/RVI) or gelsolin function, but enhances ROS generation, agonist-mediated increase in [Ca$^{2+}$], and granule exocytosis.

According to Downey et al, in neutrophil-like cells, cytochalasin primarily prevents polymerization rather than promoting its disassembly. The mechanism of action of cytochalasin is not fully understood. It is classically described as toxin binding to the barbed end of F-actin, thus preventing addition of subunits and favoring dissociation from the pointed end. A critical review of the data on cytochalasin reveals a more complex picture, where its effects vary according to the cell type, concentration of cytochalasin and ionic strength. At low concentration, 2μM, cytochalasin B inhibits actin monomer addition to the barbed end of the filaments (elongation) but has little or no effect on either the addition of monomers to the pointed end or on the rate of filament annealing. At this concentration, it probably reduces rather than prolongs the lag phase thus promoting nuclei formation. Higher concentrations of cytochalasin B might cause loss of stress fibers.

Other in vivo effects include a strong reduction of low shear viscosity, actin filament crosslinking and network formation, which for some authors might be more physiologically relevant than inhibition of F-actin polymerization. Cytochalasin B also inhibits glucose transport system of some cell membranes, which does not account for its effects on microfilaments.
Section VII. Hypothesis

Based on the preceding discussion, we developed a series of hypothesis and studies to understand the physiological mechanisms underlying the effects of osmotic stress on neutrophil function, with possible implication for the management of trauma patients in hemorrhagic shock. An algorithm of the sequence in reasoning and the pivotal findings of the following studies is displayed on the next section.

Hypothesis 1 (Chapter 2). Hypertonic saline resuscitation from hemorrhagic shock lessens the development of lung injury. This hypothesis was tested in a rodent model wherein resuscitated hemorrhagic shock primes for acute lung injury in response to a small dose of LPS. This study showed that hypertonic saline resuscitation from shock markedly reduced lung injury by preventing neutrophil sequestration. This effect was transitory and could be restored with repeated infusion of hypertonic saline. This effect appeared to be predominantly due to an effect on neutrophil expression of adhesion molecules.

Hypothesis 2 (Chapter 3). Osmosensitive kinases are involved in osmotically-induced change of neutrophil expression of adhesion molecules. In this study we specifically investigated which kinases are activated by osmotic stress in neutrophils and their potential contribution to L-selectin shedding. This study demonstrated that the trigger for the osmotic effect on L-selectin is cell shrinkage, which initiates a p38 mitogen-activated protein kinase-dependent, metalloprotease-mediated cleavage of L-selectin. P38 seem important in L-selectin shedding evoked by classical inflammatory stimuli as well.

Hypothesis 3 (Chapter 4). Hypertonicity interferes with the normal polymerization depolymerization cycle and/or distribution of actin in neutrophils, and this is an important common mechanism whereby many neutrophil functions are altered. This study demonstrated that hypertonicity causes a marked increase in actin polymerization, and alters intracellular F-actin distribution, independent of tyrosine phosphorylation. Concomitant with these changes, hyperosmolarity abolishes exocytosis of all granule types, independent of the stimulus. Pharmacological inhibition of osmotic actin polymerization restores exocytosis in shrunken cells, and induction of actin polymerization mimics the effect of hypertonicity under iso-osmotic conditions. These studies provide evidence that the osmotically triggered changes in the actin skeleton are key contributors to the neutrophil-suppressive effect of hypertonicity.
Section VIII. Schematic Summary

Chapter 2: Hypertonicity suppresses neutrophil function and prevents neutrophil-mediated lung damage.

*In vivo*
- hypertonic saline/ model of acute lung injury

**Clinical level:**
- decreased neutrophil lung sequestration
- decreased lung damage

**Cellular level:**
- decreased endothelial ICAM-1
- decreased neutrophil L-selectin/CD11b
- NO effect on chemoattractant CINC

*In vitro*
- cells exposed to 350/500 mOsM NaCl

**Human neutrophil:**
- caused shedding of L-selectin
- prevented upregulation CD11b
- decreased adhesion to endothelium

**Human endothelium:**
- decreased ICAM-1

Chapter 3: Hypertonicity causes tyrosine phosphorylation of Src- and p38-dependent pathways, but only p38 mediates shedding of L-selectin.

Chapter 4: Hypertonicity causes remodeling of the actin cytoskeleton, which is responsible for the inhibition of many neutrophil function.

**NEUTROPHIL SHRINKAGE**

**Tyrosine phosphorylation**
- Src-dependent and Hck, Syk and Pyk2
- stress-activated MAPK p38

- Altered response to inflammatory mediators
- L-selectin shedding metalloprotease-dependent
- Apoptosis

- **Actin cytoskeleton polymerization**
- inhibited by latrunculinB
- mimicked by jasplakinolide

- Impaired:
  - exocytosis
  - upregulation adhesion molecules
  - release proteolytic enzymes
  - ROS generation
  - migration/chemotaxis
CHAPTER 2. Immunomodulatory Effects of Hypertonic Resuscitation on the Development of Lung Inflammation Following Hemorrhagic Shock

Summary

Hypertonic resuscitation fluids are known to be effective in restoring circulating volume in the hypovolemic trauma patient. Previous studies have suggested that hypertonicity might exert effects on immune cells leading to an altered host response. The present studies evaluated the effect of hypertonic resuscitation on the development of lung injury in a hemorrhagic shock model in which antecedent shock primes for increased lung neutrophil sequestration in response to intratracheal LPS. Resuscitation with hypertonic saline significantly reduced albumin leak, bronchoalveolar lavage fluid neutrophil counts and the degree of histopathological injury compared to resuscitation with Ringer’s lactate. Both in vivo and in vitro data suggest that this beneficial effect may be related to altered adhesion molecule expression by the neutrophil. Specifically, hypertonicity induced shedding of L-selectin and prevented LPS-stimulated expression and activation of CD11b, both of which might contribute to reduced sequestration in the lung. Impaired upregulation of lung ICAM-1 may have also participated, although ex vivo studies suggest that alterations in neutrophils was sufficient to account for the effect. Lung cytokine-induced neutrophil chemoattractant did not differ between animals resuscitated with hypertonic saline versus Ringer’s Lactate. Considered together, these studies demonstrate a possible novel approach to inhibiting organ injury in disease processes characterized by neutrophil-mediated damage.

Introduction

The major objective of fluid resuscitation in the traumatized patient with hemorrhagic shock is the restoration of effective circulating intravascular volume. The use of crystalloid solutions for this purpose is generally considered to be optimal given their effectiveness, low cost and minimal morbidity. A metaanalysis of studies comparing the use of crystalloid to colloid solutions in patients with hypovolemic shock demonstrated a 12% reduction in overall mortality when patients were resuscitated with a crystalloid-based approach. Resuscitation with small volumes of hypertonic saline (HTS) solutions has been shown to restore circulating volume and hemodynamics, in part through imbibement of intracellular fluid into the vascular space. The ability to resuscitate with reduced fluid volume has been considered advantageous, in that it might reduce the potential consequences of third space fluid sequestration such as the development of cerebral edema in the head injured patient or pulmonary edema in the patient with contused lung. Further, some studies have reported improved cardiac contractility with HTS resuscitation regimens. Both experimental and clinical trials have suggested a more favorable outcome following HTS resuscitation from hemorrhagic shock, although the mechanisms underlying this perceived benefit remain poorly elucidated.
Recent studies have suggested that hyperosmolar solutions exert alterations in immune cell function which may contribute to improved outcome. Junger and colleagues reported the ability of HTS resuscitation to reverse hemorrhage-induced suppression of splenocyte proliferation in a murine model. In subsequent experiments, these investigators demonstrated that hyperosmolarity can enhance mitogen-stimulated T-cell proliferation in vitro through a mechanism involving tyrosine phosphorylation and activation of the p38 mitogen activated protein kinase. The reversal of trauma-induced immunosuppression by HTS may represent a mechanism whereby this resuscitation strategy may lessen septic complications and thus enhance survival.

Significant morbidity in the trauma patient also results from the development of multiple organ dysfunction, even in the absence of infection. This process is predominantly mediated by the sequestration of activated neutrophils within the microvasculature and subsequent release of their injurious products such as reactive oxygen species and proteolytic enzymes. The lung appears to be a particularly prone target with the appearance of the Acute Respiratory Distress Syndrome (ARDS) in up to 30% of the traumatized patient population. Hemorrhagic shock is felt to contribute to the pathogenesis of ARDS by rendering the patient more susceptible to a second, seemingly trivial, inflammatory stimulus, the so-called "two-hit" model. In this regard, previous studies have suggested that the process of ischemia-reperfusion might mediate this effect by priming circulating neutrophils for increased superoxide production and therefore enhanced cytotoxicity once they are sequestered in the lung.

Previous reports have documented the ability of hyperosmolar solutions to inhibit a variety of neutrophil functions including the generation of reactive oxygen species. We therefore hypothesized that HTS resuscitation from hemorrhagic shock might lessen the development of subsequent lung injury. To test this possibility, we used a rodent model wherein resuscitated hemorrhagic shock primes for increased pulmonary leukosequestration and albumin leak in response to a small dose of intratracheal LPS. The data demonstrate that HTS resuscitation from shock markedly reduced lung injury in response to LPS by preventing neutrophil sequestration. This effect was transitory and could be restored with repeated infusion of HTS. This effect appeared to be predominantly mediated via an effect on neutrophil adhesion molecule expression.

**Results**

**Hypertonic saline resuscitation restores blood pressure and increases serum osmolarity**

Animal survival at the end of the experimental period in both resuscitation groups exceeded 90% with deaths due to tracheal obstruction secondary to the presence of the intratracheal cannula. Hypertonic saline resuscitation effectively restored mean arterial pressure to preshock levels. Figure 1 illustrates the serum osmolarity at various time points following resuscitation with HTS or RL. As shown, HTS caused
a rapid increase in osmolarity that peaked at approximately 500 mOsm at 5 min and then returned to approximately 350 mOsm by one hour. This was sustained over the ensuing 3 hours.

**Hypertonic saline resuscitation reduces lung injury following LPS**

Our laboratory has previously reported that resuscitated shock primes the lung for increased transpulmonary albumin leak in response to LPS. As demonstrated in Figure 2, both shock alone and LPS alone caused small increases in albumin leak. The administration of LPS following shock resuscitated with RL caused a further significant rise in albumin leak. However, when shocked animals were resuscitated with HTS, the increase in albumin leak was totally prevented.

Neutrophil sequestration is a hallmark of acute lung injury. BAL fluid was evaluated for neutrophil counts. Figure 3A shows alveolar neutrophil counts under each of the conditions. Compared to shock/LPS animals resuscitated with RL, the use of HTS caused >50% reduction in the number of neutrophil recovered in the BAL. As a measure of overall lung sequestration of neutrophils, lung MPO levels were studied. As demonstrated in Figure 3B, HTS resuscitation caused reduced levels of MPO compared to animals resuscitated with RL. Reduced lung inflammation with HTS resuscitation was also seen in the histopathology of the lung (Fig. 4). Shock animals resuscitated with RL prior to LPS administration (Left Panel) exhibited neutrophilia in the interstitium and in the alveolar space, while shock/LPS animals repleted with HTS demonstrated lung histology comparable to sham animals with few neutrophils and minimal alveolar hemorrhage and edema. Considered together, these data suggest that the protective effect of HTS resuscitation may be related to the reduced pulmonary leukosequestration.

**Effect of HTS Resuscitation on Lung Chemokine expression**

We recently showed that augmented pulmonary neutrophil sequestration in this model was mediated by increased expression of the C-X-C chemokine CINC (cytokine-induced neutrophil chemoattractant) in the lung. To determine whether HTS might exert its effect by suppressing CINC expression, we studied CINC mRNA in whole lungs of animals resuscitated with RL or HTS. As shown in Figure 5, the shock/LPS-induced increase in CINC mRNA did not differ between RL and HTS infusion. A representative blot is shown in Panel A and the mean of multiple studies normalized for G3PDH is shown in Panel B. These data suggest that altered CINC does not account for the attenuation of neutrophil accumulation following HTS.
Figure 1: Serum osmolarity at various time points after shock/resuscitation and LPS.

Following hemorrhagic shock, rats were resuscitated with either Ringer’s lactate or hypertonic saline and serum osmolarity measured at the times indicated. The data represent the mean ± SEM for 3 animals per point. *=p<0.01 versus Ringer’s lactate at same time points; **=p<0.05 versus Ringer’s lactate at same time points.

Figure 2: Transpulmonary albumin leak index following shock/resuscitation with either RL or HTS.

Six hours after intratracheal LPS, BAL fluid $^{125}$I-albumin was measured by gamma counting and normalized for blood counts as indicated in METHODS. The data represent the mean ± SEM for 5 animals per group. *=p<0.05 versus sham, **=p<0.01 versus sham, ***=p<0.01 versus shock/LPS/RL.
Figure 3: Neutrophil sequestration in the lungs following shock/resuscitation and LPS.

3A: BAL neutrophil counts. Four hours after intratracheal LPS, neutrophils in the bronchoalveolar lavage fluid were counted using a hemocytometer. The data represent the mean ± SEM for six animals per group. * = p < 0.01 versus sham, ** = p < 0.01 versus shock/LPS/RL.

3B: Lung myeloperoxidase. Lungs were harvested at four hrs following LPS administration and evaluated for total myeloperoxidase as described in METHODS. The data represent the mean ± SEM for 3 animals per group. * = p < 0.01 versus sham, ** = p < 0.01 versus shock/LPS/RL.

Figure 4: Lung histology after shock/resuscitation and LPS.

4A: Following Ringer's lactate resuscitation, histology of the lungs showed edema, intra-alveolar hemorrhage and extensive neutrophil accumulation. Representative picture of 3 animals per group, hematoxylin-eosin, X40.

4B: Following resuscitation with hypertonic saline, the histological assessment of the lungs demonstrated marked improvement. Representative pictures of 3 animals per group, hematoxylin-eosin, X40.
Figure 5: Effect of resuscitation regimen on lung CINC mRNA expression.

A.

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<td>RL</td>
<td>HTS</td>
</tr>
</tbody>
</table>

B.

Normalized CINC mRNA levels

<table>
<thead>
<tr>
<th>sham</th>
<th>LPS</th>
<th>RL</th>
<th>HTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>600</td>
<td>800</td>
</tr>
</tbody>
</table>

Four hours after LPS, lungs were recovered and processed for Northern blot analysis.

5A: Total lung RNA was obtained at sacrifice, probed for CINC mRNA and detected by autoradiography. G3PDH reprobing confirmed equal RNA loading. Representative Northern blot of 5 animals per group.

5B: Densitometric analysis of CINC mRNA normalized for G3PDH. The data are the mean ± SEM for 5 animals per group. *p<0.05 versus sham.

Figure 6: Effect of ex vivo hypertonicity on neutrophil sequestration in the lung.

A.

$^{51}$Cr-labeled PMN lung accumulation

6A: Sequestration of $^{51}$Cr neutrophils in animals following incubation in isotonic or hypertonic medium.

After isolation, donor rat neutrophils were labeled with $^{51}$Cr, treated in either isotonic (isoPMN) or hypertonic medium (350 mOsm, htPMN), returned to isotonic medium and injected into shock/LPS animals resuscitated with RL. Lungs were recovered and analyzed in the gamma spectrometer.

The degree of neutrophil labeling was unaffected by incubation in either isotonic or hypertonic medium. The data are the mean ± SEM for 3 animals per group, *p<0.01 versus all other groups. Freshly isolated cells exhibited similar sequestration as those reported for cells incubated in isotonic medium for 4 hours (Fresh cells: SHAM 2.5%±1.1 versus SHOCK 17.4%±0.4, n=3).

6B: Membrane potential of neutrophils following incubation in either isotonic medium or hypertonic medium (500 mOsm) for 4 hours at 37°C. Membrane potential was assessed by using the anionic fluorescent probe diS-C3 as described in METHODS. Where indicated, valinomycin (val, 2μM) and gramicidin (grami, 5μM) were added to the cuvette. Two representative traces of three separate experiments.
**Ex-vivo treatment of neutrophil with hypertonicity prevents lung neutrophil sequestration**

To discern whether the changes in neutrophil function were sufficient to account for the reduced pulmonary leukosequestration, neutrophils were labeled ex vivo and exposed to isotonic or hypertonic medium for 4 hours and then resuspended in isotonic medium. They were then reinfused into sham animals or animals following RL resuscitation from shock, immediately prior to LPS administration (Fig. 6A). As expected, sham animals given isotonically treated neutrophil or hypertonically treated neutrophil did not exhibit neutrophil accumulation in the lung. Further, shocked animals resuscitated with RL showed increased neutrophil counts in the lung when isotonic-treated neutrophil were given. However, neutrophil exposed to hypertonicity ex vivo failed to sequester in the lung. The viability of cells following in vitro treatment was confirmed by their capacity to exclude trypan blue. The integrity of the cell membrane (i.e. low permeability to monovalent ions) was also demonstrated by the fact that hypertonically treated cells maintained their transmembrane potential and were able to hyperpolarize after the addition of the potassium ionophore valinomycin (Fig. 6B). Considered together, these data support the concept that hypertonicity may exert its protection by a direct effect on neutrophils, which precludes their sequestration in the lung.

**Hypertonicity alters neutrophil surface expression of CD11b and L-selectin**

The initial steps leading to neutrophil sequestration in the vasculature involve interactions between neutrophil surface receptors and counter-ligands on endothelial cells. To evaluate whether HTS might impair neutrophil adhesion molecule expression and thus contribute to the reduced neutrophil sequestration, blood was obtained from animals following each resuscitation protocol and evaluated for adhesion molecule expression. Figure 7 demonstrates CD11b expression in vivo in animals resuscitated with RL or HTS. RL animals showed a significant increase in CD11b at the end of resuscitation compared to preshock. This was sustained over the ensuing 4 hours until the time of sacrifice. By contrast, in the HTS-treated animals, CD11b levels did not increase compared to the preshock time point over this time period. Figure 8 shows levels of L-selectin on blood neutrophils following the two resuscitation regimens. In animals resuscitated with RL, L-selectin levels remained stable over time, while in HTS-treated animals, there was a significant loss of surface L-selectin expression.

To determine whether the effect of HTS on adhesion molecules was a direct one, we studied their expression on human neutrophil in vitro following exposure to various levels of hyperosmolarity. While HTS had no effect on CD11b itself, a four-hour incubation in HTS prevented the increase in response to LPS, FMLP and the phorbol ester PMA (Fig. 9A). CD11b expression on freshly isolated cells stimulated with FMLP or LPS did not differ from the expression observed after a four-hour incubation in isotonic medium. The level of CD11b expression on PMA-stimulated freshly isolated cells was ~35% higher than following a four hour incubation in isotonic medium (Fig. 9A). Several studies have suggested that increased CD11b expression is not necessary for augmented function. To
discern whether exposure of neutrophils to hypertonicity alters CD11b function, adhesion to endothelial cells was evaluated (Fig. 9B). As expected, LPS-stimulated neutrophils exhibited increased adhesion to an endothelial cell monolayer pretreated with TNF-α. However, this rise was inhibited by pretreatment with hypertonic medium. Considered together, these findings suggest that hypertonicity is able to impair both LPS-induced upregulation of CD11b surface expression and its activation. In addition, incubation in 350 mOsM in vitro caused a time dependent reduction in L-selectin levels, an effect which was augmented by increasing the osmolarity to 500 mOsM (Fig. 9C). L-selectin levels on cells incubated for 4 hours in isotonic medium did not differ from fresh cells and were significantly higher than cells incubated for 4 hours in either 350 mOsM or 500 mOsM (Fig. 9C).

**Hypertonicity alters endothelial cell ICAM-1 expression**

Endothelial cell ICAM-1 is an important ligand for neutrophil CD11b and has been implicated in mediating neutrophil-endothelial interaction at sites of inflammation. While HTS-induced alterations in neutrophils were shown to be sufficient to account for the reduced leukosequestration, this did not rule out the possibility that endothelial cells might themselves be affected by this regimen. To determine the effect of HTS resuscitation on ICAM-1 expression, we examined ICAM-1 mRNA and protein expression in the lung of animals following shock/LPS and either RL or HTS resuscitation. Four hours after LPS infusion, the RL resuscitated animals showed a marked increase in ICAM-1 mRNA expression as shown in a representative blot (Fig. 10A). Densitometric evaluation of several studies revealed ~50% reduction in ICAM-1 mRNA expression in animals resuscitated with HTS (Fig. 10B). Similar reductions in whole lung ICAM-1 protein were observed in HTS-resuscitated animals compared to RL-treated animals (Fig. 10C).

**Late effect of hypertonic resuscitation on neutrophil sequestration within the lungs**

To determine the duration of the priming effect of hemorrhagic shock and the hypertonic-effect on pulmonary leukosequestration, LPS instillation was delayed for 18 hrs. As shown in figure 11, late administration of LPS to animals primed with hemorrhagic shock resulted in a less marked influx of neutrophils into the lungs compared to when LPS was given 1 h following resuscitation. However, the increased accumulation of neutrophils in the lungs, occurred independent of the type of fluid resuscitation regimen used 18 hrs earlier. These results suggest that, while the priming effect of hemorrhage persists, the protective effect of hyperosmolarity is lost by 18 hours.

Subsequent studies were performed to discern whether the protection could be restored by a second dose of HTS. As shown on figure 11, when a second infusion of HTS was given at 18 hrs, the LPS-induced leukosequestration was dramatically reduced, reaching levels comparable to animals that were not even manipulated. Considered together, these data support the concept that the protective effect of hypertonicity is transient but fully reversible and that a repeated application, may restore the inhibition of neutrophil sequestration.
Hypertonicity reduces rat neutrophil expression of CD11b in parallel to reducing leukosequestration

In neutrophils, β2-integrins (CD11b/CD18) play a major role in neutrophil adherence to endothelial cells. We tested whether HTS might impair neutrophil adhesion molecule expression, which may contribute to the reduced leukosequestration. For this end, we used blood from animals following each resuscitation protocol and evaluated for CD11b expression.

Figure 12 demonstrates that RL-resuscitated animals had a significant increase in CD11b at the end of resuscitation compared to preshock. This was reduced but still significantly higher than sham animals at 18 hours. Following administration of LPS, CD11b expression increased to peak levels observed following resuscitation. By contrast, in HTS-treated animals, CD11b levels following hemorrhage and resuscitation were initially reduced when compared to the preshock time point. Paralleling the accumulation of neutrophils in the lungs, the CD11b expression was similar in RL- versus HTS- treated rats by 18 hrs, and both underwent a similar increase following LPS (Fig. 12).

A second bolus of HTS significantly reduced CD11b expression after LPS infusion, compared to both RL and single-HTS bolus treatment suggesting that HTS prevents the LPS-induced CD11b upregulation transiently and reversibly and these changes parallel the neutrophil influx into the lungs.

The transient effect of hypertonicity on neutrophil function

Having found that the effect of hypertonicity on pulmonary leukosequestration in vivo was transient, we tested whether the same occurred at the cellular level. Diverse neutrophil functions are affected by hypertonicity. We studied neutrophil spreading capability because this function could be directly observed by confocal microscopy. Human neutrophils were allowed to spread over a glass surface while being monitored on a confocal microscope (Fig. 13). While isotonically-treated neutrophils rapidly spread over the glass (Fig. 13A), virtually none spread when the medium was made hypertonic (Fig. 13B). This data clearly shows the complete loss of spreading function induced by osmotic shock. Interestingly, the return of hypertonia-challenged cells to isotonic conditions, even after hours of hypertonic exposure, was accompanied by the restoration of the ability to spread (Fig. 13C), showing that also at the cellular level, the effect of hypertonicity on neutrophil function is temporary. The inhibitory effect appeared to last exclusively while under osmotic stress. Furthermore, the fact that the neutrophils regain the ability to spread suggests that the neutrophils remain viable but incapable of functioning. Considering the anti-inflammatory effect of hypertonicity on neutrophil-mediated organ injury described earlier in this chapter, we especulate that hypertonicity might exert a similar potent but transient inhibitory effect in many other pro-inflammatory neutrophil functions.
Figure 7: Hypertonic saline resuscitation on CD11b expression on rat neutrophils.

Blood was sequentially collected from an animal at the beginning of the study, one hour after resuscitation immediately prior to LPS administration and 4 hours after LPS. Whole blood was incubated with FITC-labeled anti-CD11b antibody and after erythrocyte lysis, analyzed by flow cytometry. The data are the mean ± SEM for 5 animals per group. *p<0.05 versus sham and versus hypertonic saline at the indicated time points. For comparison, the starting control value for each animal was normalized to 100 and subsequent readings were compared relative to this.

Figure 8: Effect of hypertonic saline resuscitation on neutrophil L-selectin in vivo.

Blood was sampled from animals at the beginning of the protocol prior to shock/resuscitation as well as at 4 hours after LPS administration. Neutrophils were isolated on a Ficoll gradient and incubated with FITC-labeled anti-L-selectin antibody prior to analysis by flow cytometry. The data represent the mean ± SEM of 3 animals at each time point. *p<0.05 versus sham and versus Ringer's lactate. For comparison, the starting control value for each animal was normalized to 100 and subsequent readings were compared relative to this.
Figure 9: The effect of hypertonicity on the expression of adhesion molecules and adhesive function of human neutrophils in vitro.

9A: CD11b expression on human neutrophils. Cells were incubated in iso or hypertonic medium (350 mOsm) for 4 hours. LPS (1 μg/ml, 1 h), PMA (50 nM, 20 min) or FMLP (100 nM, 20 min) was added immediately after isolation or 4 h later. The data in all studies represent the mean ± SEM, n=3 studies at each time point, *p<0.01 versus no treatment in isotonic medium for the same time point, **p<0.05 versus same treatment under iso conditions, #p<0.05 versus PMA stimulation 4 h later. No significant difference between FMLP- or LPS-treated neutrophils under iso conditions, immediately or 4 h following isolation.

9B: Neutrophil adhesion assay. Neutrophils were incubated in either iso or hypertonic medium (350mOsm) for 4 h, then left untreated or treated with LPS (1 μg/ml, 1h). Next, all neutrophils were resuspended in isotonic medium and layered on top of a TNF-stimulated endothelial cell monolayer (ECV 304) for 30 minutes. After repeated washings with PBS, adherent cells were counted by optical microscopy. *p<0.05 versus isotonic, **p<0.001 versus isotonic.

9C: L-selectin expression on human neutrophils. Cells were incubated in iso or hypertonic medium (350 and 500 mOsm) for up to 4 h and analyzed by flow cytometry. n=6 to 10 studies at each time point, *p<0.01 versus isotonic at the same time point, **p<0.001 versus isotonic at the same time point.
Figure 10: Effect hypertonic saline resuscitation on ICAM-1 expression in rat lungs.

A. ICAM-1 mRNA levels

B. Normalized ICAM-1 mRNA levels

C. ICAM-1 protein expression

10A: Total lung RNA was obtained at sacrifice, probed for ICAM-1 mRNA and detected by autoradiography. Reprobing for G3PDH confirmed equivalent RNA loading. Representative northern blot of 3 animals per group.
10B: Densitometric analysis of ICAM-1 mRNA normalized for G3PDH. The data are the mean ± SEM for 5 animals per group, * = p<0.05 versus sham and versus HTS.
10C: ICAM-1 protein expression. After sacrifice, whole lungs were homogenized in 1% triton, equal amounts of protein were separated on 15% SDS-PAGE, transferred to PVDF membranes and probed for total ICAM-1. Representative Western blot of 5 independent studies.

Figure 11: The transient effect of hypertonicity on neutrophil sequestration in the lungs following shock, resuscitation and LPS.

BAL neutrophil count (X10⁶)

The animals were manipulated as described in METHODS and LPS given intratracheally 18 h following resuscitation. Neutrophils were counted in the bronchoalveolar lavage (BAL) fluid 4 h after LPS instillation. The data represent the mean ± SEM for 5 animals per group. * = p<0.05 versus sham, ** = NOT significantly different than Sham animals. RL = Ringer's lactate, HTS = hypertonic saline (7.5% NaCl), x1 = single bolus, x2 two boluses of hypertonic saline.
**Figure 12:** The transient effect of hypertonic saline resuscitation on CD11b surface expression on rat neutrophils. The effect of a second bolus.

The animals were manipulated as described in METHODS. LPS was given intratracheally either at 1 hour (LPS 1 h) or 18 hours (LPS 18 hrs) following resuscitation. Animals were sacrificed 4 hrs after LPS instillation. CD11b expression was measured in whole blood using a monoclonal antibody and flow cytometry. The data represent the mean ± SEM for 5 animals per group. *p<0.05 lower than sham. HTS=hypertonic saline (7.5% NaCl), x2 two boluses of hypertonic saline.

**Figure 13.** The transient osmotic inhibition of neutrophil spreading.

A. Isotonic condition

B. Hypertonic condition

C. Hypertonic then isotonic condition

Freshly isolated human neutrophils were allowed to spread over a glass surface for 5 to 10 minutes while being monitored on a confocal microscope.

13A: Neutrophils suspended in isotonic saline buffer (290 mOsM).

13B: Neutrophils suspended in hypertonic saline buffer (500 mOsM).

13C: Neutrophils suspended in hypertonic saline buffer for up to 4 hours then quickly sedimented and resuspended in isotonic saline buffer.
Discussion

Hypertonic saline resuscitation from hemorrhagic shock is known to be an effective means of restoring circulation in the traumatized patient. The present studies demonstrate that this resuscitation protocol may have additional effects that may serve to prevent the development of lung injury. Specifically, using a "two-hit" model of lung injury in the rat, hypertonic resuscitation from hemorrhagic shock was shown to lessen transpulmonary albumin leak, reduce pulmonary leukosequestration and prevent histopathological changes associated with lung injury compared to animals resuscitated with Ringer's lactate.

Neutrophils transiently incubated in hyperosmolar solution *ex vivo* failed to sequester in lungs of shock/LPS animals resuscitated with RL. This finding suggests that hypertonicity-induced changes in circulating neutrophils may be sufficient to account for the salutary effect of HTS resuscitation in this model. In this regard, blockade of surface integrins or selectin molecules has been shown to prevent neutrophil accumulation in various models of inflammation. In the present studies, both *in vivo* and *in vitro* data suggest that hypertonicity might exert its effect on neutrophils through altering adhesion molecule expression: L-selectin via increased shedding and CD11b through impaired LPS-stimulated upregulation and activation. The mechanisms underlying these alterations warrant further investigation. In principle, both the induction of volume-sensitive signaling cascades as well as shrinkage-related changes in the cytoskeleton might contribute to the overall effect. For example, osmotic shrinkage has been reported to cause extensive tyrosine phosphorylation in neutrophils and fibroblasts. This may be caused by clustering of surface receptors related to osmotic shrinkage, a process that has been shown to stimulate various receptors on the surface of HeLa in the absence of their ligands. Relevant to the present studies, clustering of the Leu-13 receptor on lymphocytes has been shown to cause L-selectin shedding through a tyrosine kinase-dependent mechanism. While cellular activation might be expected to lead to concomitant upregulation of CD11b and activation, hypertonicity alone did not alter surface CD11b expression and actually inhibited its stimulated upregulation *in vivo*. *In vitro*, both the level of CD11b expression and its activation in response to LPS were impaired by hypertonicity. Using an *in vitro* system, Hampton and colleagues reported that hyperosmolarity in the range of 500-700 mOsM significantly impaired FMLP-stimulated degranulation of secondary granules in neutrophils as evidenced by reduced release of the granular enzyme lysozyme. A comparable mechanism for impaired LPS-induced upregulation of CD11b might be invoked, given that CD11b is also present to some extent in secondary granules as well as in secretory vesicles in these cells. Recent studies testing the effect of the actin polymerizing agent jasplakinolide on neutrophil function revealed that this agent was able to prevent FMLP-induced expression of CD11b in neutrophils, suggesting that the state of the actin cytoskeleton might influence exocytic translocation of receptors to the cell surface. Cellular F-actin content is known to be increased following hypertonicity-induced cell shrinkage and
may therefore have similarly contributed to the impaired LPS-stimulated increase in CD11b both in vivo and in vitro.

In the present studies, the investigations regarding altered neutrophil sequestration focused on the effect of hypertonicity on the expression of CD11/CD18 and L-selectin. The rationale for choosing these particular adhesion molecules was based on previous studies demonstrating their role in lung neutrophil accumulation and subsequent tissue damage in models of acute lung injury. For example, inhibitory anti-CD18 monoclonal antibody was shown to markedly reduce neutrophil emigration into the alveolar space of rabbits following intratracheal instillation of LPS \(^{528}\). Similarly, anti-L-selectin antibodies diminished lung neutrophil accumulation in a rodent ischemia/reperfusion model as well as in an injury model induced by intrapulmonary instillation of IgG complexes \(^{529,530}\). However, particularly in the first model, inhibition of leukocyte accumulation was found to be incomplete, suggesting a role for CD18-independent mechanisms. Recent studies using adhesion molecule-deficient mice have provided significant support for this concept. In both L-selectin-deficient \(^{238,239}\) as well as CD18-deficient mice \(^{239}\), neutrophil emigration into the lung in response to intratracheal challenge with either live *Escherichia coli* or *Streptococcus pneumoniae* was actually increased compared to wild type animals. Whether intratracheal LPS in these animals will result in comparable findings of CD11- and L-selectin-independent neutrophil sequestration or findings similar to those reported in the aforementioned antibody studies remain to be tested. However, since the requirement for a particular adhesion molecule in pulmonary leukocyte sequestration is markedly dependent on the inflammatory stimulus \(^{528,530}\), previous work would suggest that, in the shock/LPS model used in the present studies, a prominent role for CD11/CD18 might be predicted. Based on this consideration, it seems plausible that the ability of hypertonic medium to inhibit upregulation and activation of this adhesion complex might contribute significantly to the inhibition of neutrophil sequestration following hypertonic resuscitation. The \(~30\%\) reduction in L-selectin levels in hypertonic resuscitated animals may be contributory, although alone it is unlikely to have been responsible for the observed protection. Other adhesion molecules may also be important and require further study.

We found that the osmotic-induced changes in CD11b expression correlated with the sequestration of neutrophils within the lungs. This finding further strengthens the notion that one major mechanism underlying the protective effect of hypertonicity is related to its ability to inhibit CD11b upregulation induced by inflammatory stimuli. However, the beneficial effect of hypertonicity was transient and lost over time (18 hrs) in the animal model. LPS failed to induce CD11b upregulation for as long as the cells are kept under hypertonic conditions. Therefore, the most plausible explanation for the reversibility of the hypertonic effect is that, once serum osmolarity returns to normal, neutrophils may regain their function and, in addition, new cells enter the circulation that had not been exposed to hypertonicity.
Moreover the duration of the hypertonic exposure seems to influence the neutrophil function, even after returning to isotonic conditions. Specifically, cells resuspended in isotonic medium after 2 hours of hypertonic treatment immediately regained their ability to upregulate CD11b in response to LPS. In contrast, 4-h pretreatment with hypertonicity resulted in the complete loss of an immediate response to LPS. The length of hypertonic exposure did not affect neutrophil spreading over a glass surface. When suspended in a hypertonic medium, the spreading was totally prevented. However, this function was immediately reestablished, even after prolonged (4 hrs) hypertonic treatment, once the cells are suspended in an isotonic medium (Fig. 13).

An important consideration is that the protective effect of HTS resuscitation is shorter in time than the priming effect of hemorrhagic shock. Thus, we hypothesized that repeated infusions of HTS would be required in order to maintain the protective effect. In fact, a second infusion HTS was at least as effective as the first one in protecting the lung. The clinical implication of our findings is that the successful modulation of the patient’s inflammatory response is likely to require repeated boluses of HTS. On the other hand, the reversibility of the hypertonic effect allows a quick and safe modulation of neutrophil functions, without causing undesirable, long-lasting immunosuppression. The molecular and biochemical mechanisms underlying the transient inhibitory effect of hypertonicity on CD11b expression are unknown.

During the process of transmigration, neutrophils have been shown to swell by up to 60%. Preventing this volume increase in vitro using hyperosmolar solutions (350 mOsM) inhibited the chemotactic index by approximately 50%. In the present studies, we did not specifically evaluate the relative effects of hypertonicity on leukocyte sequestration in the lung vasculature versus transmigration of neutrophils into the alveolar space. However, two lines of evidence suggest that reduced sequestration may have accounted for the effect. First, lung MPO levels, a measure of overall neutrophil sequestration, were lowered concomitant with the reduction in BAL neutrophils. Second, neutrophils exposed to a hypertonic incubation ex vivo failed to accumulate in the lung tissue. While these data together implicate reduced sequestration as a mechanism for the effect of HTS, we cannot rule out the possibility that reduced transmigration may have additionally contributed to the diminution of BAL neutrophil counts in HTS resuscitated animals.

Angle and colleagues recently reported that hypertonic saline resuscitation in a mouse hemorrhagic shock model caused a modest reduction in the lung injury score as well as an improvement in the histopathological appearance of the lung. Neutrophil CD11b levels did not differ between control and HTS resuscitated animals, while L-selectin, chemokine and endothelial cell adhesion molecules were not examined. Importantly, the murine model differs significantly from the “two-hit” model in the rat presented herein in that hemorrhage/resuscitation alone in the mouse suffices to induce inflammation in the lung concomitant with increased expression of pro-inflammatory and
immunoregulatory cytokines. In man, hemorrhage/resuscitation alone rarely initiates such a profound response, suggesting that the “two-hit” model may be more representative of human disease.

Nevertheless, the murine model may prove valuable in dissecting out some of the mechanistic aspects of the salutary effects of hypertonic resuscitation.

In summary, the present studies demonstrate a beneficial immunomodulatory effect of hypertonic resuscitation on the development of lung injury. This effect appears to be due to an inability of circulating neutrophils to sequester at the site of injury. These findings suggest the possibility that the induction of a transient serum hyperosmolarity might minimize tissue injury in other inflammatory processes, which are characterized by neutrophil infiltration and damage, such as those which occur following ischemia/reperfusion.
CHAPTER 3. Cell Volume-Dependent Regulation of L-selectin Shedding in Neutrophils: A Role for p38 Mitogen-Activated Protein Kinase

Summary

Neutrophil-mediated organ damage is a common feature of many disease states. We previously demonstrated that resuscitation with hypertonic salt solutions prevented the endotoxin-induced leukosequestration and consequent lung injury, and this effect was partially attributed to an altered surface expression of adhesion molecules, CD11b and L-selectin. In this study we investigated the mechanisms whereby osmotic stress evokes L-selectin shedding.

The metalloprotease inhibitor RO 31-9790 prevented the osmotic down-regulation of L-selectin, indicating that this process was catalyzed by the same "sheddase" responsible for L-selectin cleavage induced by diverse inflammatory stimuli. The trigger for hypertonic shedding was cell shrinkage and not increased osmolarity, ionic strength or intracellular pH. Volume reduction caused robust tyrosine phosphorylation and its inhibition by genistein and erbstatin abrogated shedding. Shrinkage stimulated tyrosine kinases Hck, Syk and Pyk2, but prevention of their activation by the Src-family inhibitor PP1, failed to affect the L-selectin response. Hypertonicity elicited the Src family-independent activation of p38, and the inhibition of this kinase by SB203580 strongly reduced shedding. P38 was also essential for the FMLP- and lipopolysaccharide- but not the phorbol ester-induced shedding.

Thus, cell volume regulates L-selectin surface expression in a p38-mediated, metalloprotease-dependent manner. Moreover, p38 has a central role in shedding induced by many inflammatory mediators.

Introduction

The regulated expression of adhesion molecules on neutrophil surface plays an essential role in the interactions of these phagocytes with the endothelium and their subsequent transmigration into the tissues. The initial attachment followed by the slow rolling of neutrophils along the vascular endothelium is mediated by the binding of leukocyte L-selectin (CD62L) to its endothelial ligands. Once this early contact has been achieved, L-selectin is shed from the cell surface by proteolytic cleavage by an as yet unidentified membrane-bound metalloprotease ("sheddase") and firmer adhesion develops as a result of the ensuing upregulation of another class of adhesion molecules, the β2 integrins (e.g. CD11b).

Although neutrophils are indispensable for efficient host defense against invading microorganisms, their uncontrolled or excessive activation is a major contributor to disease. Thus, neutrophil-mediated tissue damage is a key pathologic factor in a variety of conditions including overwhelming infection, ischemia-reperfusion injury or post-hemorrhage adult respiratory distress syndrome. It is therefore not surprising that a variety of approaches have been tried to mitigate neutrophil/endothelium interactions and neutrophil activation. Potential strategies for intervention
include the blockade of adhesion molecules with specific antibodies \textsuperscript{530}, the neutralization of the released tissue-toxic molecular species such as reactive oxygen intermediates (e.g. by antioxidants) \textsuperscript{539}, and the interference with signaling pathways or membrane traffic involved in the potentially deleterious activation and degranulation of the cells. A novel example for this latter approach is the application of hypertonic salt solutions which brings about an efficient, safe and reversible suppression of numerous neutrophil functions \textsuperscript{138,439,444,447}. Using an animal model, we have recently shown that an elevation of serum osmolarity protected against neutrophil-mediated post-hemorrhage lung injury \textsuperscript{138}. Importantly, hypertonicity prevented the lipopolysaccharide (LPS)-induced upregulation of CD11b and caused adhesion-independent shedding of L-selectin \textit{in vivo} and \textit{in vitro} \textsuperscript{138}. Interference with proper neutrophil adhesion appears to have contributed significantly to the protective, anti-inflammatory effect of hypertonicity.

To date, the cellular mechanisms underlying the neutrophil-suppressive effects of hyperosmolarity remained largely undefined. The primary aim of the present study was to explore the signaling pathways that might participate in the osmotic shedding of L-selectin. Since we and others have shown that hypertonicity triggers robust tyrosine phosphorylation in various cell types including neutrophils \textsuperscript{138,158,392-394}, we therefore hypothesized that osmosensitive kinases might be involved in this process. We therefore investigated which kinases are activated by osmotic stress in neutrophils and to assess their potential contribution to L-selectin shedding. Moreover, the signaling mechanisms leading to L-selectin cleavage upon exposure of the cells to inflammatory stimuli, such as formyl peptides (FMLP) or LPS are also poorly understood. The involvement of protein kinase C-dependent and independent, presumably tyrosine kinase-dependent, mechanisms have been documented \textsuperscript{345}, but the relevant tyrosine kinases have not been identified. Thus, in addition to the specific osmolarity- or cell volume-related regulation of L-selectin expression, the present studies were performed to gain further insight into the general mechanisms responsible for the control of the surface expression of this adhesion molecule.

Our results demonstrate that the trigger for the osmotic effect on L-selectin is cell shrinkage, which initiates a p38 mitogen-activated protein kinase-dependent, metalloprotease-mediated cleavage of this adhesion molecule. In addition, p38 seem to play an important role in L-selectin shedding evoked by classical inflammatory stimuli as well.

Results

\textit{The effect of hypertonicity on surface expression of L-selectin}

To study the effect of hyperosmolarity on L-selectin expression, the tonicity of the medium containing $1 \times 10^6$ neutrophils was increased from 290 mOsM to 500 mOsM by the addition of extra NaCl. After various times of hypertonic exposure, the cells were resuspended in isotonic medium and stained for L-selectin analysis by flow cytometry. Hypertonicity caused a significant, time-dependent reduction in L-selectin expression on the neutrophil surface (Fig. 14). In contrast to the rapid shedding of
L-selectin which occurs within minutes following receptor ligation or chemokine activation\textsuperscript{219, 222, 231, 538}, the hypertonicity-elicited response was a slower process, which was clearly detectable in 30-60 minutes and progressed over time, reaching almost 100% loss of L-selectin from the surface by 4 hours.

Upon stimulation, L-selectin is proteolytically cleaved yielding a soluble fragment\textsuperscript{224, 244}. To confirm that the reduction in L-selectin expression was due to shedding, we determined soluble L-selectin in the supernatant of hypertonicity challenged cells, using ELISA. Hypertonicity (for 4 hours) caused essentially the same increase in soluble L-selectin as a 20-min treatment with phorbol ester, a condition known to cause an almost complete shedding of L-selectin from neutrophils\textsuperscript{244}. Taken together, these data show that hypertonicity induces a gradual shedding of L-selectin that results in decreased cell surface expression.

\textit{The effect of the metalloprotease inhibitor RO 31-9790 on the hypertonicity-induced shedding of L-selectin}

It has been proposed that a membrane-bound protease, referred to as L-selectin "sheddase", is the common final pathway catalyzing the cleavage of L-selectin after exposure of the cells to a variety of inflammatory stimuli\textsuperscript{224, 225, 243, 244, 535-537}. To determine whether hypertonicity-induced shedding of L-selectin is also mediated by this enzyme, a newly developed zinc-chelating hydroxamic acid derivative (RO 31-9790), reported to be a potent "sheddase" inhibitor was used\textsuperscript{244}. Consistent with its known activity, pre-incubation of cells with RO 31-9790 completely abolished the shedding of L-selectin induced by LPS, FMLP or the PKC-stimulator PMA. Importantly, the drug was equally effective in preventing the hypertonicity-triggered L-selectin shedding. Specifically, the number of L-selectin-positive neutrophils remained at the level observed in the isotonically treated cells even after prolonged hypertonic incubation (Fig. 15).

Since RO 31-9790 has been shown to exert inhibitory effects on matrix proteases\textsuperscript{244, 536, 540}, one possible mechanism of its effect on shedding might have been through inhibition of soluble proteolytic factor(s) released in response to hypertonic exposure. To test this possibility, 1.66x10\textsuperscript{6} neutrophils were incubated in 1 ml of hypertonic solution for 4 hours, pelleted and the supernatant collected. After the supernatant was diluted 1.66-fold with water to reestablish isotonicity, it was then added to 1x10\textsuperscript{6} fresh neutrophils for 4 hours. The supernatant of hypertonically treated cells caused no shedding of L-selectin from the test neutrophils, arguing against the possibility that hypertonicity induced the release of a soluble proteolytic factor which cleaved L-selectin. To further demonstrate that shedding was not mediated by a matrix proteinase, we added EDTA to the medium, an intervention that selectively inhibits these enzymes without affecting L-selectin "sheddase"\textsuperscript{535, 541}. Incubation of the cells with 5 mM EDTA had no effect on the hypertonicity-induced shedding. Together, these findings strongly support the notion that the L-selectin shedding caused by osmotic shrinkage is mediated by the same membrane-bound protease as that responsible for shedding after exposure to other stimuli.
The role of cell shrinkage in the hypertonic shedding of L-selectin

In addition to causing cell shrinkage, exposure to a hypertonic environment results in changes of intra- and extracellular osmotic concentration, ionic strength and intracellular pH (pH_i). The following experiments were designed to establish which of these variables is critical to trigger shedding. To separate the effect of cell shrinkage from that of medium osmolarity, we used incubation conditions that allowed manipulation of these parameters. Initially, the medium osmolarity was increased while maintaining the cell volume constant. This was achieved by the addition of 200 mM urea to the medium. Urea is a freely cell-permeant compound and thus rapidly equilibrates across the cell membrane, causing no cell shrinkage but increasing both intra- and extracellular osmolarity. In contrast to hypertonic NaCl, incubation of cells in urea did not alter neutrophil volume (Fig. 16B). Under the hyperosmolar but isovolemic conditions caused by urea, there was no shedding of L-selectin (Fig. 16A). To discern whether a rise in Na⁺ or Cl⁻ concentration or ionic strength of the medium triggered L-selectin shedding, neutrophil shrinkage was induced by the addition of a non-ionic osmolyte in the form of 200 mM sucrose. As shown in Figure 16B, hypertonicity caused by the addition of 200 mM sucrose resulted in cell shrinkage of similar magnitude to that observed with hypertonic NaCl (500mOsM) and also provoked a comparable degree of L-selectin shedding (Fig. 16A). These observations indicate that L-selectin shedding is related to a reduction in cell volume and not an increase in extracellular NaCl concentration or ionic strength.

Shrinkage is known to stimulate NHE1, the isoform of the Na⁺/H⁺ exchanger expressed in neutrophils leading to a rise in the intracellular pH (pH_i) of the shrunken cells. To evaluate whether NHE1-mediated cytosolic alkalization contributes to L-selectin shedding, we compared the effect of hypertonicity in the presence and absence of HOE694, a potent and highly selective inhibitor of the antiporter. As shown on Figure 17A, hypertonicity caused a sizable cytosolic alkalization that was completely abolished by HOE694. However, HOE694 did not influence the hypertonicity-induced shedding of L-selectin (Fig. 17B), suggesting that the latter process is not caused by the shrinkage-induced alkalization. The combined results of these experiments demonstrate that volume reduction (shrinkage) is the critical factor triggering the osmotic shedding of L-selectin, regardless of changes in osmolarity, ionic strength or in the intracellular pH.
Figure 14. **Hypertonicity-induced shedding of L-selectin.**

Neutrophils were suspended in isotonic (290 mOsM) or hypertonic medium (500 mOsM) for up to 4 hours. L-selectin surface expression was measured using a monoclonal anti-CD62L antibody and flow cytometry. The data represents the mean ± SEM, n=10 studies/group, *=p<0.05 versus control, **=p<0.001 versus control.

Figure 15. **The effect of the "sheddase" inhibitor RO 31-9790 on the downregulation of surface L-selectin by hypertonicity and other stimuli.**

Neutrophils suspended in isotonic medium (290 mOsM) were treated in the presence or absence of the metalloprotease inhibitor RO 31-9790 (30 μM) for 30 min. The medium was then supplemented with either an extra 100 mM of NaCl for 2 h, or LPS (1 μg/ml) for 1 h, or FMLP (100 nM) for 20 min or PMA (50 nM) for 20 min. The data represent the mean ± SEM, n=5 separate studies, *=p<0.001 versus control, **=p<0.01 versus control. HT=hypertonic treatment.
Figure 16. *Cell shrinkage but not hyperosmolarity triggers L-selectin shedding.*

A. Changes in cell volume and/or medium osmolarity were achieved by adding an extra 100 mM NaCl (NaCl), 200 mM urea (UREA) or 200 mM sucrose (SUC) for 2 hours as described in the text.

16A: L-selectin surface expression was measured using a mAb and flow cytometry. The data represents the mean ± SEM, n=3 separate studies, *=p<0.001 versus control isotonic (ISO).

16B: Cell volume was determined by electronic sizing using Coulter Counter and a Channelyzer. The data represent the mean ± SEM, n=3 separate studies, *=p<0.001 versus control isotonic (ISO).

Figure 17. *Shrinkage induces L-selectin shedding independent of cytosolic alkalization.*

A. 17A: Neutrophils loaded with the pH-sensitive fluorescent indicator BCECF were incubated in the presence or absence of the NHE1 inhibitor HOE694 (10 μM) for 1 min. Cells were then challenged with hypertonicity by adding an extra 100 mM NaCl and intracellular pH monitored by fluorometry. One representative pair of traces is shown.

17B: Neutrophils suspended in isotonic (290 mOsm) medium (ISO) were treated with or without HOE694 (10 μM) for 1 h then challenged with hypertonicity (500 mOsm). L-selectin surface expression was measured using a mAb and flow cytometry. The data are presented as the mean ± SEM for 3 separate studies, *=p<0.001 versus ISO, HOE=compound HOE694, HT=hypertonic treatment.
The role of tyrosine phosphorylation in L-selectin shedding

Protein tyrosine phosphorylation in response to various inflammatory stimuli has been shown to be important in the regulation of surface adhesion molecule expression. Recent studies have reported the ability of hypertonicity to induce extensive tyrosine phosphorylation in different cell types, including neutrophils. We therefore hypothesized that tyrosine phosphorylation might be involved in shrinkage-induced L-selectin shedding. To address this possibility, we initially used pharmacological means. Specifically, the effect of two broad-spectrum tyrosine kinase inhibitors, genistein and erbstatin, with different mechanisms of action was studied. Figure 18A shows that hypertonicity causes a large increase in accumulation of phosphotyrosine residues on many proteins, and this effect was substantially inhibited by both inhibitors. In addition, genistein and erbstatin analog significantly reduced the hypertonicity-stimulated shedding of L-selectin (Fig. 18B). This effect was not due to a direct inhibition of the “sheddase” because the drugs did not affect the PKC-mediated L-selectin shedding (Fig. 19). These findings therefore suggest that tyrosine kinase activation is involved in the shedding of L-selectin following osmotic shrinkage. Further investigation addressed the identity of the tyrosine kinases involved in this process.

Cell shrinkage has been shown to induce tyrosine phosphorylation of important signal transducing molecules in various cell types. These include certain non-receptor tyrosine kinases (e.g. Hck, Syk), members of the focal adhesion kinase family (Pyk2) and the MAP kinase superfamily (ERK, JNK, p38). Studies were performed to determine whether specific members of these groups might be involved in shrinkage-induced shedding of L-selectin in human neutrophils.

We first investigated Hck, an abundant Src family kinase expressed in neutrophils. Hck was immunoprecipitated from lysates obtained from iso- and hypertonically treated cells, and probed with anti-phosphotyrosine antibody to confirm its phosphorylation following cell shrinkage. As shown on Figure 20A, shrinkage induced a marked increase in the tyrosine phosphorylation of Hck. This effect was prevented by genistein and completely abolished by PP1, a newly developed pyrazolo pyrimidine-type compound, shown to be a Src family-specific inhibitor. Reprobing the same blots with anti-Hck antibodies confirmed that similar amount of Hck was precipitated from the iso- and hypertonic samples (lower panel). Thus, Hck is activated by shrinkage and its phosphorylation is either due to autophosphorylation or phosphorylation by other Src-like kinase(s). Interestingly however, PP1 failed to inhibit the hypertonic shedding (Fig. 20D). The differential effect of genistein and PP1 was used to investigate the identity of the tyrosine kinases involved in the shrinkage-induced shedding of L-selectin.

The possible role ofSyk was then examined. This non-receptor tyrosine kinase has been shown to be rapidly phosphorylated and activated in B-cells by both osmotic and oxidative stress, although its osmotic sensitivity has not been examined in neutrophils. As shown in Figure 20B, Syk was strongly phosphorylated upon cell shrinkage. This increase was impaired by PP1, suggesting that
hypertonicity-activated Syk through heterophosphorylation catalyzed by Src-type kinases, since PP1 does not inhibit Syk itself. These findings suggest that Syk is similarly not involved in L-selectin shedding. Further, piceatannol, a Syk-selective kinase inhibitor, also failed to inhibit L-selectin shedding by hypertonicity (Fig. 19).

One further candidate kinase which might contribute to L-selectin shedding is Pyk2, a calcium-dependent member of the FAK family, which has been shown to be activated by osmotic stress in rat liver epithelial cells and reported to be involved in the integrin-related signaling of hematopoetic cells. As shown on Figure 20C, Pyk2 was constitutively phosphorylated to a modest extent in control isotonic neutrophils and shrinkage induced further accumulation of phosphotyrosine in this protein. PP1 completely abolished not only the hypertonicity-induced increase but also the basal tyrosine phosphorylation of this kinase.

To rule out that the lack of effect of PP1 on shedding was not simply due to its metabolism or spontaneous decomposition, we tested whether PP1 could inhibit phosphorylation even after 2 hours of hypertonic treatment (i.e. when the shedding was measured). As shown on figures 20A, B and C (panels labeled as 2 h), hypertonicity induced sustained tyrosine phosphorylation in all three kinases. This response was however completely prevented in the presence of PP1, confirming that the drug exerted a long-lasting effect.

Therefore, each of the three kinases evaluated proved to be osmo-sensitive in neutrophils and their phosphorylation was strongly inhibited by PP1, a finding suggesting that the Src family catalyzed the phosphorylation. However, despite their activation, these kinases do not seem to be responsible for the hypertonicity-induced L-selectin shedding.
**Figure 18.** The role of tyrosine phosphorylation in the hypertonic shedding of L-selectin.

**A:** Neutrophils were treated in the presence or absence of either genistein (40 µM) for 20 min or erbstatin analog (30 µM) for 1h then challenged with hypertonicity (100 mM extra NaCl) for 10 min. Next, the cells were lysed, subjected to SDS-PAGE and immunoblotted using an anti-phosphotyrosine (4G10) antibody. Representative blot of 5 separate experiments.

**B:** Neutrophils were treated in the presence or absence of either genistein (40 µM) for 20 min or erbstatin analog (30 µM) for 1h then treated with hypertonicity (100 mM extra NaCl) for 2h. L-selectin surface expression was measured by flow cytometry. The data represents the mean ± SEM, n=4 separate studies. * = p<0.001 versus control, genistein and erbstatin, ** = p<0.05 versus control, genistein=genistein, erb=erbstatin analog.
Figure 19. The effect of diverse tyrosine kinase inhibitors on the downregulation of surface L-selectin by hypertonicity and other stimuli.

Neutrophils suspended in isotonic medium (290 mOsm) were treated in the presence or absence of piceatannol (100 μM), PP1 (10 μM), staurosporine (100 nM) or genistein (40 μM) for 20 min. The medium was then supplemented with either extra 100 mM of NaCl for 2 h, or LPS (1 μg/ml) for 1 h, FMLP (100 nM) or PMA (50 nM) for 20 min. The data represent the mean ± SEM, n=5 separate studies. *p<0.05 versus the stimulus alone, pic=piceatannol, staur=staurosporine, gen=genistein.
Figure 20. Osmotic responsiveness and inhibitory sensitivity of non receptor tyrosine kinases Hck, p72Syk and Pyk2 in the neutrophils.

A. Hck

B. Syk

C. Pyk2

D.

%Neutrophils positive for L-selectin

20A, 20B, 20C: Neutrophils were treated with either genistein (40 μM) or PP1 (10 μM) for 20 min then stimulated with hypertonicity (100 mM extra NaCl) for either a short term (1 min for Hck, 5 min for Syk and 2 min for Pyk2) or long term (2 h). Using specific monoclonal antibodies, the kinases were immunoprecipitated as described in METHODS, separated by SDS-PAGE and probed with anti-phosphotyrosine (4G10) mAb. The same membranes were stripped and re-probed with the corresponding antibodies to verify equal loading. One representative experiment of 3 similar ones is shown for each group. Gen=genistein, PY=tyrosine phosphorylation.

20D: The lack of effect of PP1 on hypertonicity-induced shedding of L-selectin. Neutrophils were treated with PP1 (10 μM) for 20 minutes prior to and during exposure to hypertonicity (100 mM extra NaCl) for 2 h. The data represents the mean ± SEM, n=10 separate studies, *=p<0.001 versus control, HT=hypertonic treatment.
Figure 21. The role of p38 MAPK in the shedding of L-selectin induced by hypertonicity and other stimuli.

A. Immunoblot

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<th>Phospho p38</th>
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<td>Control</td>
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B. Kinase assay

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<td>Control</td>
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<td>genistein</td>
<td>ATP-2</td>
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<td>PP1</td>
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C. Neutrophils were treated with either genistein (40 μM) or PP1 (10 μM) for 20 min then challenged with hypertonicity (100 mM extra NaCl) for 10 min or 2 h. Cells were lysed, subjected to SDS-PAGE and immunoblotted using polyclonal anti-phospho-p38. The same blot was then stripped and re-probed with an anti-p38 antibody.

21B: Following the same treatment as in 7A and exposure to hypertonicity for 2 hours, p38 was immunoprecipitated with a specific anti-p38 antibody. In vitro kinase assay was then performed using ATP-2 as a substrate and the results visualized with autoradiography.

21C: Neutrophils were treated in the presence or absence of the p38 inhibitor SB203580 (20 μM) for 20 min. The medium was then supplemented with either an extra 100mM NaCl for 2 h (HT), or LPS (1 μg/ml) for 1h, or FMLP (100 nM) or PMA (50 nM) both for 20 min. L-selectin surface expression was measured using a mAb and flow cytometry. The data represents the mean ± SEM, n=6 separate studies, *p<0.001 versus control (CON), **p<0.05 versus control, ***p<0.01 versus control, genist=genistein, HT=hypertonic medium.
The role of MAPK p38

Three distinct families of mitogen-activated protein kinases have been identified in mammalian cells: p42/44 ERKs, JNK/stress activated protein kinases and p38. While it has been established that in neutrophils neither ERKs nor JNK/SAPK (except in extreme hypertonicity) are activated by osmotic stress, the studies concerning osmotic activation of p38, have reported conflicting results.

To investigate whether shrinkage triggers phosphorylation of p38, the cells were subjected to hypertonic stress, lysed and immunoblotted with a specific anti-phospho-p38 antibody. As shown in Figure 21A, hypertonic exposure of the cells for 10 minutes caused a modest but readily detectable phosphorylation of p38. Since the effect of hypertonicity on L-selectin was a slow process, studies were performed to discern whether more prolonged hypertonic treatment might induce a persistent and stronger response. Figure 21A shows that by 2 hours of hypertonic exposure, there was a marked increase in p38 phosphorylation, a time course comparable to that of L-selectin shedding. This observation supports the involvement of this kinase in the shrinkage-induced shedding of L-selectin (Fig. 21A and B). To verify whether the increased phosphorylation was associated with an increased kinase activity even after 2 hours of treatment, p38 activity was determined in immunocomplex kinase assays using ATF-2 as exogenous substrate. Figure 21B shows that the kinase activity of p38 obtained from hypertonically treated cells was significantly increased compared to the isotonic controls. In addition, treatment of the cells with genistein completely prevented p38 phosphorylation while PP1 had absolutely no effect on this process (Fig. 21A and B). This inhibitory profile correlated with the differential ability of genistein and PP1 to affect the hypertonicity-induced shedding of L-selectin (Fig. 18B and 20D, respectively).

To determine whether p38 activation is in fact involved in L-selectin shedding, neutrophils were incubated with the inhibitor SB203580 before exposure to hypertonicity. SB203580 is a highly specific inhibitor of the p38 kinase activity with no effect on p42/44 ERKs and JNK/stress activated protein kinases, even at 100 µM. Since in suspension of human neutrophils substantial inhibition of p38 was attained at 10-30 µM of SB203580, we routinely applied 20 µM. As demonstrated in figure 21C, SB203580 inhibited the hypertonic shedding of L-selectin suggesting that p38 activation is required for this process. This novel finding led us to investigate whether p38 might play an important signaling role in the shedding of L-selectin induced by other stimuli such as LPS, FMLP and PMA. LPS and FMLP are strong stimulators of p38 in neutrophils, while PMA acts through the activation of PKC and has little effect on p38. Inhibition of p38 by SB203580 abrogated the shedding caused by LPS and FMLP, but not by PMA (Fig. 21C). Conversely, the PKC inhibitor staurosporin prevented the PMA-induced shedding but had no effect on the LPS- or hypertonicity-induced response (Fig. 19). These data suggests that p38 plays a major role in the hypertonicity-, LPS- and FMLP-mediated shedding of L-selectin, while the direct stimulation of PKC induces shedding in a p38-independent manner.
Figure 22. Differential inhibitor sensitivity of hypertonicity-induced L-selectin shedding and apoptosis.

Neutrophils were treated with the metalloprotease inhibitor RO 31-9790 (30 μM) for 30 min or the caspase-3 inhibitor DEVD-fmk (100 μM) for 1 h, then kept under hypertonic conditions (100 mM extra NaCl) for 18 h.

22A: Apoptosis was measured after resuspension in propidium iodide for 30 min and flow cytometry to determine DNA fragmentation. The data represents the mean ± SEM, n=4 separate studies, *p<0.05 versus control, **p<0.05 versus hypertonicity.

22B: L-selectin surface expression. The data represents the mean ± SEM, n=4 separate studies, *p<0.05 versus control, HT=hypertonic treatment, RO = RO 31-9790.
The relationship between apoptosis and the hypertonic shedding of L-selectin

Aging neutrophils undergo apoptosis spontaneously, a process which is accompanied by shedding of L-selectin. Frasch et al. recently reported that p38 is involved in the signaling of the stress-activated neutrophil apoptosis. It was therefore conceivable that L-selectin shedding might be a consequence of the hypertonicity-induced apoptosis or vice versa. To investigate the relationship between these events, we examined whether the inhibition of apoptosis interferes with shedding, and conversely, whether the inhibition of shedding interferes with apoptosis.

To abrogate apoptosis we used DEVD-fmk, a potent caspase-3 inhibitor. Neutrophils suspended overnight in isotonic DMEM/FCS underwent spontaneous apoptosis that was significantly increased by hyperosmolar stress (Fig. 22A). Treatment with DEVD-fmk protected the cells from the hypertonicity-induced apoptosis but did not inhibit the hypertonicity-induced shedding of L-selectin (Fig. 22A and B). Further, treatment with the "sheddase" inhibitor RO 31-9790 prevented the hypertonic shedding (Fig. 22B), but had no effect on the hypertonicity-induced increase in apoptosis (Fig. 22A and B). These data suggest that the hypertonicity-induced shedding of L-selectin is not a consequence of apoptosis. Although p38 is involved in the upstream signaling of both processes, shedding and apoptosis are not necessarily coupled to each other.

Discussion

The present study provides evidence that hypertonicity induces shedding of L-selectin from the surface of neutrophils. This process is triggered by a decrease of cell volume, is mediated by a RO 31-9790-sensitive protease ("sheddase") and involves tyrosine kinase activation. While several candidate tyrosine kinases appear to be osmo-sensitive, our studies determined that p38 stress kinase plays an important role in L-selectin shedding in response to hypertonicity.

Other pro-inflammatory stimuli including bacterial peptides, LPS, and cytokines have been previously reported to activate p38 in neutrophils, although the role of this kinase in mediating the functional responses to these stimuli has not been fully elucidated. Activation of p38 appears to participate in the regulation of phospholipase A2, in the FMLP- or TNF-α-induced superoxide production, in β2-integrin-dependent adhesion and oxidative burst and in stress-induced apoptosis. To our knowledge, the present studies are the first to implicate p38 as an essential mediator for the shedding of L-selectin. The central role of this stress kinase is underlined by our finding that its inhibition completely abolished the shedding triggered by both by LPS and by FMLP and strongly abrogated the process induced by hypertonic stress. Further, activation of p38 (e.g. by LPS or hypertonicity) can trigger L-selectin shedding in the presence of PKC inhibitors, suggesting independence from the PKC signaling pathway. In support of this notion, L-selectin shedding elicited via the direct stimulation of PKC by phorbol esters was insensitive to the inhibition of p38. PKC-independent, but tyrosine kinase-dependent shedding of L-selectin has been recently reported in
lymphocytes after ligation of the Leu-13 surface antigen \textsuperscript{245}. Considered together, these results indicate that the activation of PKC and p38 represent two distinct signaling pathways contributing to the regulation of L-selectin shedding.

The molecular mechanisms by which p38 activation leads to cleavage of L-selectin remain to be elucidated. It is conceivable that p38 is involved in the activation or membrane insertion (exocytosis) of the “sheddase”, or it may be necessary for rendering L-selectin susceptible for proteolytic cleavage. In this regard, the cytosolic tail of L-selectin has also been shown to bind to various cytoskeletal molecules \textsuperscript{223}. Phosphorylation of these interacting proteins may result in a conformational change that exposes the membrane proximal ecto-domain of L-selectin to a constitutively active “sheddase” \textsuperscript{231,244}. Alternatively, L-selectin could be a direct substrate of p38. Direct phosphorylation of L-selectin on serine residues has been shown to occur after activation of PKC in rat basophilic leukemia cells \textsuperscript{221}. However, this reaction seemed to be independent of the subsequent shedding, since no reduction was observed in the cleavage of L-selectin variants in which target serines were exchanged for alanines.

Hypertonicity has been shown to stimulate p38 in many different cells types \textsuperscript{158,285,392}. Although an earlier study reported that p38 may not be affected by hypertonicity in neutrophils \textsuperscript{393}, recent data, including those described herein, unambiguously show that this kinase is both phosphorylated and stimulated by osmotic shock in neutrophils \textsuperscript{158,294}. However, there are striking differences in the kinetics of p38 activation by the various stimuli, a finding that provides a plausible explanation for the apparent controversy. Thus, FMLP provokes a robust activation within a minute, while LPS requires approximately 20 minutes for maximal action. In contrast, the effect of hypertonicity, although detectable after 10 minutes, continues to increase for as long as 2-3 hours. For each stimulus, the time-dependence of p38 activation correlates well with the kinetics of L-selectin shedding, further confirming the causal relationship between the two phenomena. An interesting and possibly functionally important feature of this slow kinetics is that the slight p38 activation induced by hypertonicity at early time points may render the kinase refractory to the much stronger stimulatory effects of FMLP and LPS. For example, Junger \textit{et al} demonstrated that hypertonicity impaired FMLP-induced exocytosis \textsuperscript{158}, while our group recently reported that osmotic shrinkage inhibited LPS-stimulated CD11b upregulation \textsuperscript{140}. In both studies, exposure to hypertonicity inhibited ligand-induced activation of p38 stress kinase, without altering the interaction of the stimulus with the surface ligand. Further studies are warranted to elucidate the mechanisms contributing to the refractoriness.

The present studies showed that two important tyrosine kinases Syk and Pyk2 are osmotically activated in neutrophils and are probably downstream to the Src family. Rather surprisingly, however, these kinases do not seem to be responsible either for the osmotic activation of p38 or for the osmotic shedding of L-selectin. This finding does not exclude the possibility that the activation of Src family members may play a role in the shedding induced by other stimuli. In fact, the LPS- and to a lesser extent
the FMLP-triggered L-selectin release was partially sensitive to PPI suggesting that the Src family may be involved in p38 stimulation induced by these agonists.

Hypertonicity has been previously shown to interfere with several neutrophil functions including superoxide production \(^{447}\), phagocytosis \(^{439}\), adhesion and transmigration \(^{138, 444}\). In addition to these inhibitory effects on neutrophil function, the present studies show that shrinkage actively promotes the premature loss of an important surface adhesion molecule. This loss may impair not only the initial endothelium-neutrophil adhesive interactions, but also the L-selectin-mediated intracellular signaling, which helps prime cells for augmented superoxide production and attachment induced by subsequent stimuli \(^{222}\). Further, since hydroxamate-sensitive metalloprotease(s) are involved in the shedding of other surface molecules including TNF- and Fc-receptors \(^{554}\), it is conceivable that these proteins may also be lost from the surface of shrunken cells, rendering them unresponsive to their corresponding ligands. When considered together, these findings suggest that hypertonic solutions may exert a net anti-inflammatory effect, in addition to their resuscitative effects. The therapeutic application of hyperosmotic fluids may represent a safe, reversible and inexpensive means of lessening organ dysfunction in disease processes characterized neutrophil-mediated tissue injury such as ischemia/reperfusion and ARDS.

In summary, we show that cell volume regulates the expression of L-selectin on the surface of neutrophils. This observation led us to the recognition that p38 stress kinase plays an important general role in the control of L-selectin shedding and thereby in the regulation of adhesive interactions of neutrophils with their environment.
CHAPTER 4. Hypertonic Inhibition of Exocytosis in Neutrophils: Central Role for the Osmotic Remodeling of the Actin Skeleton

Summary

To gain insight into the cellular mechanisms whereby hypertonicity abrogates neutrophil functions, we investigated the osmotic inhibition of exocytosis triggered by diverse stimuli (formyl-methionyl-leucyl-phenylalanine, lipopolysaccharide, phorbol ester, and Ca\(^{2+}\) ionophore). Hyperosmolarity inhibited the mobilization of all four granule types independent of the agonist used, suggesting that it exerts a general block on the process of exocytosis itself, rather than on individual signaling pathways. Concomitantly, osmotic stress elicited a 2-fold increase in F-actin content and abolished depolymerization that normally follows agonist-induced F-actin assembly. Hypertonically polymerized actin formed a thick submembranous ring. The effect was reversible, and the pre-stimulus F-actin level was inversely proportional to the stimulus-induced granule release.

Cytochalasin had no effect on the hypertonic polymerization and inhibition of exocytosis. In contrast, the monomer-sequestering drug, latrunculin B prevented the F-actin response and entirely restored exocytosis. Latrunculin B did not affect the hypertonicity-induced shrinkage, the osmotic tyrosine phosphorylation of Syk and p38, or the activation of the Na\(^+\)/H\(^+\) exchanger. Inhibition of osmosensitive kinases did not alter the actin response.

The actin-polymerizing toxin jasplakinolide inhibited exocytosis under iso-osmotic conditions, without causing shrinkage or tyrosine phosphorylation. Actin polymerization, together with p38 activation, was also essential for the osmotically induced L-selectin shedding. Thus, osmotic shock induces cytoskeletal remodeling, and this process is a central mechanism whereby hypertonicity exerts many of its neutrophil-suppressive effects.

Introduction

Neutrophils play a pivotal role in antimicrobial host defense, but their uncontrolled activation is a key pathologic factor in a variety of inflammatory conditions, such as ischemia-reperfusion injury and the adult respiratory distress syndrome. Recent studies from this and other laboratories have shown that hyperosmotic solutions can exert strong anti-inflammatory effects in vivo, primarily by interfering with neutrophil transmigration into the tissues. Hypertonicity has been shown to inhibit a plethora of neutrophil functions, including superoxide production, release of certain granular contents, expression of surface attachment molecules, phagocytosis and migration. Although these effects are well documented, the underlying cellular mechanisms have remained largely undefined.

To find a link between volume change and the ensuing functional consequences, increasing attention has been focused to identify signaling pathways activated by osmotic shock. One of the most prominent responses to cell shrinkage is a dramatic increase in protein tyrosine phosphorylation.
We and others have shown that shrinkage results in the activation of at least two kinase pathways in neutrophils. It leads to the stimulation of members of the Src family (e.g. Hck), which in turn propagates the signal to other non-receptor tyrosine kinases (e.g. Syk, Pyk-2), and also initiates the Src family-independent activation of certain mitogen-activated protein kinases (MAPK), especially of p38 MAPK. The relationship between these kinase cascades and the alteration in the various functions have not been elucidated. So far, the only functional consequence that has been conclusively associated with hypertonicity-induced kinase activation is the p38-dependent cleavage of L-selectin from the neutrophil surface. The attempt to correlate the activation of the different kinase pathways with the inhibition of the above-mentioned neutrophil functions is further complicated by the fact that these cascades are generally activated by most of the stimuli that are strong enhancers of these same neutrophil functions. Thus, it is not clear whether the hypertonically treated neutrophils remain suppressed because of, or rather in spite of the activation of the various kinase cascades.

Another key target of the hypertonic effect might be the cytoskeleton, the integrity of which is a prerequisite for several neutrophil functions. Surprisingly, little is known about the effect of cell shrinkage on the actin skeleton, and the potential correlation between such a change and the osmotic inhibition of neutrophils has not been addressed. Earlier studies dealing with this issue have concentrated on the potential role of actin in regulatory volume changes. Neutrophil swelling was associated with a decrease in filamentous actin (F-actin) content, and an inverse relationship between volume and polymerized actin was observed in HL-60 cells. Based on these facts, we therefore hypothesized that hypertonicity interferes with the normal polymerization/depolymerization cycle and/or distribution of actin in neutrophils, and this effect may be an important common mechanism whereby many neutrophil functions can be altered.

The primary aim of the present study was to characterize the effect of hypertonicity on actin remodeling, and to examine the role of these changes in cellular function. We have selected exocytosis as an important functional representative, because the actin network is known to affect degranulation. In addition, the presence of four different granule types, whose exocytosis is regulated by a multitude of signaling pathways, offers an excellent opportunity to establish whether hyperosmolarity causes a general block in membrane traffic or whether it selectively interferes with specific signaling routes. We also intended to discern whether the activation of osmosensitive kinases may lay upstream or downstream of the observed cytoskeletal effects.

The present studies show that hypertonicity causes a marked increase in actin polymerization, and alters intracellular F-actin distribution, independent of tyrosine phosphorylation. In addition, we show that osmotic shock interferes with the process of depolymerization that normally follows stimulus-induced polymerization. Concomitant with these changes, hyperosmolarity abolishes exocytosis of all granule types, independent of the stimulus. Further, pharmacological inhibition of osmotic actin
polymerization restores exocytosis in shrunken cells, and induction of actin polymerization mimics the effect of hypertonicity under iso-osmotic conditions. These studies provide evidence that the osmotically triggered changes in the actin skeleton are key contributors to the neutrophil-suppressive effect of hypertonicity.

Results

The effect of hypertonicity on neutrophil granule exocytosis

Multiple signaling pathways trigger the fusion of the various neutrophil granules with the plasma membrane. To assess whether hypertonicity can exert a universal inhibition on the process of exocytosis per se, or whether its effect is restricted to certain granule types and/or to particular signaling routes, we exposed the cells to a variety of stimuli known to act through distinct mechanisms, and measured the exocytosis of each four granule type, under iso- and hypertonic conditions. To ascertain specificity, we used granule markers stored exclusively in one vesicle type and inserted into the membrane solely by exocytosis. Thus, to detect the insertion of primary granules, secondary granules and secretory vesicles, the surface expression of CD63, CD66b and CD35 were followed respectively. Surface labeling was detected on non-permeabilized cells using flow cytometry. To follow the fusion of tertiary granules that do not contain one exclusive marker, we measured the release of gelatinase B (MMP9) into the extracellular space by ELISA. The agents applied to trigger exocytosis included both receptor-mediated and receptor-independent stimuli. Specifically, we used the bacterial peptide FMLP, that requires both Ca\(^{2+}\)-signal and the activation of tyrosine kinases to elicit exocytosis; lipopolysaccharide, whose effect depends on the activation of p38 MAPK; the protein kinase C activator phorbol ester that induces granule release in a Ca\(^{2+}\) signal- and tyrosine kinase independent manner and the ionophore A23187, which acts via the direct elevation of intracellular Ca\(^{2+}\). Fig. 23 shows that under isotonic conditions, each of these agents was able to induce significant exocytosis, although their efficiency in the mobilization of the different vesicles varied, in agreement with earlier findings. Each of the four stimuli caused similar and substantial exocytosis of secretory vesicles and secondary granules, whereas tertiary granules were best mobilized with PMA, and the primary granules were most responsive to A23187, and resistant to LPS, confirming earlier findings. When these stimuli were added to hyperosmotically shrunken cells, a dramatic reduction in exocytosis was observed. This phenomenon was not due to altered epitope recognition by the corresponding antibodies, since flow cytometry was performed after resuspending the cell in the same isotonic solution. The osmotic prevention of exocytosis was essentially complete and affected all four granule types, suppressing their mobilization to near-baseline levels. Importantly, hypertonicity abrogated exocytosis regardless of the applied stimulus. Both Ca\(^{2+}\)-dependent and independent responses were prevented, and even the direct, A23187-elicited granule release was essentially abolished.
Figure 23. Hypertonicity inhibits exocytosis of all four neutrophil granules triggered by diverse stimuli.

Neutrophils were suspended in isotonic (290 mOsM, clear bars) or hypertonic medium (500 mOsM, hatched bars), stimulated by FMLP (100 nM for 20 min), PMA (10 nM for 10 min), A23187 (1 μM for 10 min) or LPS (1 μg/ml for 1 hour). Exocytic insertion of granule markers into the cell membrane was measured using FITC- or PE-labeled antibodies and flow cytometry. For primary granule determination, cells were pretreated with CB (10 μM). MCF = mean channel fluorescence. Data are mean ± SEM of at least 3 separate experiments, *=p<0.05 versus control.

23A: secretory vesicle (marker CD35);
23B: tertiary granule (marker gelatinase); measured with a commercially available ELISA kit;
23C: secondary granules (marker CD66b) and
23D: primary granules (marker CD63).
Figure 24. The effect of hypertonicity on F-actin content and distribution in resting and stimulated neutrophils.

24A and B: 1x10⁶ neutrophils were exposed to hypertonicity (500 mOsM, ▲), FMLP alone (100 nM, ▲) or both hypertonicity and FMLP (○). In the represented experiments extra 100 mM NaCl was added 5 min before FMLP challenge, but similar results were also obtained with the reverse addition. The time scale shows the time elapsed after the final stimulus. Cells were then fixed at the indicated times, permeabilized with 0.1% triton X-100 and labeled with 0.33 μM rhodamine-phalloidin. After a final wash, the intracellular filamentous-actin (F-actin) content was quantified by flow cytometry (A), and its distribution monitored by fluorescent microscopy (B). Data are mean ± SEM of at least 5 separate experiments per time point.

24C: Cytoskeleton-associated actin was assayed in neutrophils suspended in saline buffer (Iso), then exposed to hypertonicity (H, 500 mOsM for 5 min), FMLP (F, 100 nM for 1 min) or both (H+F, extra NaCl for 5 min then FMLP for 1 min). Cells were then lysed in ice-cold Triton lysis buffer and triton-insoluble portion dissolved and boiled in equal amounts of 2X Laemmli buffer, subjected to electrophoresis and probed with a polyclonal anti-actin antibody. Representative Western blot (WB) of 3 different experiments.

24D: Electron microscopic pictures of iso- and hypertonically treated cells were obtained as described under METHODS. Smaller frames depict details of the submembranous area.
Since osmotic stress did not alter the ionophore-induced Ca\(^{2+}\) rise (as reported later in this chapter), mechanisms other than the previously suggested hyperosmotic distortion of the Ca\(^{2+}\) signal must be contributing to the observed effects.

Taken together, our data show that hyperosmotic exposure of neutrophils results in a universal block of granule exocytosis. This inhibition is dependent neither on granule type nor on stimulus, suggesting that the underlying mechanisms may affect a common step in the exocytic process.

**The effect of hypertonicity on the actin cytoskeleton**

The actin skeleton is known to play a crucial role in most neutrophil functions, including exocytosis. It is well established that agents that inhibit actin polymerization (e.g. cytochalasin B) potentiate stimulus-induced degranulation, and polymerized actin has been suggested to act as a barrier against exocytosis in other cell types. It was therefore conceivable that hypertonicity might exert its general inhibitory effect on exocytosis through the perturbation of the actin skeleton.

To test this hypothesis, we initially characterized the shrinkage-induced changes in the amount and distribution of filamentous actin in neutrophils using rhodamine phalloidin staining. Exposure of the cells to hypertonicity (500 mOsM) caused a sizeable and persistent increase in F-actin, as verified by flow cytometry (Fig. 24A). The phalloidin fluorescence of shrunken neutrophils increased by more than a 100% within 2 minutes, and remained at this elevated level or continued to slightly rise further as long as hyperosmolarity was maintained. This kinetics is markedly different from the FMLP-elicited two-phase response, which is characterized by a rapid and even greater initial increase, followed by a gradual return to baseline values (Fig. 24A). When the cells were exposed to both hypertonicity and FMLP (as in the exocytosis experiments), the ensuing rapid rise in F-actin was of similar magnitude as that observed with FMLP alone, however the depolymerization phase was entirely missing. Thus, while FMLP remained capable of inducing actin polymerization even in the presence of hypertonicity, the transient nature of the response was abolished. We observed similar changes in cytoskeleton-associated actin by Western blotting, confirming that the fluorimetric findings were not due to optical artifacts that might be caused by cell shrinkage (Fig. 24B).

While these observations clearly indicate that hyperosmolarity alters the normal actin dynamics both in resting and in stimulated cells, the increased amount of F-actin cannot, by itself, explain the inhibition of exocytosis, since most stimuli that trigger granule release also increase F-actin levels. Therefore, we tested whether the spatial distribution of the newly polymerized actin is different in hypertonically treated and FMLP-stimulated cells. Fig. 24C shows that FMLP caused a characteristic polarized accumulation of polymerized actin. Similar uneven F-actin distribution was observed when cells were stimulated with PMA or A23187 (not shown). In sharp contrast, hypertonicity induced the formation of a thick and continuous cortical F-actin ring, apparently under the entire surface of the plasma membrane (Fig. 24C). Corresponding observations were made by electron microscopy. While
under control conditions vesicles and organelles frequently abutted right up to the plasma membrane, in hypertonically-treated neutrophils, a thick and homogenous area appeared under the membrane that was completely devoid of intracellular granules and organelles (Fig. 24D).

Thus, these data demonstrate that hypertonicity has multiple effects on cytoskeleton dynamics: it elicits a substantial rise in polymerized actin in the absence of any other stimulus, and it distorts the stimulus-induced actin response by inhibiting net depolymerization.

**Hypertonicity-induced actin polymerization and the inhibition of exocytosis**

To assess whether the osmotically induced actin changes and the concomitant block of exocytosis might be causally linked, we applied two approaches: first, we attempted to set differential pre-stimulus actin levels and measure whether this parameter correlated with the subsequent stimulus-induced exocytosis; and second, we tested whether pharmacological interference with the hypertonic actin response would restore degranulation.

To obtain varying basal actin levels in resting neutrophils, we took advantage of the reversibility of the hyperosmotic actin polymerization. To determine the kinetics of the post-hypertonic decay of F-actin levels, cells were treated with a 500 mOsM solution for 5 minutes, followed by rapid return to isotonic medium. Fig. 25A shows that, following reestablishment of isotonic conditions, the intracellular F-actin level gradually reverted to near-baseline values over the course of 5 to 10 minutes. At various time points during this actin recovery, the same sample of cells was challenged with FMLP, and the subsequent exocytosis was quantified. To minimize the time necessary to detect exocytosis itself, we followed the expression of CD66b, since using FMLP as stimulus, 3 minutes were sufficient to induce a sizable mobilization of the secondary vesicles. As shown in figure 25A, 3 minutes after returning to isotonic medium, the total F-actin content was still elevated and only a slight increase in exocytosis was measured. After 5 minutes in the isotonic treatment, actin depolymerized to near normal levels, and the FMLP-induced exocytosis was gradually resumed. Thus, the curves depicting pre-stimulus F-actin levels and post-stimulus exocytosis were almost mirror images of each other, with a slight time delay occurring in the degranulation response compared to the cytoskeletal change. To better visualize this relationship, Fig. 25B shows the percent change in F-actin content plotted against the percent change in CD66 expression. Clearly, the two phenomena are inversely related in a near-hyperbolic manner. Essentially similar observations were made with PMA or A23187 as stimuli and CD66 or CD35 as exocytosis markers. Taken together, both the hypertonicity-triggered actin polymerization and the inhibition of exocytosis are fully reversible under isotonic conditions, and their recovery follows a similar time course.

While these data are suggestive of a possible link, they do not define whether the actin change is responsible for the inhibition, or a common upstream process regulates both phenomena. To address this issue, we used two cytoskeletal drugs, cytochalasin B (CB) and latrunculin B (LB), which are known to
interfere with normal F-actin assembly through different modes of action. CB binds to the barbed end of the actin filaments thereby preventing further polymerization from this side \(^{422,518,521}\). As shown on Fig. 26A, this drug did not affect the amount of polymerized actin in resting cells. Rather surprisingly, it also failed to interfere with the hypertonically provoked actin polymerization, although it substantially inhibited the FMLP-induced intracellular F-actin formation, verifying that the compound was biologically active. Although somewhat unexpected, this finding is agreement with an earlier observation made on HL60 cells, where CB reduced the basal F-actin content but did not affect the changes of this parameter during regulatory volume changes \(^{368}\). We then opted for the use of latrunculin B (LB), a powerful depolymerizing fungal toxin, which acts mainly by sequestering monomeric actin, and is at least 100 times more potent than CB \(^{518,519}\).

Latrunculin B exerted a robust depolymerizing action as demonstrated by measuring F-actin content with flow cytometry (Fig. 26A). Incubation with 5 \(\mu\)M LB virtually abolished F-actin measured in non-stimulated cells. Importantly, in the presence of LB, neither hypertonicity nor FMLP was able to induce actin polymerization (Fig. 26A). This effect was confirmed by measuring cytoskeleton-associated actin using Western blots: in the presence of LB, the amount of sedimentable actin was lower than baseline, even after FMLP, hypertonicity or any other agent we tested (Fig. 26B).

Having established the differential effects of these drugs on hypertonic actin polymerization, we tested their ability to influence exocytosis under iso- and hyperosmotic conditions. As an overall marker of the process, we followed the surface expression of CD11b, which is delivered to the surface through the insertion of three granule types. Both cytochalasin B (CB) and LB caused a substantial potentiation of the FMLP-induced exocytosis, in agreement with the well-known fact, that prevention of actin polymerization promotes degranulation. The two drugs, however, had a dramatically different influence on the hypertonic inhibition of granule fusion: while CB was not able to modify the hypertonic block, LB completely prevented it. It is important to note that in the presence of LB, the restoration was essentially complete. This suggests that the increase in exocytosis was not simply due to potentiating effect of LB added to the inhibitory effect of hypertonicity, but the hypertonic inhibition per se was abolished by the drug. Identical findings were attained when the expression of the secondary vesicle marker CD66 was examined (Fig. 26D). Moreover, LB was equally effective in reestablishing exocytosis triggered by other stimuli such as PMA or A23187. Thus, the differential effect of the two drugs on actin polymerization was perfectly mirrored by their differential capability to prevent the hypertonic effect on exocytosis. Taken together, these data clearly suggest a strong relationship between the hypertonicity-induced actin polymerization and the concomitant inhibition of granule exocytosis. Once the osmotic effect on the actin cytoskeleton is neutralized, the osmotic inhibitory effect on exocytosis is eliminated.

To avoid misinterpretation of data, it was essential to verify that latrunculin B (LB) exerted its effect through the inhibition of actin polymerization and not via other mechanisms. First, we examined
whether the effect of the drug was not merely due to the prevention of the hypertonicity-induced volume change per se, e.g. by increasing the permeability of the plasma membrane. To this end, the volume of neutrophils was determined using electronic sizing under iso- and hypertonic conditions, in the presence and absence of LB. Pretreatment with LB had no effect on the resting neutrophil volume and it failed to alter the hypertonicity-induced shrinking of the cells, as well (Fig. 27A). To further investigate the specificity of the action of LB, we tested whether other osmotic responses remained intact in the presence of the drug. First we studied the hypertonicity-induced activation of the Na⁺/H⁺ exchanger by following the changes in intracellular pH. This function is strictly dependent on low permeability for Na⁺ and H⁺ and stimulated exclusively by volume reduction and not by hyperosmolarity itself. Addition of 100 mM NaCl caused a large cytosolic alkalization that was completely abolished by the specific Na⁺/H⁺ antiport inhibitor, HOE 694, indicating that the response was due to the volume-dependent activation of the exchanger (Fig. 27B). Importantly, the osmotically induced alkalization remained completely intact in the presence of LB, and in fact its initial rate seemed to be slightly higher than in non-treated controls (Fig. 27B lower panel). These findings showed that cells remained viable and responsive to the volume change. Furthermore, the osmotic actin polymerization is not involved in the shrinkage-induced activation of the antiporter.

Another characteristic response to shrinkage is a robust increase in protein tyrosine phosphorylation, and the related activation of various protein kinases, including Hck, Syk, Pyk-2 and p38 MAPK. Since it was conceivable that the osmotic tyrosine phosphorylation plays a role in the alteration of exocytosis, we tested whether LB could interfere with this phenomenon. As shown on Fig. 27C, LB neither caused tyrosine phosphorylation, nor prevented the hypertonicity-triggered general phosphotyrosine accumulation as verified by probing whole cell lysates with anti-phosphotyrosine antibodies. Moreover, LB did not prevent the hyperosmotic tyrosine phosphorylation of p38 MAPK or Syk. Rather, a potentiation of the Syk response was again observed. These findings therefore suggest that LB does not act by preventing the volume change or any of the tested functional consequences thereof, other than actin polymerization. Thus the hypertonic alterations of the cytoskeleton seem to play a predominant role in the osmotic inhibition of exocytosis.
Neutrophils were suspended in a hypertonic saline buffer for 5 minutes, then rapidly pelleted and resuspended in isotonic saline buffer. F-actin content was measured at the indicated times (3, 5, 10 and 20 min) following return to isotonic conditions by flow cytometry and rhodamine phalloidin labeling. Recovery of the ability to give rise to exocytosis was measured after a 3-minute stimulation with 100 nM FMLP, immediately prior to terminating the experiment.  
25A: Relative levels of F-actin content and exocytosis (CD66b surface expression) are shown as a function of time after resuspension in isotonic medium. 100% corresponds to values measured under isotonic conditions in unstimulated cells.  
25B: Correlation between the relative changes in F-actin and CD66b exocytosis. Data are mean ± SEM of 5 separate experiments.
Neutrophils, suspended in isotonic saline buffer (Iso), were incubated in the presence or absence of either latrunculin B (LB, 5μM) or cytochalasin B (CB, 10μM) for 20 min. The medium was then supplemented with either FMLP alone (100 nM), extra NaCl alone (100 mM) or NaCl followed by FMLP. After the indicated times, F-actin content was determined by flow cytometry (26A). To measure exocytosis, control, CB- or LB-pretreated cells were either left without further treatment, or challenged with FMLP, or 100 mM NaCl, or the combination of these. Twenty minutes later, exocytosis was quantified by measuring the changes in surface expression of CD11b (26B) and the secondary granule marker CD66b (26C) using specific FITC-labeled antibodies and flow cytometry. Data are mean ± SEM of at least 3 separate experiments, *p<0.05 versus FMLP, **p<0.05 versus hypertonicity.
Figure 27. Latrunculin B does not affect the hypertonicity-induced cell shrinkage, the activation of the $Na^+/H^+$ exchanger or tyrosine phosphorylation.

27A: Cell volume was measured by electronic sizing under isotonic (290 mOsM, clear bars) and hypertonic conditions (H, 500 mOsM, hatched bars) in cells pre-treated with or without latrunculin B (LB, 5 μM for 20 min). Data are expressed as means ± SEM of at least 3 separate experiments, *=p<0.05 versus isotonic control.

27B: Intracellular pH was monitored spectrofluorimetrically. BCECF-loaded neutrophils were incubated with or without (control) 5 μM of latrunculin B (LB) and the basal fluorescence recorded. When indicated by the arrows, the medium was rapidly made hypertonic by adding 100 mM NaCl. The pH was also monitored in the presence of the NHE-1 inhibitor HOE694. The lower panel depicts the initial rate of the hypertonicity-induced alkalization in the absence or presence of LB. Data are expressed as means ± SEM of at least 3 separate experiments.

27C: Whole cell lysate obtained from cells treated as in A were probed with either anti-phospho tyrosine (PY, 4G10) or anti-phospho p38 antibody. Molecular weight markers are in kDa. Syk was immunoprecipitated (IP) as described under METHODS, and the precipitates probed with anti-phospho tyrosine (PY). Equal loading was established by stripping the blots and reprobing with anti-p38 or anti-Syk antibody (not shown). Representative Western blots (WB) of 3 independent studies are shown.
Drug-induced actin polymerization and exocytosis under isotonic conditions

The following experiments were performed to discern whether the induction of strong actin polymerization is sufficient to inhibit exocytosis even in an isotonic environment. To address this question, we used jasplakinolide (JK), a membrane-permeable sponge toxin that elicits robust and irreversible actin polymerization. Initially we assured that 10 μM JK caused monomeric actin to polymerize into F-actin in neutrophils. Since JK and phalloidin compete for the same binding sites, the JK-induced F-actin response cannot be quantified by flow cytometry. Therefore, we assayed actin polymerization by measuring cytoskeleton-associated actin using Western blotting. As expected, exposure to JK under isotonic conditions resulted in a marked increase in the cytoskeleton-associated actin, even higher than the rise caused by hypertonicity (Fig. 28A). Moreover, JK was reported to induce submembranous F-actin deposition, similar to the effect of hypertonicity. Fig. 28B-D shows that the drug completely inhibited the FMLP-triggered exocytic release of markers from all four neutrophil granules. Similar to the effect of hypertonicity, the JK-induced block of exocytosis was independent of the stimulus, be it FMLP, PMA, A23187 or LPS. However, in contrast to hyperosmolarity, JK did not elicit any change in the neutrophil volume (Fig. 28E), nor did it influence the basal or hypertonicity-triggered overall tyrosine phosphorylation (Fig. 28F), or the phosphorylation of specific osmosensitive kinases such as Hck, Syk, Pyk-2 or p38 MAPK. Thus, JK mimicked the effect of hypertonicity on actin polymerization and on exocytosis, without affecting cell volume or the shrinkage-related signaling. These data indicate that extensive actin polymerization is sufficient to prevent exocytosis under isotonic conditions.

The polymerizing state of the cytoskeleton affects the bidirectional transmembrane traffic

Having established that the redistribution of actin into a tight cortical layer can prevent the transmembrane inside-out traffic of granule contents, we investigated whether these cytoskeletal changes would also modify the outside-in traffic. To this end we measured Mn²⁺ influx through store-operated or depletion-activated channels (Fig. 29). Fura-2 loaded neutrophils suspended in Ca²⁺-free medium were depleted of intracellular Ca²⁺ by thapsigargin leading to the opening of membrane store-operated channels while Mn²⁺ was added to the Ca²⁺ medium. As shown in Fig. 29, disassembly of the actin cytoskeleton by latrunculin B resulted in substantial increase in Mn²⁺ influx while actin polymerization by either jasplakinolide or hypertonicity reduced Mn²⁺ entry by Ca²⁺-store depletion. These results suggest that the actin cytoskeleton modifies both exit and entrance traffic through the plasma membrane.

Tyrosine phosphorylation and the hypertonic actin polymerization

In subsequent experiments, we tested whether the hypertonicity-induced tyrosine phosphorylation could be an upstream signal for the osmotic actin polymerization. Osmotic shock results in extensive tyrosine phosphorylation in neutrophils, including activation of various members of the Src-family, as well as Syk, Pyk-2 and the p38 MAPK. To test the potential involvement of these osmosensitive enzymes in the actin response, we used a variety of kinase inhibitors. Specifically, we
applied the broad-spectrum tyrosine kinase inhibitor genistein, the Src family inhibitor PP1, that has been shown to prevent the osmotic activation of Syk and Pyk-2 as well \(^{141}\), and SB203580, a highly specific p38 MAPK blocker. As shown on Fig. 30A, none of these compounds affected the magnitude or the kinetics of the osmotically elicited increase in F-actin. Moreover, although protein tyrosine phosphorylation is known to play a crucial role in many FMLP-induced functions, the FMLP-triggered increase in F-actin remained intact in the presence of the applied inhibitors (Fig. 30B and see \(^{477}\)). These data suggests that protein tyrosine kinase activation is not an upstream mediator of the osmotically elicited actin response, nor it is a prerequisite for the FMLP-induced effect.

**The role of ionic strength and cell shrinkage in the hypertonic effect on actin**

Actin polymerization is strongly influenced *in vitro* by concentration of various cations, including, K\(^+\), Na\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\). Increasing ionic strength strongly facilitates actin polymerization *in vitro*, and this effect is promptly reversed upon reestablishing the original ionic composition. A decrease in cell volume causes a proportional rise in the concentration of the poorly-buffered intracellular ions, such as K\(^-\) and Na\(^-\). It was conceivable that the elevation in K\(^-\) (or Na\(^-\)) and/or in the total ionic strength of the cytosol could affect actin polymerization. Moreover, in shrunken cells, the concentration of monomeric actin also increases, which may further promote F-actin assembly. Therefore, we designed experiments in which the potential involvement of the major ions, the ionic strength, and cell volume can be separately evaluated.

In order to establish conditions when intracellular ionic strength (or species) and cell volume can be manipulated independently, we used KCl-based, intracellular-like media, and nystatin, an ionophore that permeabilizes the membrane selectively to monovalent ions. The basal F-actin levels were not significantly different in cells suspended in isotonic NaCl or KCl medium, and induction of hyperosmolarity by the addition of extra KCl, NaCl, or sucrose was equally effective to induce actin polymerization (Fig. 31). Since all these osmolytes are poor permeants, this treatment resulted in equal volume reduction and a parallel rise in intracellular ionic strength. Then cells were incubated in the same KCl medium, supplemented with nystatin. In the presence of the ionophore, the unbalanced intracellular colloid osmotic pressure would drive water entry and consequent cell swelling. To counteract this effect we added 60 mM sucrose to the medium, which was shown to balance the colloid osmotic pressure and maintain near-normal neutrophil volume, under these conditions \(^{392; 560}\). The basal F-actin content of the cells suspended in this medium was somewhat higher than without permeabilization. More importantly, the addition of 100 mM extra KCl caused substantial increase in the F-actin level. This effect was not specific for K\(^-\), since addition of NaCl induced a similar response (Fig. 31). It is important to note that under these conditions the intracellular ionic strength has increased while the cell volume remained constant since the membrane did not pose any barrier against the movement of the added ions. In contrast, the impermeant sucrose, which does not alter ionic strength but causes shrinkage, failed to
elevate the F-actin level. Taken together, we conclude that elevated ionic strength may be an important contributor to the effect, whereas reduced cell volume itself does not seem to be sufficient for actin remodeling.

**Linking actin polymerization with other neutrophil-suppressive effects of hypertonicity**

We have shown so far that osmotically induced actin polymerization is a key factor in the inhibition of exocytosis. Since hyperosmolarity abrogates many other neutrophil functions and signaling pathways, we investigated the possibility that cytoskeletal changes might also be important for these effects. We examined the potential involvement of the cytoskeletal change in the hypertonic alteration of three tonicity-sensitive functions, namely, superoxide production, the FMLP-induced Ca\(^2+\)-signal and the shedding of L-selectin.

Linking actin polymerization with other neutrophil-suppressive effects of hypertonicity

Reactive oxygen species generation is a complex process that requires the assembly of the NADPH oxidase in the membrane from cytosolic factors and membrane-embedded cytochrome components. We followed the release of hydrogen peroxidase by flow cytometry using dihydrorhodamine (DHR) as a probe (Fig. 32). Hypertonic environment abolished the FMLP-triggered increase in DHR fluorescence, while both cytochalasin B (CB) and latrunculin B (LB) caused a robust potentiation. In the presence of LB, FMLP remained capable of inducing some ROS production under hypertonic conditions. However, this response was much less than the level observed in LB-treated isotonic cells, indicating that high osmolarity exerted strong inhibition on this function even in the absence of actin polymerization. CB had no restorative effect. Collectively, these findings indicate that the FMLP-induced actin polymerization is a negative regulator of ROS production. On the other hand, while the actin-polymerizing effect of hypertonicity may contribute to the overall inhibition of ROS production, the major part of the block is attributable to other cellular effects of high osmolarity.

Hyperosmolarity has been shown to alter the stimulus-induced Ca\(^2+\)-signal \(^{158, 440}\). High osmolarity in the absence of other stimuli induced a rise in the basal Ca\(^2+\) from 88±9 nM to 138±17 nM, in agreement with previous reports \(^{440}\). After FLMP stimulation, the Ca\(^2+\) peak (246.9±9 nM) was not significantly different in iso- and hypertonically treated cells, although the rise was slower in the hypertonic samples. LB, under isotonic conditions, caused a drop in the basal level (30±7 nM), and a significant elevation in the peak value (457±40 nM). Moreover, the high Ca\(^2+\) level persisted much longer, and often a plateau phase was observed without significant drop for as long as 5 minutes. When hypertonicity and LB were applied together, the basal level was slightly lower than, whereas the peak and the kinetics of the signal were not different from the isotonic control. Thus, stimulus-induced actin polymerization contributes to changes in the Ca\(^2+\) signal: depolymerization of actin seems to facilitate Ca\(^2+\) removal (pumping) under resting conditions, and appears to increase the stimulus-induced Ca\(^2+\) entry. However, in the presence of LB the Ca\(^2+\) response is still different under iso- and hypertonic conditions, suggesting that high osmolarity alters this parameter by other mechanisms, as well.
We have recently demonstrated that exposure to hypertonicity resulted in extensive shedding of L-selectin from the neutrophil surface\textsuperscript{138, 141}. To discern whether a change in actin polymerization plays a role in this response, we tested the effect of jasplakinolide (JK) and latrunculin B (LB) on L-selectin expression. Under isotonic conditions, JK induced a significant loss of L-selectin from the cell surface. This response was similar to that obtained by short-term hypertonic exposure, but it did not progress in time, so the overall shedding was much less in JK-treated cells than in the neutrophils challenged with hypertonicity for 2 hours or longer (Fig. 33A). Since our previous studies showed that p38 MAPK activation was required for the hypertonicity-induced shedding\textsuperscript{141}, we tested whether the JK-induced shedding occurred through the activation of this enzyme. As a sensitive marker of p38 MAPK activation, cells lysates were probed with a phospho-specific anti-p38 antibody. Control cells exhibited low level of p38 MAPK phosphorylation and this was not augmented by JK, indicating that the drug does not act through the stimulation of the kinase (Fig. 33B). Together, these findings suggest that actin polymerization may facilitate L-selectin shedding, but it may not be sufficient for the full-blown response.

Finally, we asked whether actin polymerization might be necessary for the hypertonic effect. While LB did not influence L-selectin expression under isotonic conditions, the drug almost completely prevented the hypertonicity-induced shedding (Fig. 33C). This observation clearly suggests that the hyperosmotic actin polymerization is an essential contributor to the osmotically elicited shedding (Fig. 33C). As shown in Fig. 27C, LB did not affect the hypertonic p38 MAPK activation. These data are consistent with the possibility of dual signaling, i.e. both actin polymerization and p38 MAPK activation are necessary for the induction of L-selectin shedding by hypertonicity.
Figure 28. The polymerizing drug jasplakinolide (JK) mimics the hypertonic inhibition of exocytosis without inducing shrinkage and tyrosine phosphorylation.

E. Cell volume

A. WB: actin

F. WB: anti-PY

B. CD11b

C. CD35

D. CD66b

Neutrophils were treated with or without JK (10 μM) for 1 h or hypertonicity (H, 500 mOsM) for 5 min.

28A: Cytoskeletal-associated actin was measured as in Fig. 20, and hypertonic stimulation was maintained for 1 h.

28E and F: Cell volume and general tyrosine phosphorylation were determined as described in Fig. 23.

28B, C and D: Exocytosis was induced by FMLP (F, 100 nM for 1 min). Surface expression of CD11b (B), secretory vesicle marker CD35 (C) and 2ary granule marker CD66b (D) were measured by flow cytometry. Data are mean ± SEM of at least 5 separate experiments, * = p<0.05 versus isotonic control (Iso). MCF = mean channel fluorescence, PY = phosphotyrosine. Each Western blot (WB) shown represents 3 separate studies.
Figure 29. The polymerizing state of the cytoskeleton affects the Mn$^{2+}$ entry through store operated channels.

Measurement of Mn$^{2+}$ influx through store-operated channels was estimated in neutrophils suspended in Ca$^{2+}$-free medium and loaded with 4 μM fura-2 AM for 30 min at 37°C, then washed and resuspended in isotonic saline. Intracellular Ca$^{2+}$ stores were depleted by incubation with 100 nM thapsigargin for 5 minutes, followed by the treatment with 5 μM latrunculin B, 10 μM jasplakinolide or extra 100 mM NaCl for 2 minutes. 5 mM of MnCl$_2$ was then added to the medium as marked and Mn$^{2+}$ influx estimated by measuring the fluorescence quenching at 360 nm.
Figure 30. Diverse tyrosine phosphorylation inhibitors fail to prevent actin polymerization.

A. Hypertonicity

30A: 1×10^6 neutrophils were left untreated (control, O), or challenged with 100 mM extra NaCl (■) in the absence of any inhibitors, or after pre-incubation for 20 min with genistein (40 μM, ▲), PP2 (10 μM, ◊) or SB203580 (20 μM, ○). F-actin was measured as in Fig. 20 at the indicated times after hypertonic challenge.

30B: Equal manipulation as A was performed using 100 nM FMLP (■) instead of hypertonicity. Data are mean ± SEM of at least 3 separate experiments per time point.
Figure 31. *The effect of an increase in ionic strength on the intracellular F-actin polymerization.*

Neutrophils were suspended in isotonic KCl-based solution with similar ionic concentration as the cytosol (iso KCl medium). For part of the experiment, the cells were permeabilized in this same medium supplemented with 400 units/ml of nystatin and 60 mM sucrose (KCl medium + nystatin). Medium osmolarity was increased to 500 mOsM by the addition of extra 100 mM of KCl (K), NaCl (Na) or 200 mM of sucrose (Suc) for 5 min. Intracellular filamentous-actin content was quantified by flow cytometry as described under Fig 20. Data are mean ± SEM of at least 5 separate experiments, *p<0.05 versus Iso KCl medium, **p<0.05 versus Iso KCl medium + nystatin.

Figure 32. *The effect of Latrunculin B (LB) on FMLP-induced superoxide production under iso- and hyperosmotic conditions.*

Neutrophils (1x10⁶) suspended in isotonic DMEM (Iso), were initially treated with or without latrunculin B (5µM) or cytochalasin B (CB, 10µM) for 20 min. Extra 100 mM of NaCl was added to some samples (Hyper, 500 mOsM) for 5 min. The cells were then resuspended in isotonic DMEM supplemented with 10 µM DHR for 5 min at 37°C, then exposed for 10 min to 10 µM FMLP (hatched bars), and subsequently analyzed on a FACScan. Data are mean ± SEM of 4 separate experiments, *p<0.05 versus no FMLP.
Figure 33. Osmotic remodeling of the cytoskeleton is involved in the hypertonicity-induced shedding of L-selectin.

33A: Actin polymerization was induced by treating 1x10^6 neutrophils with either 10 μM jasplakinolide (O) or 500 mOsM hypertonicity (▼) for the indicated times. Surface expression of L-selectin was measured using a monoclonal FITC-labeled antibody and flow cytometry.

33B: Cells were treated with 10 μM JK or 500 mOsM hypertonicity (H) for 1 h after which whole cell lysate were obtained and probed with anti-phospho p38.

33C: Actin polymerization was prevented by incubating the neutrophils with latrunculin B (LB, 5 μM) for 20 min then the medium osmolarity was increased to 500 mOsM (H) for 1 h and L-selectin assayed as in A. The number of positive cells was determined by comparison of histograms with those obtained using FITC-conjugated non-reactive antibody and the data expressed as percent of positive cells for L-selectin. Data are mean ± SEM of 3 separate experiments, *=p<0.05 versus isotonic control (Iso).
Discussion

The present studies report two novel findings regarding the effect of hypertonicity on neutrophils: 1) Hypertonicity induces perturbation of the cytoskeleton that is manifested by increased actin polymerization occurring predominantly in the submembranous area, and complete abolition of depolymerization that normally follows stimulus-induced F-actin assembly; 2) Actin polymerization is a key contributor to the osmotic inhibition of a variety of neutrophil functions.

*The mechanism of hyperosmotic inhibition of exocytosis*

Previous studies by Kazilek *et al* have suggested that the impaired degranulation upon hypertonic treatment was primarily due to distortion of the Ca\(^{2+}\) signal. However, these authors correctly noticed that this cannot be the exclusive mechanism, since the ionomycin-promoted release of β-glucuronidase (primary granules) was also abrogated. Several additional observations argue against the primary role of this mechanism. First, relatively mild hypertonicity which fails to abolish the FMLP-induced Ca\(^{2+}\) signal completely eliminates exocytosis (present work, and 158). Shrinkage itself induces elevation in the resting Ca\(^{2+}\) without causing exocytosis. Most importantly, hypertonicity prevents exocytosis triggered by stimuli that do not elicit or require Ca\(^{2+}\) signal (e.g. PMA). Our systematic investigation has clearly indicated that osmotic shock interferes with the release of all four granule types, independent of the stimulus applied. This observation supports the notion that hypertonicity brings about a general block in the exocytic process itself that affects step(s) distal to the various signaling events initiating granule release.

As an alternative mechanism, the osmotic perturbation of the membrane fusion was suggested. According to the so called "osmotic hypothesis of exocytosis", vesicular swelling might be a crucial factor whereby membrane stress, necessary for the fusion event, is generated in the contact area. In agreement with this prediction, vesicular swelling was sufficient to induce the fusion of liposomes with planer bilayers in *vitro*, and the volume expansion of vesicles is known to precede their release in some cellular systems. However, vesicular swelling does not necessarily accompany exocytosis, and membrane fusion can occur in the absence of any fusogenic stress. Our finding that pharmacological inhibition of actin polymerization with latrunculin B, which does not affect the osmotically driven water efflux and the consequent neutrophil shrinkage completely restores exocytosis, clearly implies that the shrinkage-related change in membrane tension is not a determinant factor in the osmotic block of exocytosis. This conclusion is further strengthened by electron microscopic observations, showing that hyperosmolarity inhibited exocytosis in sea urchin eggs by interfering with matrix disassembly and not by preventing fusion, *per se*.

We propose that the crucial element in the inhibition of exocytosis is osmotically-induced actin remodeling. This view is supported by the following findings: 1) There is an inverse relationship between the pre-stimulus level of osmotically polymerized actin and the subsequent stimulus-induced
exocytosis. 2) LB prevents the osmotic actin change, and concomitantly abolishes the inhibition of exocytosis. 3) The induction of submembranous, annular actin polymerization by jaspaklinolide, a drug that in this respect mimics the effect of hypertonicity, also leads to a general block in granule release. The realization that actin polymerization plays a major role in the inhibitory effect of hypertonicity has been delayed by the lack of suitable pharmacological tools. In agreement with Downey at al.422, we have found that CB does not change significantly the basal actin level, and, in addition, it is not capable of preventing the alteration of exocytosis, either. The differential effects of cytochalasin B and latrunculin B may permit some insight into the underlying mechanism. Since CB acts primarily by binding to the fast-growing or barbed end of the actin filament, a conceivable scenario is that hypertonic actin polymerization occurs at the pointed end. Alternatively, under hyperosmotic conditions, CB may not be able to bind to the barbed end, and therefore polymerization can proceed from this site. Although further work is required to answer this question, the second possibility appears to be more likely, because under hyperosmotic conditions CB was not able to abolish the FMLP-induced extra actin polymerization, which is known to occur at the barbed end, and is highly CB-sensitive in isotonic cells. Hypertonicity might similarly interfere with the binding of cellular capping proteins to the filament, thereby promoting polymerization. In any case, sequestration of monomeric actin by LB is undisturbed in osmotically challenged cells, and this is sufficient to prevent polymerization by any stimulus.

The pioneering studies by Watts and Howard revealed that neutrophils contain three different actin pools: stable, or Triton X-insoluble F-actin, that is predominantly localized to the membrane; labile, or soluble F-actin, that is composed of short oligomers and is rich in gelsolin; and G-actin. FMLP was shown to induce a transitional rise in stable F-actin to the expense of the other two pools. Using both biochemical and morphological criteria, we have found that hypertonicity promotes actin accumulation in the Triton-X insoluble, membrane-associated pool. This however does not exclude the possibility that the labile pool may also grow, as was reported for HL-60 cells. This pool may also contribute to the encasement of vesicles into an actin meshwork that hinders exocytosis.

The negative effect of actin polymerization on exocytosis is well documented in many systems. It has been proposed that subplasmalemmal actin can act as a physical barrier against exocytosis. Depolymerizing agents facilitate exocytosis, whereas polymerizing drugs, such as phalloidin inhibit the process. On the other hand, most secretagogues induce actin polymerization. However, these agents elicit a transient, and spatially restricted response, as opposed to the sustained effect of hypertonicity that is not confined to specific membrane areas.

**The impact of hypertonic actin polymerization on other functions**

The dramatic effect of hypertonicity on the physiologic polymerization depolymerization cycle is expected to influence other functions as well. For example, this phenomenon may be responsible for the hypertonic inhibition of locomotion. Pseudopodium formation is believed to require the initial
disassembly of the cortical skeleton at a single locale, followed by the appearance of F-actin in the newly formed protrusion. Further, rigid cortical actin organization may affect receptor signaling too, and actin polymerization was suggested to separate the FMLP receptors from the G-proteins, thereby facilitating the termination of the signal \textsuperscript{310}. The alteration of the Ca\textsuperscript{2+}-signal may also be related to this phenomenon. We observed that the Ca\textsuperscript{2+} release as well as the store-operated Ca\textsuperscript{2+} entry pathways was directly regulated by the cytoskeleton. Latrunculin B substantially prolonged the FMLP-triggered Ca\textsuperscript{2+} rise and enhanced Mn\textsuperscript{2+} influx, while jasplakinolide had the opposite effect. These data suggest that both the efflux and influx traffic through the plasma membrane is specifically sensitive to depolymerization. Our findings are in good agreement with the novel concept that a secretion-like, JK-sensitive process mediates the capacitative Ca\textsuperscript{2+} influx \textsuperscript{359}. 

Actin polymerization seems to play a key role in the hypertonic shedding of L-selectin, as suggested by the LB-sensitivity of the process. We found earlier that the p38 MAPK inhibitor SB203580 also abrogates this process. Since our results suggest that actin polymerization and p38 MAPK activation are independent, shedding seems to require the parallel occurrence of both phenomena. Actin polymerization may be necessary to induce a cleavable conformation of the cytoskeleton-associated L-selectin \textsuperscript{223}, while p38 MAPK may be necessary for sheddase activation. While this work was in preparation, Middelhoven et al reported that JK induces substantial loss of L-selectin from neutrophils \textsuperscript{242}. In contrast, we found that JK alone caused only a moderate release. It is conceivable that their cells were slightly pre-activated, and this resulted in a higher basal p38 MAPK activity.

Taken together, the hypertonic actin polymerization appears to be an important common mechanism that accounts for a variety of hypertonic effects including impaired exocytosis, chemotaxis, rolling, adhesion, transmigration. However, a number of hypertonic responses, such regulatory volume changes, the activation of Na\textsuperscript{+}/H\textsuperscript{+} exchanger, tyrosine phosphorylation, or the inhibition of the NADPH oxidase are fully or partially independent of the actin changes.

\textit{What is the mechanism of the osmotically-triggered actin polymerization?}

Although the answer remains to be elucidated, two mutually non-exclusive mechanisms can be considered: 1) direct or indirect physico-chemical effects, and 2) activation of signaling pathway controlling actin dynamics. Under in vitro conditions, the dehydration of actin \textit{per se} has been reported to facilitate polymerization \textsuperscript{425}. Increased ionic strength has a similar effect. During osmotic shrinkage, both of these conditions occur, and may contribute to the overall polymerization. Nevertheless, direct physico-chemical effects on actin are unlikely to account for the \textit{in situ} polymerization, because: a) the ends of the filaments are capped, and the vast majority of monomeric actin is sequestered by specific proteins; b) actin polymerization occurs preferentially in membrane-associated areas. It is conceivable that changes in ionic conditions (and in hypertonically induced signaling pathways) induce the dissociation of actin capping proteins such as gelsolin and CapZ \textsuperscript{566}, whose binding affinity was shown to
depend on both KCl and PIP₂, concentration, and therefore may be primarily affected at near-membrane areas. Similar mechanisms may alter the major sequestering proteins (profilin, thymosin), as well.

Considering the role of hypertonicity-induced signaling, tyrosine phosphorylation seemed as a plausible candidate, since many osmosensitive kinases (e.g. Src family, Syk, Pyk-2 and p38 MAPK) are known to act on the cytoskeleton. Moreover, we have recently shown that osmotic stress induces the Src-family-dependent phosphorylation of cortactin, a prominent cortical actin crosslinker, and hypertonicity has been reported to induce robust tyrosine phosphorylation of actin in Dctiostelium. Nevertheless, activation of the osmosensitive tyrosine kinases proved to be neither sufficient nor necessary for the hypertonicity-induced actin polymerization. Clearly, osmotic shock simultaneously initiates these two parallel responses. We observed that the originally inhibited degranulation was often enhanced after reestablishing isotonicity. Presumably, the facilitating effect of tyrosine phosphorylation was able to manifest, after the sterical hindrance was removed.

Other alternative signaling pathways include changes in phosphoinositide metabolism and activation of small G proteins. Osmotic stress was suggested to generate various phosphoinositides, which can in turn induce actin polymerization. Moreover, changes in the membrane structure might expose previously hidden PIP₂ molecules. It is noteworthy that hypo-osmotic shock induced the rapid dissociation of Pleckstrin Homology domain-containing proteins from the membrane.

Small G-proteins (e.g. Rac or CDC42) are major regulators of the actin skeleton and recent studies indicate that they can be translocated to the membrane upon shear stress, or shrinkage (A. Kapus, unpublished observation). Moreover, some of their downstream targets, such as PAK kinase, are known to be stimulated by hypertonicity. Recent work indicates that small G-protein-mediated signaling affects profilin and cofilin, which in turn induce actin polymerization and inhibit depolymerization. Future studies should address whether these intriguing mechanisms participate in the hypertonic actin response.
Section IX. Future Directions

"Science becomes dangerous only when it imagines that it has reached its goal."
George Bernard Shaw (preface to The Doctor’s Dilemma, 1911)

We used an integrated approach, with both cellular and whole animal systems, to understand the diverse molecular, cellular and physiological mechanisms underlying the altered neutrophil inflammatory response to a hypertonic challenge. Our utmost goal while elucidating the physiological effects of hypertonicity was to develop a therapy based on this knowledge. Therefore, the greatest challenging for the near future is to test whether hypertonic saline resuscitation of human subjects in hemorrhagic shock will attenuate neutrophil-mediated inflammatory organ damage. Decade-old clinical studies proved that hypertonic saline resuscitation is at least as safe as traditional resuscitation, the goal now is to find out whether hypertonicity is able to reduce post-shock morbidity in clinical practice. For this purpose, we have made arrangements with the Trauma Unit at Sunnybrook Health Science Centre and a clinical study will start over the next few months.

The present studies identified that shrinkage leads to both extensive intracellular tyrosine phosphorylation and actin cytoskeleton remodeling, which impaired neutrophil exocytosis, expression of adhesion molecules, release of proteolytic enzymes, respiratory burst, migration, chemotaxis but stimulated apoptosis. Now we are able to ask further and more complex questions: what is the mechanism underlying the shrinkage-induced cytoskeletal and signal transduction changes; and how does biochemical and mechanical molecular functions cooperate to give rise to this aberrant but mostly transient behavior of neutrophils?

Even though we proved that the cytoskeleton changes independently of osmosensitive tyrosine kinases, other signaling pathways such as the small G proteins and phosphoinositide metabolism could regulate the actin remodeling. Small G proteins of the Rho family are the molecular switches regulating actin organization in all eukaryotic cells. Rac and CDC42 have recently also been linked to activation of p38, possibly through PAK, a kinase activated by hyperosmotic shock in myocytes. Potentially PAK activation could explain the simultaneous but independent osmotic activation of p38 and the cytoskeletal changes. The role of Rac/CDC42 and PAK in this process certainly deserves further studies.

Another possibility is the involvement of phospholipids. As presented earlier in this dissertation, recent studies (see section V plus a study in plant cells J Biol Chem 274, 53:38232, 1999), demonstrated an osmotically-mediated regulation of PIP2 and PIP3 levels. While this effect is possibly the result of shrinkage-induced membrane deformation, the subsequent cascade of events might account for the altered neutrophil response.
Other potential mechanisms include several actin-binding proteins. The interaction between several ABP (including gelsolin, capZ, thymosin β4 and profilin) with actin is largely regulated by ionic/pH strength. It is conceivable that osmotically induced changes in intracellular ionic conditions, and possibly in signaling pathways such as phosphoinositides, could explain the functional effects. In fact, neutrophils from gelsolin-null mice behave in many ways like hypertonically challenged ones, including excessive actin polymerization.

Another actin-binding protein likely to be involved in the shrinkage-induced responses is myosin II. Many of the cellular effects of hypertonicity mimic those of myosin (see section V and VI). Myosin is responsible for the stiffening of the cortical cytoskeleton and the movement of some cell-surface proteins, while myosin light chain kinase bundles and stabilizes F-actin. In diverse non-muscle cells, hypertonicity causes phosphorylation of both myosin and MLCK. Our own preliminary experiments support an important role for both calmodulin and MLCK in diverse osmotic effects on neutrophils, including L-selectin shedding.
Section X.  Material and Methods for Chapters 2, 3 and 4

Materials

Ficoll and dextran T500 were purchased from Pharmacia Biotech Inc. Trizma (Tris base), 2-mercaptoethanol, N-formyl-methionyl-leucyl-phenylalanine (FMLP), phorbol 12-myristate 13 acetate (PMA), bovine serum albumin (BSA), citrate, lipopolysaccharide (E.coli O111: B4), propidium iodide, sarcosyl, guanidine-isothiocyanate, urea, sucrose, diisopropylfluorophosphate (DFP), cytochalasin B (CB) and nystatin were obtained from Sigma. 1-albumin from Frosst, EDTA from BDH Inc, Triton X-100 from Caledon Lab Ltd., Cr and protein G-agamse beads from Amenharn Inc. Cell Wells tissue culture chambers were from Coming Costar Co. G3PDH was from Clontech, gelatin from Bio-Rad and paraformaldehyde was from Canenco Inc. Fura 2-AM, dihydrorhodamine 123 (DHR), slowfade anti fade kit, rhodamine phalloidin, jasplakinolide (JK), diS-C3-(5) and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methylester (BCECF/AM) were from Molecular Probes while piceatannol (3,4,3',5' tetrahydroxy-trans stilbene) and human fibronectin were from Boehringer Mannheim. Human recombinant TNF-α, valinomycin, gramicidin, genistein, erbstatin analog, SB203580, PP 1, A23 187, ionomycin, latrunculin B (LB), DEVD-fmk and staurosporin were from Calbiochem. Proteinase inhibitor cocktail containing 0.8 mg/ml benzamidine HCl, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin A, and 50 mM phenylmethylsulfonyl fluoride in pure ethanol was from PharMingen and ATF2 from Santa Cruz Biotechnology. RO 31-9790 was a kind gift from Dr. W.H. Johnson from Roche Products Ltd. The anesthetic drugs were pentobarbital from MTC Pharmaceuticals, ketamine from Rogar/STB and xylazine from Bayer Inc. Rat neutrophil isolating kit NIM2 was from Cardinal Assoc. Inc. All chemicals used were of the highest purity available.

Solutions

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), Medium 199 and phosphate buffered saline (PBS) were from Gibco BRL. Ringer's Lactate (RL) was from Baxter Co and 10% buffered formalin from Fisher Chemical. Isolated neutrophils were suspended in either DMEM (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Hyclone Lab Inc) plus penicillin/streptomycin (Gibco BRL), or bicarbonate-free RPMI 1640 (Gibco BRL) buffered to pH 7.4 with 10 mM HEPES or isotonic saline buffer containing (in mM): 140 NaCl, 5 KCl, 5 glucose, 1 MgCl2, 1 CaCl2, 10 HEPES (pH 7.4). Iso-K and Hyper-K media had the same composition as isotonic saline buffer, except NaCl that was replaced by KCl.

Permeabilization media contained (in mM): 140 KCl, 10 NaCl, 1 MgCl2, 1 EGTA, 0.194 CaCl2 (100 nM free Ca2+), 5 glucose, 10 Heps (pH 7.2). To permeabilize the cells for monovalent cations, this medium was supplemented with 400 U of nystatin and 60 mM of sucrose. Sucrose was included to counterbalance the intracellular colloidosmotic pressure and thereby maintain cell volume by preventing swelling of the permeabilized cells as reported earlier 392.
When indicated, hypertonic medium (500 mOsM) was obtained by the addition of extra 100 mM NaCl, KCl or 200 mM urea or sucrose into the isotonic medium, hypertonic 350 mOsM medium was obtained by the addition of extra 25 mM NaCl. TBS contained the following: 100 mM NaCl and 50 mM TRIS pH 7.4. The Toronto Hospital Pharmacy Department prepared hypertonic saline, 7.5% NaCl for use in resuscitation of animals.

Antibodies and cell line

The following antibodies were anti-human: FITC-labeled anti L-selectin DREG 56 from Immunotech, anti CD11b (Leu-15) PE from Becton-Dickinson, anti phosphotyrosine monoclonal antibody (4G10) from Upstate Biotechnology Inc., the polyclonal phospho- and non-phospho- specific anti-p38 from New England Biolab, anti-Pyk2 from Transduction Laboratories, polyclonal anti-actin from Sigma and both anti-p72vak and anti-Hck from Santa Cruz Biotechnology. From Serotec we purchased the anti-CD63 antibody, the fluorescein isothiocyanate-conjugated (FITC) rat anti-mouse IgM heavy chain antibody, the FITC anti-CD66b and the phycoerythrin (PE)-labeled anti-CD35.

The following were anti-rat: FITC-labeled anti L-selectin HRL2 from Sekagaku Co, FITC-labeled anti CD11b OX-42 and anti-ICAM-1 both from Serotec Ltd. Horseradish peroxidase-coupled anti-rabbit, anti-mouse and anti-goat antibodies were from Amersham Inc.

ECV304 (human endothelial umbilical cord cell line) was obtained from American Type Culture Collection (ATCC). Neutrophil gelatinase B release was measured using a sandwich ELISA kit from Amersham Pharmacia Biotech.

Animal model

Adult male Sprague-Dawley rats weighting 300 to 350 g (Charles River) were anesthetized with intraperitoneal ketamine (80mg/kg) and xylazine (8mg/kg). The right carotid artery was cannulated with a 22-gauge angiocath (Becton Dickinson) for monitoring of mean arterial pressure (MAP), blood sampling and resuscitation. Hemorrhagic shock was initiated by blood withdrawal and reduction of the MAP to 40 mm Hg within 15 min. This blood pressure was maintained by further blood withdrawal if the MAP > 45 mm Hg, and by infusion of 0.5 ml of RL if the MAP was < 35 mm Hg. Shed blood was collected into 0.1 ml citrate/ml blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood and either an equivalent amount of RL or a 4 ml/kg volume of 7.5% NaCl (HTS) over a period of two hours. The catheter was then removed, the carotid artery ligated, and the cervical incision closed.

Next, 100 µg of LPS (E.coli 0111:B4) in 200 µl of saline, was administered intratracheally through a 14-gauge angiocath tracheotomy at three different times. For most of the studies, tracheotomy and LPS instillation were done 1 hour after completion of the fluid resuscitation. To study the late effects of hypertonic saline resuscitation, tracheotomy and LPS instillation were delayed until 18 hrs after resuscitation in one group of animals. In a third group, tracheotomy and LPS instillation were also
delayed for 18 hours but preceded by a second dose of 4 ml/kg of HTS. In the last group, HTS was administered 1 hour prior to LPS, through a 24-gauge angiocath placed in the tail vein. The tail vein catheter was removed after HTS infusion. For the delayed manipulation, all animals were re-anesthetized as described above.

Sham animals were instrumented but not bled and saline alone was instilled intratracheally. Animals were sacrificed by pentobarbital overdose at various time points depending on the specific study. Blood samples were collected during the experiment for serum osmolarity. Serum was separated by centrifugation and osmolarity measured in an automated osmometer Advanced Osmometer 3D3 (Two Technology Way). For histological assessment, the whole lungs were fixed in 10% neutral buffered formalin (pH 7.4) and later stained with hematoxylin/eosin and examined using an optical microscope.

Bronchoalveolar lavage (BAL)

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

Myeloperoxidase (MPO) assay

Lung samples were thawed and approximately 0.5 gm of tissue was homogenized in 25 ml of potassium phosphate (10 mM, pH 7.4) for 1 minute using a Brinkman Polytron (model PT10/35, Brinkman Instruments, Inc.). The homogenate was centrifuged at 12,000xg for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended and homogenized in 25 ml potassium phosphate (50 mM, pH 6.0) containing 0.5% HTAB. The homogenate was frozen overnight at -70°C, rehomogenized for 1 min and sonicated (model VC 50T, Sonics and Materials Inc.) at 40 W for 1 min. After centrifugation as above, the supernatant was collected and used for both MPO and protein assay. MPO activity was assessed by colorimetric assay using H$_2$O$_2$ as a substrate as previously described. The absorbance change per minute was used as a measure of MPO activity. Results are expressed as MPO activity per mg of protein. Protein concentrations were determined using the Pierce BCA protein assay.
Transpulmonary albumin flux

Rats were injected with 1 μCi of $^{125}\text{I}$-albumin into the tail vein immediately preceding i.t. LPS or saline. Six hours later, both 1 ml of blood collected by cardiac puncture and 3 samples of 1 ml of BAL fluid were analyzed by a gamma spectrometer. The $^{125}\text{I}$-albumin leak was corrected to blood cpm and expressed as the transpulmonary albumin leak index as follows:

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

Neutrophil isolation and labeling

Human neutrophils: Cells were isolated from fresh blood drawn by venipuncture into heparinized tubes. Isolation was performed using 3% dextran sedimentation and centrifugation on Ficoll-Hypaque cushions as described previously. After erythrocyte lysis in 0.84% NH$_4$Cl, the neutrophils were resuspended at a cell density of 1 X 10$^6$ neutrophil/ml in DMEM and used immediately after isolation.

Rat neutrophils: Isolation was done according to the NIM2 manufacturer's instructions. Briefly, after sacrifice, the total blood volume of the animal was obtained by cardiac puncture and collected in a citrated glass tube. The blood was layered onto two discontinuous Ficoll-Hypaque gradients (NIM2). After centrifugation at room temperature, 400xg for 45 min, the pelleted cells were washed in HBSS once and the erythrocytes lysed in erythrocyte lysing buffer (0.84% NH$_4$Cl). Finally, the cells were resuspended in DMEM. Isolated cells consisted of over 90% neutrophil with >95% viability by trypan blue dye exclusion.

Neutrophil Labeling with $^{51}\text{Cr}$: For labeling rat neutrophils, 5 X 10$^6$ neutrophils were resuspended in 0.4 ml of DMEM and incubated for 20 min with 40 μCi of $^{51}\text{Cr}$ at 38°C. Following the labeling period, cells were washed twice and incubated in either isotonic medium (isoPMN) or hypertonic medium (350 mOsM) for 4 hours (htPMN). In some studies, freshly isolated cells were used immediately following the labeling step. Cells were pelleted by centrifugation at 300xg and resuspended in isotonic DMEM. An aliquot of cells from each group was counted to ensure equivalent labeling (1480 Wizard 3" automatic gamma counter). There was no difference in the labeling between isotonic and hypertonic treatment. Next, 2 X 10$^6$ $^{51}\text{Cr}$-labeled isoPMN or htPMN, resuspended in isotonic medium, were injected into the tail vein of the rats immediately before i.t. LPS or saline instillation. At sacrifice 4 hours later, 1 ml of blood was collected by cardiac puncture. The lungs were harvested after being flushed with PBS/EDTA through a cannula inserted in the pulmonary veins until the effluent was completely clear. $^{51}\text{Cr}$-labeled neutrophil accumulation in the lung was analyzed in the gamma spectrometer and calculated as follows:

$$\text{neutrophil lung accumulation of }^{51}\text{Cr}-\text{labeled neutrophils} = \frac{\text{lung cpm/g tissue}}{}$$
**Assessment of neutrophil viability**

At the end of the preparation phase, two approaches were used to assess neutrophil viability. By trypan blue exclusion technique, neutrophil viability exceeded 95% and did not differ between isotonic and hypertonic treatment. Measurement of transmembrane potential was also tested using the anionic fluorescent probe diS-C3(5) as described.

**Measurement of neutrophil surface CD11b and L-selectin**

*In vitro studies on human neutrophil:* Flow cytometric analysis was used to evaluate surface expression of these adhesion molecules. Isolated human neutrophils were incubated in isotonic medium or hypertonic media at 350 or 500 mOsM for up to 4 hours. Neutrophils were resuspended in isotonic medium and incubated with FITC-labeled anti-L-selectin or PE-labeled anti-CD11b antibody at a 1/10 dilution for 20 min incubation at 4°C. Neutrophils were then analyzed on the FACScan (Becton-Dickison) using FL1 detector for L-selectin (488 nm excitation and 520 nm emission wavelengths) or FL2 detector for CD11b (570 nm emission wavelengths). Typically, 5,000 cells were analyzed per condition, CD11b surface expression was reported as mean channel fluorescence (MCF) and L-selectin as the number of positive cells for L-selectin, which was determined by comparison of histograms with those obtained using FITC-conjugated non-reactive antibody as described by Preece et al.

Surface expression of these molecules was was measured in resting and stimulated human neutrophil. The following stimuli were used: LPS (1μg/ml for 1 hour), PMA (50 nM for 20 min) or FMLP (100 nM for 20 min). Soluble L-selectin was measured using an ELISA kit from Bender MedSystems, according to the manufacturer's instructions.

**Detection of surface markers on rat neutrophil in whole blood:** For CD11b detection, 100 μl of whole rat blood was incubated with FITC-labeled anti-CD11b antibody at 1/10 dilution at room temperature for 20 minutes. Erythrocytes were lysed with lysing buffer (0.84% NH₄Cl), the remaining cells washed twice with DMEM and analyzed on the FACScan as above. For measurement of L-selectin, neutrophils were isolated from whole blood and incubated with FITC-labeled L-selectin at a 1/10 dilution for 20 min at 4°C. Neutrophils were then analyzed on the FACScan as above.

**Neutrophil adhesion assays**

ECV304 human endothelial cells were grown to confluence on fibronectin coated tissue culture chambers. To induce high expression of ICAM-1, the ECV304 cells were stimulated with TNF-α (1ng/ml) for 6 hours, and subsequently washed with fresh DMEM + 10% FCS. Neutrophils were treated with iso or hypertonic (350 mOsM) medium for 4 hours, then incubated in the presence or absence of LPS (1μg/ml) for 1 h. Next, all neutrophils were resuspended in isotonic DMEM +10% FCS and layered on top of the pre-stimulated endothelial cells for 30 minutes at 37°C. Nonadherent neutrophils were removed by repeated washes with PBS. The number of adherent neutrophil was determined by
microscopy by 2 investigators blinded to the manipulations. Three high-power fields were counted per well and each group was plated in 6 different wells.

**Northern blot analysis**

Total RNA from lungs was obtained using the guanidium-isothiocyanate method. Briefly, lungs were harvested and homogenized in 10 ml of 4 M guanidine-isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl and 100 mM b-mercaptoethanol. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to nylon membrane. Hybridization was carried out using either a [32P]-labeled random-primed murine ICAM-1 cDNA probe (from ATCC) or a [32P]dATP-end-labelled 30 base oligonucleotide probe for CINC with the sequence 5'-GCCGGCATCACCTTCAAACCTCTGATGTCT-3', which is complimentary to nucleotides 134 to 164 of CINC cDNA kindly provided by Dr. Timothy S. Blackwell (Vanderbilt University School of Medicine, Nashville, TN). Blots were then washed under conditions of high stringency and specific mRNA bands were detected by autoradiography in the presence of intensifying screens as previously reported. To control for loading blots were then stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), a ubiquitously expressed housekeeping gene. Expression of mRNA was quantitated using a phosphorimager and accompanying ImageQuant software (Molecular Dynamics) and was normalized to the G3PDH signal.

**SDS PAGE and immunoblotting**

To study ICAM-1 protein expression in the lung, whole rat lungs were homogenized in TBS/1% triton X-100 solution, while for the neutrophils studies, these cells were rapidly sedimented in a microcentrifuge at 4°C, and the pellet resuspended in hot Laemmli sample buffer and boiled for 10 min. Lung tissue homogenate samples were separated on a 15% SDS-PAGE under nonreducing condition while neutrophils were subjected to 10% SDS-polyacrylamide gel electrophoresis. Separated proteins were electroblotted to a nitrocellulose membrane (Schleicher and Schuell) and blocked for 1 h at room temperature in Tris-buffered saline containing 5% BSA. The membranes were then incubated with the corresponding antibodies, a 1:3000 dilution of anti-phosphotyrosine (4G10) or 1:1000 dilution for antiserum anti-ICAM-1, anti-p38, anti-Syk, anti-Pyk2, and anti-Hck at room temperature for 1 h. Antigen-antibody complexes were identified with peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody (1:4000) and visualized using the enhanced chemiluminescence detection system.

**Immunoprecipitation**

To minimize proteolysis, neutrophils were pretreated with 1mM DFP for 30 minutes, washed and resuspended at a concentration of 5x10^6 cells/ml in HEPES-buffered RPMI. After specific treatments, the same volume of ice-cold buffer of the corresponding osmolarity was added and the cells rapidly sedimented in a microcentrifuge. The cells were dissolved in ice-cold lysis buffer containing 100
mM NaCl, 30 mM Hepes, 20 mM NaF, 1mM EGTA, 1% Triton X-100, 1 mM sodium vanadate, 20 µl/ml proteinase inhibitor cocktail. The lysate was kept in ice for 5 min, centrifuged for 10 min at 4°C, the Triton-insoluble pellet discarded. The lysate was precleared and incubated with specific antibody for 2 h. Subsequently protein G-agarose beads (40 µl) were added and the lysate was incubated at 4°C for 1 h. Following centrifugation, the immune complexes were sedimented and washed 3 times with washing buffer (25 mM Tris pH 7.4, 1 mM sodium vanadate, 150 mM NaCl) then the precipitate was boiled in LSB for 10 min.

p38 kinase assay
Following immunoprecipitation, the immune complexes were resuspended in 50 µl of kinase reaction mix containing 20 mM Hepes pH 7.6, 200 µM MgCl₂, 20 µCi of [γ-³²P] ATP, 2 mM dithiothreitol, 100 µM sodium vanadate, 25 mM β-glycerolphosphate pH 7.2 and 5 µg of recombinant fragment of activated transcription factor 2 (ATF-2) for 15 min at 37°C. The reaction was terminated with hot LSB and the samples were boiled for 10 min. Proteins were separated by 15 % SDS-PAGE and the phosphorylated ATF-2 was visualized by autoradiography.

Measurement of cell volume and intracellular pH (pH)
For cell volume determination neutrophils were suspended in various media as indicated in the text and under the figures, and their volume assessed by electronic sizing using a Coulter Counter model ZM, equipped with a Channelyzer. Intracellular pH determination was done as described ⁵⁷⁵. Briefly, 1x10⁶ neutrophils suspended in isotonic saline were incubated with 2 µM of BCECF/AM for 15 min at 37°C. The cells were then washed and resuspended in isotonic saline buffer and the fluorescence was continuously monitored using a Perkin-Elmer LS50 fluorimeter, in the presence or absence of 10 µM HOE694 for 1 minute. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. Further conditions are detailed under the corresponding figure ⁵⁷⁵. After obtaining a baseline reading, neutrophils were challenged with hypertonicity by the addition of 100 mM NaCl.

Apoptosis analysis by flow cytometry
Neutrophils were incubated with specific inhibitors prior to challenge with iso or hypertonic medium for 18 hours at 37°C. The cells were then centrifuged and resuspended in propidium iodide (500 ng/ml) and stored at 4°C for 30 min. DNA fragmentation analysis was carried out by flow cytometry. Apoptosis was scored by the appearance of a sub-G₀ peak as described by Frasch et al ⁵⁹⁴.

Granule exocytosis
Exocytic insertion of CD35, CD66b and CD63 into the cell membrane were determined by flow cytometry. Neutrophils suspended in DMEM at 1x10⁶/ml were incubated with various agents under iso or hypertonic conditions as detailed under the figures. Subsequently, 100 µl aliquots of cells were incubated at 1/10 dilution of FITC- or PE-labeled antibodies for 20 min at 4°C and analyzed on a
FACScan (Becton-Dickison, Palo Alto, CA) using FL1 detector (488 nm argon laser excitation and 520 nm emission wavelengths) and FL2 detector (570 nm emission wavelength), respectively. In all experiments, 5,000 cells were typically analyzed per condition and the results expressed as mean channel fluorescence (MCF).

Primary granule exocytosis was studied in the presence of cytochalasin B (10 μM), since otherwise only a negligible release occurs. For CD63 determination, after incubation with anti-CD63 antibody, the cells were pelleted and resuspended in isotonic medium with 1/50 dilution of FITC-anti mouse IgM antibody. Exocytic release of gelatinase into the supernatant was quantified by an enzyme-linked immuno absorbent assay (ELISA). After treatment, supernatant was collected by centrifugation and gelatinase release measured by sandwich ELISA using a commercially available kit. The ELISA assay was done in accordance to the manufacturer's instructions.

**Quantification and distribution of intracellular filamentous actin**

F-actin was quantified essentially as described. A 500 μl aliquot containing 1x10⁶ neutrophils in either isotonic saline buffer or permeabilization media was incubated at 37°C with various agents under iso or hypertonic conditions as detailed under the figures. Cells were then fixed with ice-cold 4% paraformaldehyde for 15 min, pelleted and resuspended in the initial buffer supplemented with 0.1% Triton X-100 and 0.33 μM rhodamine phalloidin for 15 min at 4°C. Subsequently the cells were pelleted and resuspended again in the initial buffer. F-actin (filamentous actin) content was quantified by flow cytometry (488 nm excitation and 520 nm emission). Five thousand cells were typically measured per condition and the values expressed as percent of control samples. Occasionally, batches of cells were stained with either NBD phallacidin or rhodamine phalloidin and the results obtained with these two dyes were indistinguishable. For the investigation of F-actin distribution, fixed and rhodamine phalloidin labeled cells were centrifuged onto glass coverslips by cyto spun. The slides were mounted using the SlowFade antifade kit, and were visualized by fluorescent microscopy, using a Leica DRBM inverted fluorescence microscope connected to a Cooled CCD Video Camera (Princeton Instruments). Images were collected with the Winview software.

**Measurement of intracellular free calcium ([Ca²⁺]ᵢ) and Mn²⁺ influx**

Measurement was done as described. Briefly, for [Ca²⁺]ᵢ determination, 4X10⁶ cells were loaded with 4 μM fura-2 AM for 30 min at 37°C. Where applied, latrunculin B was continuously present from the last 10 minutes of the dye-loading period. Fura-2-loaded cell were washed and resuspended in isotonic saline and treated as described in the text. Ratio fluorescence was measured using dual excitation at 340/380 nm, and single emission at 510 nm. Fluorescence maximum and minimum were determined in the presence of 2 μM ionomycin and either 2 mM CaCl₂ or 5 mM MnCl₂, respectively. Measurement of Mn²⁺ influx through store-operated channels was estimated in neutrophils suspended in Ca²⁺-free medium and loaded with fura-2 as above. Intracellular Ca²⁺ stores were depleted by incubation
with 100 nM thapsigargin for 5 minutes, followed by the treatments specified in the text. Five mM of MnCl₂ was then added to the medium and Mn²⁺ influx estimated by measuring the fluorescence quenching at 360 nm.

**Measurement of reactive oxygen species (ROS) generation**

Determination of ROS generation was done by flow cytometry using dihydrorhodamine 123 (DHR) as oxidative probe, as reported previously. Colourless DHR is oxidized by hydrogen peroxide to a fluorescent product, which is used to monitor ROS generation. Briefly, 1x10⁶ neutrophils suspended in DMEM, were treated as described under the figures. The cells were then resuspended in fresh DMEM supplemented with 10 µM DHR for 5 min at 37°C. After a 10 min exposure to 10 µM FMLP, the cells were analyzed on a FACScan and the results expressed as mean channel fluorescence.

**Electron microscopy**

Cells were fixed for 2 hours in 2.5 % glutaraldehyde in PBS, pelleted and fixed in 1 % osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon-Araldite resin. Thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate for viewing in a Philips 400 electron microscope.

**Statistical analysis**

Data in all studies are presented as mean ± standard error of the number of experiments as indicated. When blots are shown, they are representative of at least three independent studies. Significance was assessed using Student's t test, one-way ANOVA with posthoc testing with the Tukey-Kramer Multiple Comparisons Test. A probability of p<0.05 was considered significant.
References


160


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List of Abbreviations

AA = arachidonic acid; ABP = actin binding proteins; ARDS = acute respiratory distress syndrome;
BALF = bronchoalveolar lavage fluid; BCECF = 2',7'-bis-[2-carboxyethyl]-5[and 6]carboxyfluorescein.;
CHO = Chinese hamster ovarian cells; CINC = cytokine-induced neutrophil chemoattractant; Cox-2 =
cyclooxygenase-2; CR3 = complement receptor 3; DAG = 1,2-diacylglycerol;
EGF = epidermal growth factor; ELISA = enzyme-linked immunosorbent assay; ERK = extracellular
signal-related kinase; ERM = ezrin, radixin and moesin; ENA = epithelial neutrophil activating peptide;
FiO₂ = fraction of inspired oxygen; FMLP = N-formyl-methionyl-leucyl-phenylalanine;
GDI = guanine nucleotide dissociation inhibitor; GlyCAM-1 = glycosylation-dependent cell adhesion
molecule-1; GTPyS = non hydrolyzable guanosine-5'-O-(3-thiotriphosphate);
HS = hypertonic saline; HSD = hypertonic saline dextran; HUVE = human umbilical vein endothelial;
ICAM-1 = intercellular adhesion molecule-1; ICP = intracranial pressure; IGF = insulin-like growth
factor; IL = interleukin;
Jak = Janus tyrosine kinase; JK = jasplakinolide; JNK = c-Jun N-terminal kinase;
LBP = LPS binding protein; LeuCAMs = leukocyte cell adhesion molecules; LFA-1 = lymphocyte
function-associated antigen-1 (CD11a/CD18); LPS = lipopolysaccharide; LTB4 = leukotriene B4;
MAPK = mitogen-activated protein kinase; MCP-1 = monocyte chemotactic protein-1; MDCK = Madin-
Darby canine kidney cells; MEKK = MAPK kinase kinase; MIP-2 = macrophage inflammatory protein
2; MMP = metalloproteinase; MKK = MAPK kinase; MODS = Multiple Organ Dysfunction Syndromes
NHE = Na⁺/H⁺ exchanger; NSF = N-ethylmaleimide-sensitive fusion protein;
PAF = platelet-activating factor; PAK = p21-activated kinase; PaO₂ = partial pressure of arterial oxygen;
PDGF = platelet-derived growth factor-B; PECAM-1 = platelet-endothelial cell adhesion molecule;
PGE₂ = prostaglandin E₂; PTP = protein tyrosine phosphatases; PTK = protein tyrosine kinases; PIP₂ =
phosphatidylinositol 4,5 biphosphate; PIP₃ = inositol 1,4,5-triphosphate; PKC = protein kinase C; PMA =
phorbol 12-myristate 13-acetate; PM = plasma membrane; p90rsk = 90 kDa ribosomal S6 kinase; Pyk =
prolene-rich tyrosine kinase;
ROS = reactive oxygen species; RVD = regulatory volume decrease; RVI = regulatory volume increase;
SAPK = stress-activated protein kinase; SCR = short consensus repeat; SEK1 = SAPK/ERK kinase 1;
SIRS = systemic inflammatory response syndrome; sLE⁺ = sialyl Lewis X; SNAP = soluble NSF-
attachment protein; SNARE = SNAP-receptor; Syk = spleen tyrosine kinase; STAT = signal transducers
and activators of transcription;
TACE = TNF-α converting enzyme; TGF-β = transforming growth factor-β; TNF-α = tumor necrosis
factor alpha; TPK = tyrosine protein kinase;
VCAM-1 = vascular cell adhesion molecule 1; VLA = very late activation antigen family

197