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DISSERTATION

THE ROLE OF ATRIAL NATRIURETIC PEPTIDE IN AMELIORATING HIGH ALTITUDE PULMONARY EDEMA

Submitted by

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In partial fulfillment of the requirements

For the degree of doctor of philosophy

Colorado State University

Fort Collins, Colorado

Fall, 2004

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DAVID CHARLES IRWIN ENTITLED *THE ROLE OF ATRIAL NATRIURETIC PEPTIDE IN AMELIORATING HIGH ALTITUDE PULMONARY EDEMA* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSPHY.

Committee on Graduate work

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ABSTRACT OF DISSERTATION

THE ROLE OF ATRIAL NATRIURETIC PEPTIDE IN AMELIORATING HIGH ALTITUDE PULMONARY EDEMA.

Atrial natriuretic peptide (ANP) can reduce high altitude pulmonary edema (HAPE), but no explanation of a mechanism has been offered other than its vasodilatory and natriuretic actions. Therefore, we sought to determine if ANP could inhibit HAPE by superceding the counteracting actions of endothelin-1 (ET-1) in neutral endopeptidase (NEP) gene deficient mice and inhibit vascular leak in pulmonary endothelial cells.

Plasma ANP and ET-1 concentrations, right ventricular pressure (P_{RV}) and indices of lung injury were measured in wild type (NEP +/+) mice and mice in which the NEP gene was deleted (NEP -/-) on the same genetic background (C57BL/6J). Mice were exposed to a simulated altitude (HA) of 22,000 ft (6728 m; P_B= 328 mm Hg) for 24 h. At HA lung wet weight-to-body weight increased in all animals, but greatest in the NEP (+/+) mice. Vascular leak as measured by Evans blue dye was increased only in the NEP (+/+) mice at HA. P_{RV} was lower in NEP (-/-) mice at LA, but increased in both genotypes at HA. Plasma ANP concentrations increased at HA, but plasma ET-1 concentrations were elevated only in the NEP (-/-) mice at HA. There were negative correlations between plasma ANP concentration, lung wet weight-to-body weight and P_{RV}. We conclude that NEP (-/-) mice showed increased ANP concentration and decreased pulmonary vascular pressure at HA, preventing HAPE.

Bovine pulmonary microvascular (MVEC) and macrovascular (LEC) endothelial cell monolayers were stimulated with hypoxia, TNF-α or bacterial endotoxin (LPS) in the presence or absence of ANP, and albumin flux, NF-κB activation, TNF-α secretion, p38

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MAPK and F-actin formation were assessed. In transwell cultures ANP reduced hypoxia-induced permeability in MVEC and TNF-α-induced permeability in MVEC and LEC. ANP inhibited hypoxia and LPS increased NF-κb activation and TNF-α synthesis in MVEC and LEC. Hypoxia decreased activation of p38 MAPK in MVEC, but increased activation of p38 MAPK and stress fiber formation in LEC. TNF-α had the opposite effect. ANP inhibited an activation of p38 MAPK in MVEC or LEC. These data indicate ANP has a direct cytoprotective affect on the pulmonary endothelium other then its vasodilatory and natriuretic properties.

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IN MEMORY TO

Dr. Alan Tucker, my advisor who graciously accepted me into the graduate program in the department of physiology and instilled a love for science and the study of hypoxia.

Dennis Irwin, My father whom instilled in me a love of the mountains, encouraged me on many climbing expeditions worldwide, and was greatly relieved when I was accepted to graduate school.

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TABLE OF CONTENTS

1. **INTRODUCTION:** 1
Statement of Hypothesis: 2
Specific Aim 1 2
Specific Aim 2: 3
Specific Aim 3: 3
References: 3
2. **LITERATURE REVIEW** 5
2.1 Noncardiogenic pulmonary edemas. 5
2.1.1 High altitude pulmonary edema 5
2.1.2 Neurogenic pulmonary edema. 8
2.1.3 Acute respiratory distress syndrome (ARDS). 9
2.2 Mediators of pulmonary vascular leak: natriuretic peptides, endothelins, neutral endopeptidase
and TNF-α 11
2.2.1 The natriuretic peptides. 11
2.2.1.1 Overview 11
2.2.1.2 History 12
2.2.1.3 Structure of natriuretic peptides. 12
2.2.1.4 Distribution. 15
2.2.1.5 Synthesis and release 15
2.2.1.6 Natriuretic peptide receptors. 16
2.2.1.7 ANP mediated effects. 16
2.2.2 Endothelins 17
2.2.2.1 Overview. 17
2.2.2.2 History 17
2.2.2.3 Structure 18
2.2.2.4 Distribution 18
2.2.2.5 Synthesis and release 18
2.2.2.6 Endothelin receptors 20
2.2.2.7 Endothelin mediated effects. 20
2.2.3 Neutral endopeptidase 21
2.2.3.1 Overview. 21
2.2.4 Tumor Necrosis Factor 24
2.2.4.1 Overview 24
2.2.4.2 Signal transduction by TNF-α 27
2.2.4.3 TNF-α mediated effects on the endothelium. 27
2.3 Pulmonary vascular endothelium. 28
2.3.1 The cytoskeleton. 28
2.3.1.1 Overview 28
2.3.1.2 Signal transduction pathway. 29
2.3.1.3 Gap formation 30
2.3.2 Endothelial cell heterogeneity. 34
2.3.2.1 Overview. 34

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2.3.2.2 Embryogenesis 34
2.3.2.3 Morphological and structural differences. 34
2.3.2.4 Endothelial cell heterogeneity and acute lung injury. 35
2.4 References: 36
3. HIGH ALTITUDE-INDUCED PULMONARY VASCULAR LEAK IS REDUCED IN NEUTRAL ENDOPEPTIDASE GENE DELETED MICE 43
3.1 Abstract 43
3.2 Introduction 44
3.3 Methods. 46
3.4 Results. 50
3.5 Discussion 65
3.6 References 72
4. DIRECT ANP INHIBITION OF HYPOXIA-INDUCED INFLAMMATORY PATHWAYS IN PULMONARY MICROVASCULAR AND MACROVASCULAR ENDOTHELIAL MONOLAYERS 76
4.1 Abstract 76
4.2 Introduction 77
4.3 Materials and methods 78
4.4 Results. 86
4.5 Discussion 99
4.6 References: 105
5. SUMMARY AND SPECULATIONS 110
5.1 Summary of Specific aim 1 110
5.2 Summary of specific aim 2: 111
5.3 Summary of specific aim 3: 113
5.4 Conclusion: 113
5.5 Speculations and future research: 115
6. APPENDICES 117
6.1 Pulmonary microvascular endothelial cell line 117
6.2 Macrovascular endothelial cell line (LEC) 119

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LIST OF FIGURES AND TABLES

Figure 2.1: Schema of proposed pathophysiological process of high altitude illness Hackett and Roach. 7
Figure 2.2: Overview of natriuretic peptide actions on maintaining vascular homeostasis. 13
Figure 2.3: Natriuretic peptide family. 14
Figure 2.4: Endothelin family. 19
Table 2.1: Pathological conditions endothelin may be implicated in 22
Figure 2.5: Neutral endopeptidase (NEP) action on ANP and ET-1. 23
Figure 2.6: Neutral endopeptidase regulation of peptides. 25
Figure 2.7: Endothelial cell activation by TNF-α. 26
Figure 2.8: Three tiered mitogen activated protein kinase (MAPK) system. 31
Figure 2.9: Adapted from Baldwin and Thurston [6]. Forces on endothelial cells. 32
Figure 2.10: Endotheliale cell gap formation from passive contraction. 33
Figure 3.1: Lung wet weight-to-dry weight ratio between NEP -/- and NEP +/+ mice 52
Figure 3.2: Lung wet weight-to-body weight ratio of NEP -/- and NEP +/+ mice. 53
Figure 3.3: Lung wet weight-to-body weight ratio normalized to low altitude means. 54
Figure 3.4: Evans blue dye leak in NEP -/- and NEP +/+ mice at low and high altitude. 55
Figure 3.5: Photomicrograph of lung parenchyma of mice at low and high altitude. 57
Figure 3.6: Right ventricular pressure in NEP -/- and NEP +/+ mice. 58
Figure 3.7: Plasma ANP levels in NEP -/- and NEP +/+ mice. 59
Figure 3.8. Plasma ET-1 concentration in NEP -/- and NEP +/+ mice. 61
Figure 3.9: Plasma ANP/ ET-1 ratio. 62
Figure 3.10: Relationship between LWW/BW and right ventricular pressure. 63
Figure 3.11: Relationship between right ventricular pressure and ANP concentrations. 64
Figure 3.12: Relationship between LWW/BW and plasma ANP concentrations 66
Figure 3.13: Relationship between NEP, ANP and ET-1 and HAPE. 71
Figure 4.1: Permeability. 87
Figure 4.2: ANP effect on Permeability. 88
Table 4.1: Summary table of ANP effect on pulmonary endothelial cells. 89
Figure 4.3: ANP inhibition of MVEC and LEC TNF-α secretion. 91
Figure 4.4: ANP inhibition of NF-κB activity. 92
Figure 4.5: ANP inhibition of activated p-38 MAPK. 94
Figure 4.6: F-actin formation. 95
Figure 4.7: ANP inhibition on F-actin formation. 96
Figure 4.8. Morphology. 97
Figure 4.9: ANP reduces F-actin formation 98
Figure 4.10: Schema proposing where ANP exerts its effect on endothelial cells. 104
Table 5.1: Summary table of NEP -/- mice vs. NEP +/+. 111
Table 5.2: Summary table of effects on pulmonary endothelial cells. 111
Figure 5.1: Schematic of proposed ANP inhibition of gap formation. 112
Figure 5.2: Schematic of proposed intracellular actions of ANP inhibition 114
Figure 6.1: Flow chart of microvascular (MVEC) cell isolation 117
Figure 6.2: Flow chart of "cell line 2" microvascular MVEC cell isolation 118
Figure 6.3: Flow chart of macrovascular (LEC) cell isolation 119

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CHAPTER 1

1. INTRODUCTION:

High altitude pulmonary edema (HAPE) is a non-cardiogenic pulmonary edema that 38 million people each year are at risk for while visiting the mountainous Western states [5]. Furthermore, HAPE shares characteristics of neurogenic pulmonary edema and acute respiratory distress syndrome[11, 12, 14-17], two other non-cardiogenic, permeability-type pulmonary edemas. Thirteen million people worldwide suffer a stroke each year [18], and as many as 10 % of stroke victims may suffer secondary pulmonary edema. In addition, 75,000 deaths occur annually in North America from acute respiratory distress syndrome[3]. Therefore, it is crucial to understand the pathogenesis of "permeability" induced pulmonary edema.

HAPE involves a web of vasoactive agents released from a combination of cerebral and alveolar hypoxemia resulting in increased vascular permeability. This web includes vasoconstrictors and inflammatory agents (such as endothelin-1, bradykinin, and TNF-α) as well as vasodilators and anti-inflammatory mediators (such as NO, atrial natriuretic peptide (ANP) and IL-10) [2, 6, 11-13]. One reason the pathogenesis of HAPE remains elusive is our lack of understanding of what tips the balance towards the unfavorable vasoconstrictors and inflammatory mediators. In addition, it is increasingly recognized that endothelial cells, the primary target for vasoactive peptides, are phenotypically different in the main pulmonary artery versus small venuoles and arterioles[1, 7]. These differences in the pulmonary vasculature compound the difficulty in understanding the effect of vasoactive agents.

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Atrial natriuretic peptide (ANP) and endothelin-1 (ET-1) are two opposing peptides that play a role in the formation of high altitude pulmonary edema (HAPE) and are degraded by a common zinc metalloprotease neutral endopeptidase (NEP)[7, 10]. Recent data has indicated that ANP is elevated in HAPE, and can abrogate pulmonary vascular leak *in vivo* and *in vitro*[6, 8, 9]. However, no explanation of a mechanism by which ANP acts on the pulmonary endothelium as been offered other than its vasodilatory and natriuretic actions. ET-1 is a potent pulmonary vasoconstrictor and induces inflammatory agents such as TNF-α, and may be increased in HAPE-prone mountaineers[4, 13]. However, because ET-1 and its receptor expression is influenced by many diverse physical and biochemical mechanism, the role of ET-1 in pathological states has been difficult to define[4].

Therefore, the aim of this study was to determine the interaction between ANP, ET-1 and NEP in the development of pulmonary vascular leak at high altitude *in vivo*, and to determine if ANP inhibits hypoxia-induced pulmonary endothelial cell permeability and/or inhibits an inflammatory response *in vitro*.

Statement of Hypothesis:

Atrial natriuretic peptide prevents high altitude pulmonary edema by a combination of *vasorelaxant, natriuretic* and ***anti-permeability and anti-inflammatory*** activities in the pulmonary circulation. We will test this hypothesis by addressing the following specific aims.

Specific Aim 1.

Test the *hypothesis* that neutral endopetidase (NEP) gene knockout mice exposed to hypobaric hypoxia will have an increased plasma ANP/ ET-1 ratio as compared to

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control mice and this will negatively correlate with pulmonary vasoconstriction and edema.

Specific Aim 2:

Test the *hypothesis* that ANP exposure will decrease permeability of hypoxia-induced bovine pulmonary *microvascular* and *macrovascular* endothelial cells.

Specific Aim 3:

Test the *hypothesis* that ANP exposure will decrease NF-Kb activation, TNF-α secretion, activation of p38 mitogen activated protein kinase (MAPK) and F-actin (stress fiber) formation in hypoxia-, TNF-α- or endotoxin-induced bovine pulmonary *microvascular* and *macrovascular* endothelial cells.

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CHAPTER II

2 LITERATURE REVIEW

Noncardiogenic pulmonary edema is a life threatening condition with high morbidity and mortality, and is the result of a persistent imbalance between the fluid filtration and fluid clearance (i.e. lymphatic) forces [11, 52, 64]. The pathogenesis of noncardiogenic pulmonary edema remains unclear. Therefore, this review will briefly discuss: 1) noncardiogenic pulmonary edema syndromes, 2) mediators controlling pulmonary vascular leak, and 3) the pulmonary endothelium as a dynamic barrier. At the end of this review the reader should have an appreciation for the severity of non-cardiogenic pulmonary edema and complexity of vascular biology within the framework of pulmonary endothelial leak.

2.1 NONCARDIOGENIC PULMONARY EDEMAS.

2.1.1 High altitude pulmonary edema.

In the United States an estimated 38 million people are at risk for altitude illness, usually while visiting the Western states [23]. A principle manifestation of altitude illness is high altitude pulmonary edema (HAPE), a rare form of noncardiogenic pulmonary edema that can be fatal [26]. Despite decades of research, the pathogenesis of this condition remains unclear [7, 8, 18, 20, 83]. The prevalence of HAPE was less then 0.2% in a mountaineering population when ascent occurred in 2 to 4 days to an altitude of 4560 m, but when the same altitude was reached within 22 h, the incidence increased to 10% in mountaineers without, and 60% in mountaineers with a history of radiographically documented HAPE [7, 18, 20]. Physiologically, hypoxia elicits neurohumoral and hemodynamic responses in the lungs, that results in over perfusion of microvascular beds, elevated hydrostatic capillary pressure, capillary leakage, and subsequent edema

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(Figure. 1) [7, 18, 20]. Early symptoms of HAPE include exertional dyspnea, dry cough, and reduced exercise performance [7, 19, 20, 62]. Clinical examination reveals cyanosis, tachypnea, tachycardia, elevated body temperature [7], and hypoxic pulmonary vasoconstriction resulting in pulmonary hypertension and increased alveolar-arterial oxygen gradient [7, 18-20]. The earliest localized finding is usually the presence of crackles in the right middle lobe [83]. In advanced cases, signs of concomitant cerebral edema, such as ataxia and decreased levels of consciousness are frequent [26, 83].

Although the pathogenesis of HAPE remains unknown [10, 18, 20, 57], work over the past quarter century has revealed some common pathophysiological characteristics in victims of HAPE. Many victims have a blunted ventilatory response to hypoxia, accentuated hypoxemia, and marked hypoxic pulmonary vascular response (HPVR) [7, 18, 63], even at low altitudes. In addition, broncho-alveolar lavage (BAL) fluid measured in HAPE victims at 4400 m on Mt. McKinley contained high levels of large molecular weight proteins, and high levels of chemotactic and vasoactive mediators [19, 63]. Likewise, BAL fluid from four HAPE patients in the Japanese Alps showed increased levels of macrophages, neutrophils, interleukins IL-1β, IL-6, and IL-8, and TNF-α when compared to healthy individuals [44, 45]. However, prospective studies in the Alps have failed to demonstrate an increase in these inflammatory agents in the early stages of HAPE, suggesting that the inflammatory response may be a consequence rather then a cause of HAPE [7, 8, 18, 69]. Nevertheless, a significant body of literature suggests that hypoxia alone can alter vascular permeability [10]. Exposure to moderate hypoxia has been shown to increase lung vascular permeability in both adult and weanling rats; an effect augmented by inflammatory stimuli such as endotoxin or viral infection [9, 29, 57], although the mechanism for this vascular leak induced by hypoxia remains unclear [10].

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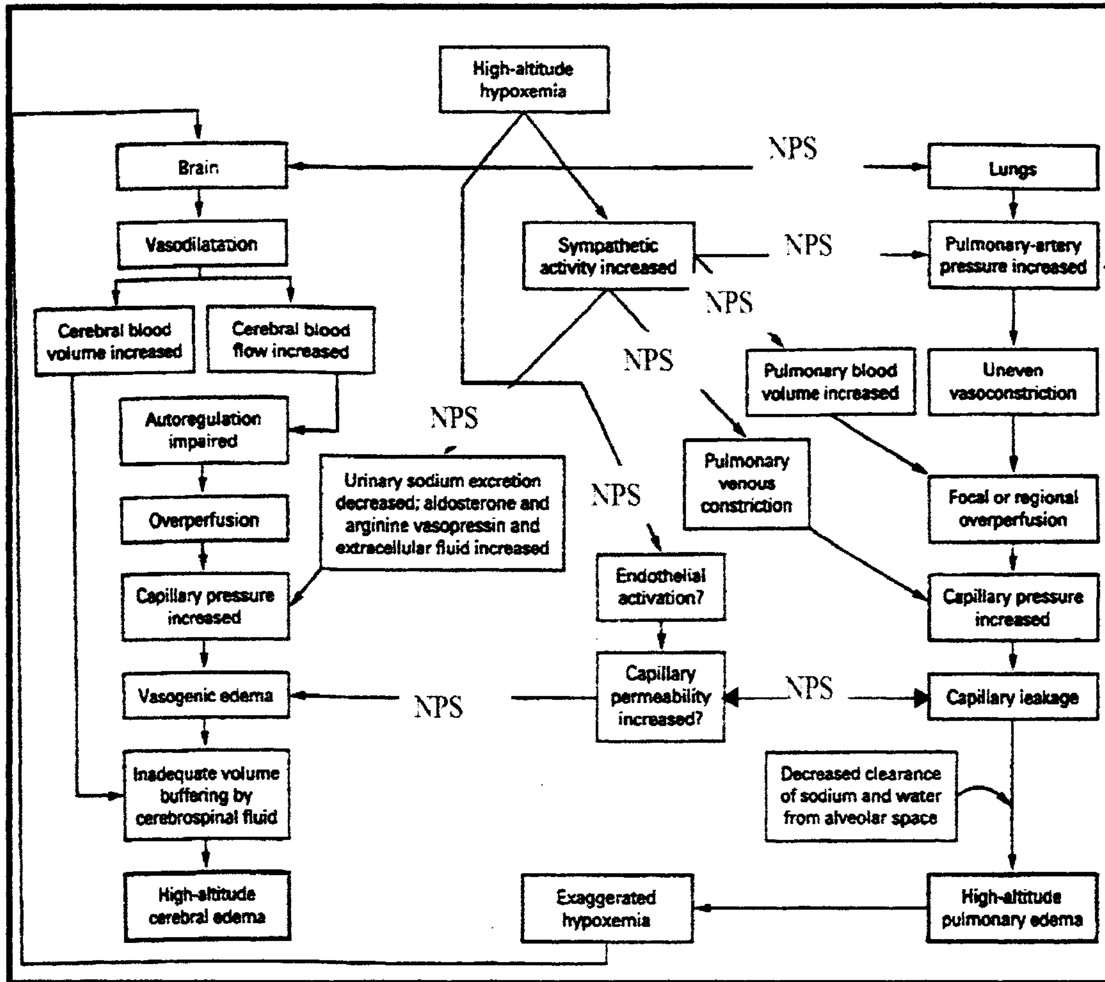

Figure 2.1: Over all schema of proposed pathophysiological process of high altitude illness Hackett and Roach, 2001 [20]. NPS = where the natriuretic peptide system can potentially prevent the onset of high altitude illness (modified from original with permission from the author).

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2.1.2 Neurogenic pulmonary edema.

Neurogenic pulmonary edema (NPE) affects individuals after stroke, head injury and seizures [52, 64, 81]. Weissman in 1939 presented the most comprehensive survey in patients with intra-cranial hemorrhage. He reported that various degrees of lung edema and congestion were found in 70% of 686 cases with intra-cranial hemorrhage [11, 80], but in contrast mild lung congestion was found in only 2% of 200 control cases without intra-cranial lesions [11]. Weisman noted that in1934, Hess [11, 80] reported several cases of pulmonary edema in patients with brain tumor, epilepsy and lesions in the medulla. In 1963, Richards reported fatal pulmonary edema in 46 of 88 patients (52%) with brain injury [60]. Recently it has been estimated that as many as 10% of the reported thirteen million people worldwide who suffer a stroke annually may develop pulmonary edema [82]. This serious clinical problem has been overlooked by clinicians because: a) attention on the cerebral problem always outweighs the lung condition, and b) the dramatic, fulminating outcomes often result in sudden death before any emergent intervention can take place [11, 52, 64].

One of the pathophysiologic mechanisms of NPE is the combination of an increase in capillary hydrostatic pressure and permeability [11, 52, 64] caused from a massive surge in sympathetic activity [11, 52, 64]. It has been postulated that this sympathetic "storm" may cause a marked rise in aortic impedance, arterial pressure and vascular resistance increasing the left ventricle after-load [11]. This, in turn, results in acute ventricular failure as manifested by a drastic and immediate fall in aortic flow, and

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a backward accumulation of blood volume within the lungs [11]. Sudden pulmonary volume loading coupled with a direct sympathetic drive to the lungs could exacerbate the degree of pulmonary hypertension, and cause disruption of the pulmonary vessels leading to fatal pulmonary edema [11]. However, the extent to which the pulmonary capillary hydrostatic pressure is affected by these changes has not been ascertained [21, 52]. Sympathetic control of tissue fluid balance may also involve direct control of endothelial permeability, although this potential mechanism is less understood [21, 52]. At present, circumstantial evidence supports a direct permeability increase as a mechanism of NPE [52]. Within 5-10 m after injection of thrombin and fibrinogen into the cisterna magna of rats and rabbits, pulmonary edema with a high protein concentration developed with only a small increase in systemic arterial pressure [52]. This small rise in pressure could not account for the protein rich edema [52]. However, it is still uncertain how direct neural control regulates pulmonary capillary permeability [11, 21, 52, 64].

2.1.3 Acute respiratory distress syndrome (ARDS).

Acute respiratory distress syndrome (ARDS) describes a form of acute lung injury characterized by permeability pulmonary edema associated with diffuse alveolar damage, an increase in vasomotor tone, and alterations in reactivity of small airways [5, 13]. ARDS is associated with a wide variety of clinical situations including, trauma, acute pancreatitis, hemorrhage, aspiration of gastric contents and systemic sepsis [5, 13, 17, 47]. The morbidity and mortality of ARDS is an estimated 75,000 deaths per year in North America [13]. Pathological changes in ARDS include pulmonary neutrophil sequestration and intravascular fibrin platelet aggregation [17]. Subsequent injury to the alveolar-capillary barrier leads to increased pulmonary vascular permeability, progressive

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lung inflammation, and pulmonary edema [17]. Progressive ARDS is characterized by increased shunt fraction, reduced lung compliance, and increased dead space ventilation as inflammatory cell and fibroblast infiltration, type II pneumocyte proliferation, and progressive obliteration of the pulmonary microvasculature evolve, [17] eventually resulting in irreversible lung damage [17].

A number of vasoactive mediators contribute to the pathological response of ARDS. Activated neutrophils release cytokines, such as TNF-α and interleukins (IL-1 and IL-8) that exacerbate the inflammatory response [5, 13, 17], and contribute to the formation of pulmonary edema. Dysfunction in the oxygen delivery system results in further cellular injury from hypoxia [5, 13, 17]. Thus begins a vicious cycle as hypoxic cellular injury increases reactive oxygen species and pro-inflammatory agents which further affect cellular homeostasis, vascular leak and edema formation.

Therapeutic interventions of ARDS have included compounds that neutralize pro-inflammatory cytokines, specifically TNF-α and IL-1B. Experimental data have established that blocking TNF-α or IL-1 with antibodies, receptor antagonist, or soluble receptors prevents lethal shock in animal models of sepsis [17, 77]. Others have reported beneficial effects of cylcooxygenase inhibitors and pharmacological doses of surfactant in animal models of diffuse lung injury [17]. Although advances in understanding of the pathophysiological mechanisms of ARDS have opened new research initiatives and introduced new clinical tools, the morbidity and mortality of ARDS remain high [5, 13, 17, 47]. Further investigation is required to find new strategies to stabilize and reduce severe acute lung injury associated with ARDS [5, 13, 17, 47].

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2.2 MEDIATORS OF PULMONARY VASCULAR LEAK: NATRIURETIC PEPTIDES, ENDOTHELINS, NEUTRAL ENDOPEPTIDASE AND TNF-α.

Natriuretic peptides and endothelins are opposing vasoactive agents [58, 62, 78]. They are increased during acute hypoxia exposure [29, 62], modulate pulmonary artery pressure and pulmonary endothelial leak [16, 48, 78] and are proteolytically controlled by neutral endopeptidase, a cell surface zinc metalloprotease [15, 43]. Therefore, these peptides seem uniquely suited for study of vascular homeostasis and the development of permeability-types of pulmonary edema such as HAPE. In addition, because atrial natriuretic peptide (ANP) inhibits and endothelin-1 (ET-1) induces tumor necrosis factor alpha (TNF-α) secretion [16, 35], and TNF-α is a central mediator in pulmonary vascular leak [13, 17] a brief review of TNF-α is also included in this section.

2.2.1 The natriuretic peptides.

2.2.1.1 Overview

The natriuretic peptide system is composed of four closely related hormones, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide and urodilatin [48, 58, 78]. Most research has focused on vasorelaxant and natriuretic actions of ANP, but it is beginning to be recognized that the natriuretic peptide system has a much broader role in maintaining vascular homeostasis then previously thought **(Figure 1)**. Recent data indicates that ANP can modulate endothelial leak *in vivo* by; 1) vasodilatory action on the pulmonary artery [27, 29, 79], and 2) decreasing capillary pressure by increasing natriuresis and controlling extracellular fluid retention by the renin-angiotensin-aldosterone system [1, 78]. Furthermore, data has indicated that when applied *in vitro* ANP can; 1) inhibit the production and secretion of ET-1 [25], 2) inhibit the production of catecholamines [48], and 3) regulate an inflammatory response [35-42]

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(Figure 2). Therefore, the natriuretic peptide system seems to be a candidate for working to maintain pulmonary vascular homeostasis during insults that can lead to dysfunction of the pulmonary endothelium by increasing vascular leak and resulting in the development of pulmonary edema.

2.2.1.2 History

Atrial natriuretic peptide (ANP) was discovered in 1981 as a biologically active substance in granules of cardiac atria [14]. A few years later a structural homologue of ANP was isolated from porcine brain homogenates and therefore named "brain natriuretic peptide" [66]. Following identification of BNP, a second natriuretic peptide was isolated from porcine brain, and named C-type natriuretic peptide (CNP) [67]. Urodilatin isolated from the human kidney completes the natriuretic peptide family [48].

2.2.1.3 Structure of natriuretic peptides.

ANP, BNP, CNP and urodilatin all have the structural characteristic of a 17 amino acid ring system, and a disulfide linkage between the two cysteine residues that was found to be essential for the pharmacologic activity [1, 31, 48, 78]. Within the cyclic structure the peptides are highly conserved, 11 of the 17 amino acids are identical, and the differences reside in the ends of the peptides **(Figure 3)**.

ANP: Comprised of 28 amino acids and differs only in 6 amino acids in the ring structure from BNP. The sequence is homologous between species differing by only 1 amino acid in humans, dogs, pigs, sheep, rats, mice and rabbits [61].

BNP: Comprised of 32 amino acids, it is less conserved between species compared to ANP retaining only 59% of the human amino acid sequence in dogs, rats, mice and rabbits [61].

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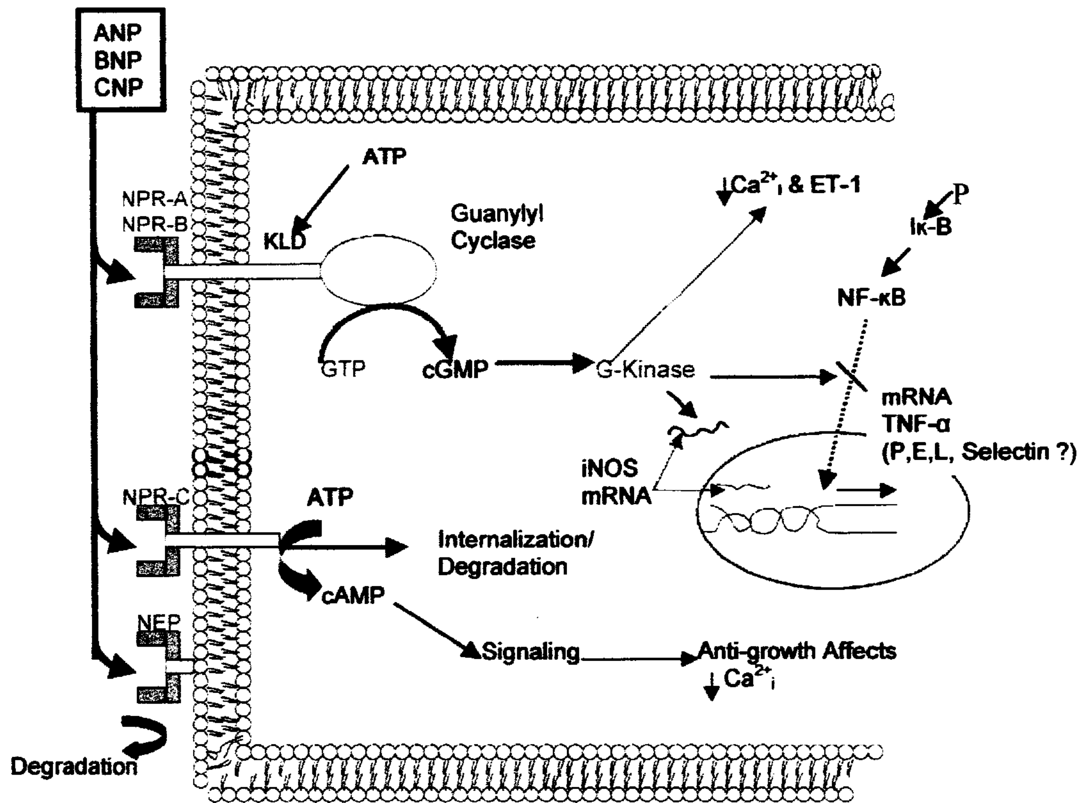

Figure 2.2: Overview of natriuretic peptide actions on maintaining vascular homeostasis.

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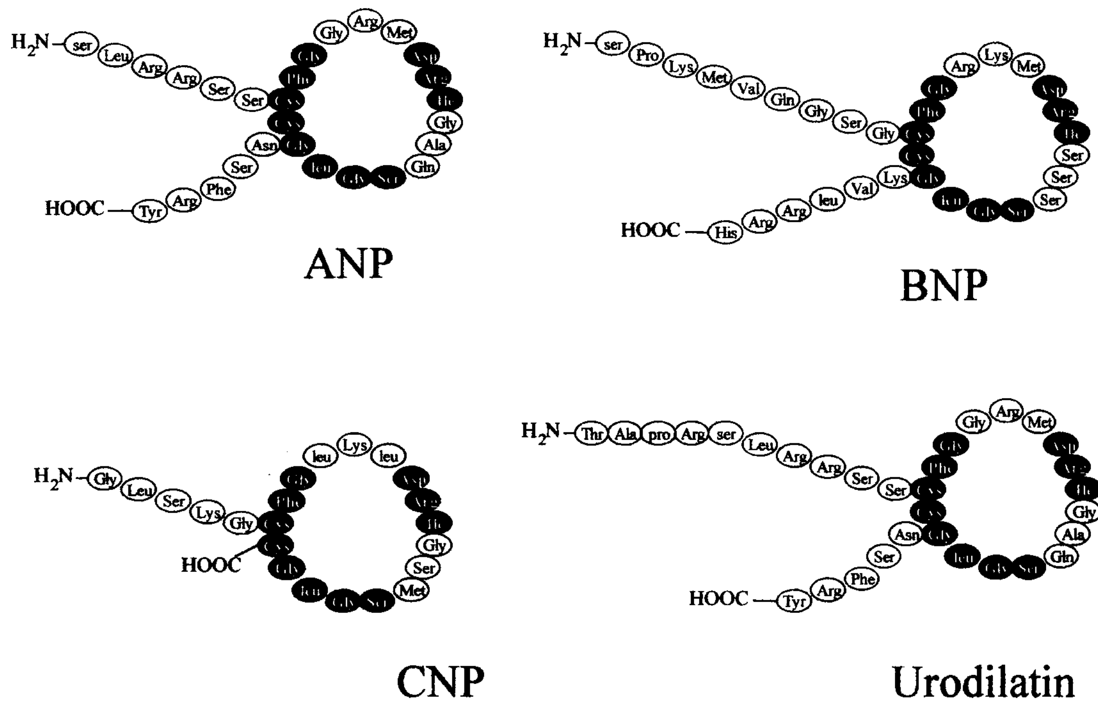

Figure 2.3: Natriuretic peptide family. Shaded regions are conserved amino acids between the four peptides (ANP, BNP, CNP and urodilatin) of the natriuretic peptide family.

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CNP: Comprised of 22 amino acids, it differs from ANP, BNP and urodilatin by abruptly terminating the C- end at the final cysteine residue in the ring structure. Similar to ANP, CNP is highly conserved between species [61].

Urodilatin: Comprised of 32 amino acids, differs from ANP by 4 additional amino acids at the N- terminus, and is highly conserved between species. However, unlike ANP, BNP and CNP, urodilatin is not degraded by neutral endopeptidase [61].

2.2.1.4 Distribution.

The highest concentrations of ANP are expressed in the left atrium; followed by the right atrium, right ventricle and then left ventricle [1, 31, 48, 58, 78]. In addition, ANP is found in lower concentrations in the lung, brain, kidney, adrenals, and the gastrointestinal tract [1, 31, 48, 58, 78]. Similarly, BNP is predominantly distributed in the heart ventricles [1, 31, 48, 58, 78], but unlike ANP and BNP, the highest concentrations of CNP are found in the brain [1, 31, 48, 58, 78]. Although in less quantity, CNP is also distributed in vascular endothelial cells and myocardial tissue [1, 31, 48, 58, 78]. Urodilatin is believed to be a kidney-derived peptide and immunohistochemical studies identified high concentrations in the cortical tubules and around the collecting ducts of the kidneys [48].

2.2.1.5 Synthesis and release

The natriuretic peptide family follows a pattern typical of protein hormone synthesis. The appropriate mRNA codes for a "prepro-peptide" and after cleavage of the N-terminal signal it is stored as a "pro-peptide".

After release, the N-terminal fragment is cleaved to form the active peptide [1]. Volume loading, atrial distension and hypoxia stimulate ANP release [3], which in turn

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induces diuresis, natriuresis, and plays an important role in body fluid and blood pressure homeostasis [70].

2.2.1.6 Natriuretic peptide receptors.

Three natriuretic peptide receptors (NPR-A, NPR-B and NPR-C) have been identified in mammalian tissues **(Figure 2)** [48]. NPR-A and B receptors are linked to the cGMP signaling cascade, and are composed of an extracellular binding site, a single membrane-spanning region and an intracellular tail composed of protein kinase like domain and a guanylyl-cyclase-like domain [12, 68]. NPR-C, the most prevalent receptor, has a similar extracellular ligand binding site, but only a short intracellular tail [12, 68]. NPR-C is involved in the clearance of peptides [48] and may be able to inhibit cAMP production via a G_I protein-coupled signaling system [22, 48]. The homology in the extracellular binding domain of the three receptors is only 30-40% [12, 68]; therefore the receptors have different ligand specificity for the natriuretic peptides. NPR-A binds ANP and BNP with a higher affinity than to CNP [48]. However, ANP is 10-fold more potent in stimulating cGMP release compared to BNP through NPR-A [48]. CNP is specific for the NPR-B, but to a lesser extent binds ANP and BNP [48]. The NPR-C receptor has a much lower specificity then NPR-A and NPR-B and binds the natriuretic peptides in the following order ANP>CNP>BNP. [48].

2.2.1.7 ANP mediated effects.

Increasingly, it is recognized that the natriuretic peptide family has a wide range of actions that can control blood pressure, salt and water balance, inflammatory agents and vascular permeability [48, 78]. Until recently most ANP research has focused on vascular tone, renal handling of sodium, and interaction with the renin-angiotensin-

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aldosterone system, due to the vasorelaxant and natriuretic properties of ANP. However, recent data suggest that ANP may inhibit endothelial vascular leak by inhibiting inflammatory agents (TNF-α and iNOS) and/or preventing the p38 mitogen activated protein kinase cascade [35-37, 39-42, 48, 78] **(Figure 2)**. Interestingly, plasma ANP levels are increased in pathophysiological conditions such as HAPE, ARDS, sepsis and chronic heart failure [24, 32, 59, 79]. In animal models of these illnesses the administration of ANP antibodies has led to deterioration in cardiovascular, renal and pulmonary function [27, 28, 30, 79]; however, the exact role of the natriuretic peptide system in these illnesses remains unclear.

2.2.2 Endothelins

2.2.2.1 Overview.

The endothelin (ET) family is composed of three closely related peptides, ET-1, ET-2 and ET-3 that have been the subject of much interest in the past decade. Endothelins are potent and long-lasting vasoconstrictors produced by vascular endothelial cells with a preferential abluminal secretion to effectively interact with the subjacent smooth muscle layer. They are rapidly removed by the pulmonary circulation of various species, suggesting that the lungs are an important site for ET clearance [16]. Data indicate that ET are increased up to five fold in various pathological conditions, including pulmonary hypertension, acute myocardial infarction and high altitude pulmonary edema [16, 33, 62]. However, the fate of circulating ET-1 and how it interacts with other vasoactive peptides is incompletely understood.

2.2.2.2 History

Endothelin-1 (ET-1) was originally discovered in 1984 from culture medium of endothelial cells. Subsequently it was isolated, sequenced, its cDNA cloned, and named

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by Yanagisawa in 1988 [16]. Within one year of its discovery, two structurally related peptides were identified and termed endothelin-2 (ET-2) and endothelin-3 (ET-3) respectively [16].

2.2.2.3 Structure

The endothelins are a family of 21 amino acid peptides, of which there are three distinct isoforms (ET-1, ET-2 and ET-3) [16]. The isoforms of ET-2 and ET-3 differ from ET-1 by two and six amino acids respectively, and share significant homology, especially at the carboxyl terminus with sarafotoxins **(Figure 4)** [16].

2.2.2.4 Distribution

ET-1 is the most abundant isoform and is predominantly expressed in the lung endothelium, smooth muscle, airway epithelium, and a variety of other cells, and circulates in the plasma [16]. ET-2 has similar biological function as ET-1 and is found on the myocardium, kidney and placental tissues. ET-3 circulates in the plasma and is found in the central nervous system, gastrointestinal tract, lung and kidney although its cellular source is not clear [16, 33, 53].

2.2.2.5 Synthesis and release

All three endothelins are synthesized as preprohormones and post-transcriptionally processed to active peptides. ET-1 processing begins with 212 prepro amino acid peptide and is then is cleaved by endopeptidases to "big" ET-1 (pro ET-1). "Big" ET-1 is cleaved by the endothelin-converting enzyme, resulting in the active form of ET-1. ET-1 is not stored in the cells, but is processed and transported through the cell in vesicles, resulting

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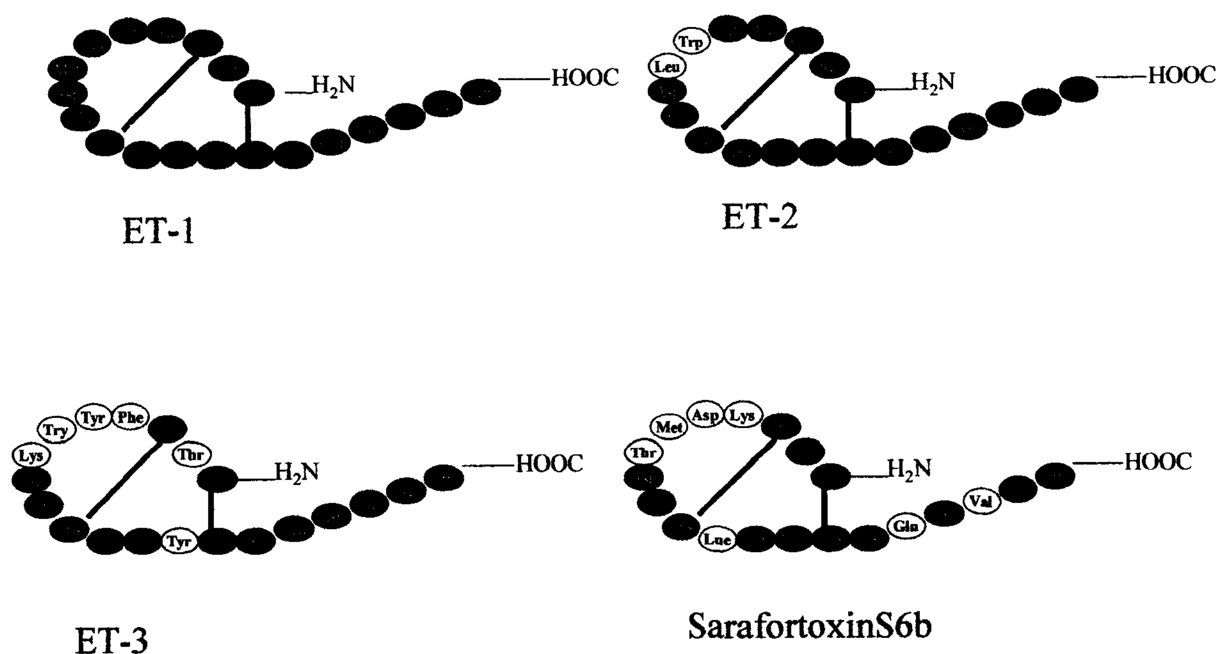

Figure 2.4: Endothelin family. Shaded regions are conserved amino acids between ET-1 and ET-2, ET-3 and sarafortoxin.

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in directional secretion (80%) toward the interstitium and smooth muscle cells and away from the luminal surface of the airway vessel [16, 40, 53]. ET-1 secretion may be increased by a variety of stimuli including cytokines, catecholamines, and physical forces such as shear stress [16, 40, 53]. Interestingly, hypoxia has been reported to increase, have no effect, or decrease ET-1 release from endothelial cells [16].

2.2.2.6 Endothelin receptors

There two distinct types of endothelin receptors, endothelin A (ET_A) and endothelin B (ET_B) receptors, which are members of the seven transmembrane G-protein-coupled rhodopsin superfamily [16]. ET_A has a higher affinity for ET-1 and ET-2 compared to ET-3, but all three share an equal affinity for the ET_B receptor [16]. In the normal lung ET_A receptors are expressed predominantly on vascular and airway smooth muscle cells, whereas ET_B receptors are found on endothelium. Clearance of ET-1 from the circulation is mediated through the ET_B receptor primarily in the lung and from degradation by neutral endopeptidase [15, 16].

2.2.2.7 Endothelin mediated effects.

In the airway, all three endothelins cause bronchoconstriction, with ET-1 being the most potent [16, 33, 53]. While ET-1 stimulates release of multiple cytokines important in airway inflammation, it does not enhance secretion of histamine and leukotrienes [16, 33, 53]. In the pulmonary vasculature, ET-1 leads to both vasodilation and vasoconstriction, depending on both the cell type and the receptor [16, 33, 53]. ET_A receptors are most abundant and are localized to the medial layer of the arteries, decreasing in intensity in the peripheral circulation, while ET_B receptors are localized in the conduit arteries. However, because ET-1 and its receptor expression are influenced by many diverse

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physical and biochemical mechanisms, the role of ET-1 in pathological states has been difficult to define **(Table 1)** [16].

2.2.3 Neutral endopeptidase

2.2.3.1 Overview.

Neutral endopeptidase (NEP) was discovered, extracted and purified from the renal brush border of the rabbit and hog [15]. NEP is a transmembrane enzyme inserted into the bilayer of the microvillar plasma membrane at its NH_2-terminal end [55, 72]. It is a membrane-bound zinc metallo-endopeptidase that is located on various tissues and organs including the lung, brain, intestine and gut in a variety of species [72]. This enzyme has a broad selectivity and is responsible for degrading various linear or cyclic peptides including ANP, endothelin (ET), insulin-B chain, substance P (SP), bradykinin, enkephalins and neurotensin [72]. NEP initiates the degradation of ANP by cleaving bonds between the amino acids ser-123 and Phe-124, and between cys-105 and Phe-106 **(Figure 5)** [72], which results in the destruction of the ring necessary for biological activity. With a recent trend in research on ANP actions in modulating the pulmonary circulation, NEP inhibition has been suggested as a method to enhance the favorable effects of ANP [71-75]. Several NEP inhibitors have been shown to reduce the development of pulmonary hypertension secondary to chronic hypoxia in rats, and reverse the cardio-pulmonary remodeling associated with this disease [72]. However, mice lacking the NEP gene have lower systemic pressures, higher vascular endothelial leak and are less likely to survive septic shock compared to wild type cohorts [50, 51]. In addition, data showed that NEP was down regulated in weanling rats exposed to hypoxia, and this correlated

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TABLE 2.1: Pathological conditions endothelin may be implicated in

Human diseases in which endothelin-1 may be involved
Airway disease
Asthma
Chronic obstructive pulmonary disease
Bronchiectisis
Parenchymal lung disease
Pulmonary fibrosis
Idiopathic
Pulmonary vascular disease
Primary pulmonary hypertension
Secondary pulmonary hypertension
Acute lung injury
Pulmonary edema
Ischemia/reperfusion
Acute respiratory distress syndrome
High altitude pulmonary edema
Lung transplant rejection

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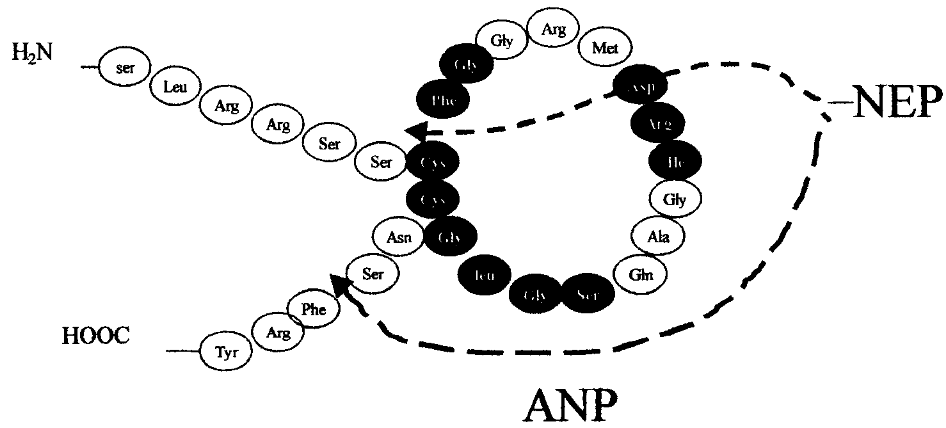

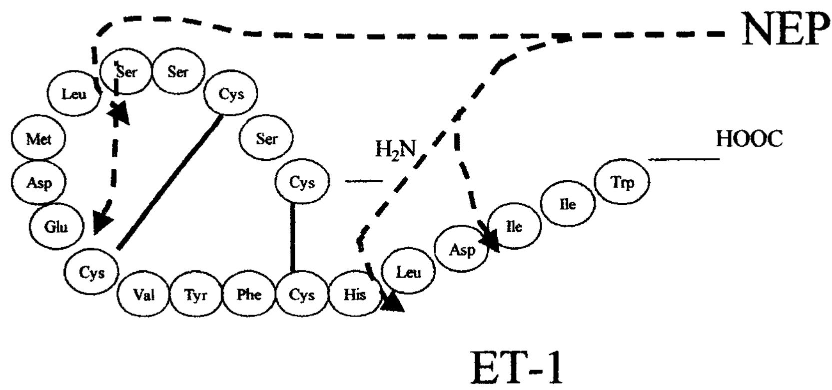

Figure 2.5: Neutral endopeptidase (NEP) action on ANP and ET-1. NEP cleaves ANP between ser-123 and Phe-124, and between cys-105 and Phe-106 and ET-1 between Ser-5 and Leu-6, Cys-11 and Val-12, His-16 and Leu-17 and Asp-18 and Ile-19.

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with hypoxia-induced pulmonary edema [50, 51]. In pathological conditions it remains unclear what role NEP plays in modulating the various counteractive vasopeptides and how this affects pulmonary vascular leak, but in human disease characterized by pulmonary hypertension, the manipulation of ANP seems increasingly viable to treat the hypoxic pulmonary vasoconstriction and the associated remodeling [72, 76]. However, it is unclear if NEP inhibition decreases, increases or has no effect on permeability-induced pulmonary edemas **(Figure 6)**.

2.2.4 Tumor Necrosis Factor

2.2.4.1 Overview

Tumor necrosis factor (TNF-α) is an inflammatory cytokine that circulates as a homotrimer with a molecular weight of 70 kDa [49]. TNF-α is synthesized by activated mononuclear phagocytes and other leukocyte types, epithelium, and endothelium [49]. Two receptors have been described for TNF-α, TNF-receptor I (TNF-RI; p55 receptor, CD120a) and TNF-receptor II (TNF-RII; p75 receptor, CD 120b) and are commonly expressed by many cell types including leukocytes and endothelium [49]. The biological actions of TNF-α are broad and affect cell proliferation, activation, differentiation and apoptosis (cell death) depending upon the receptor that is signaling, the cell type and the signal the cells receive **(Figure 7)**. However, TNF-α is mostly known for its pro-inflammatory effects on endothelial cells. Treatment of endothelial cells with TNF-α induces a dramatic increase in leukocyte adhesion and subsequent transmigration *in vivo*

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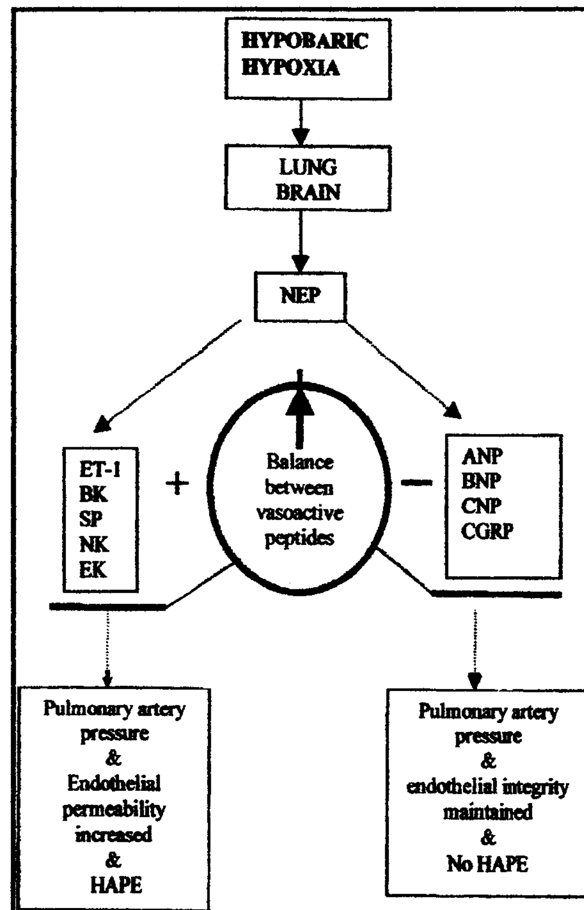

Figure 2.6: Neutral endopeptidase regulation of peptides. High altitude pulmonary edema may develop when constrictive and permeability agents take over in lack of counteracting mediators. Endothelin-1 (ET-1), bradykinin (BK), substance P (SP), neurokinins (NK), enkaphlins (EK), atrial, brain and C-type natriuretic peptides (ANP, BNP, and CNP), calcitonin gene-related peptide (CGRP).

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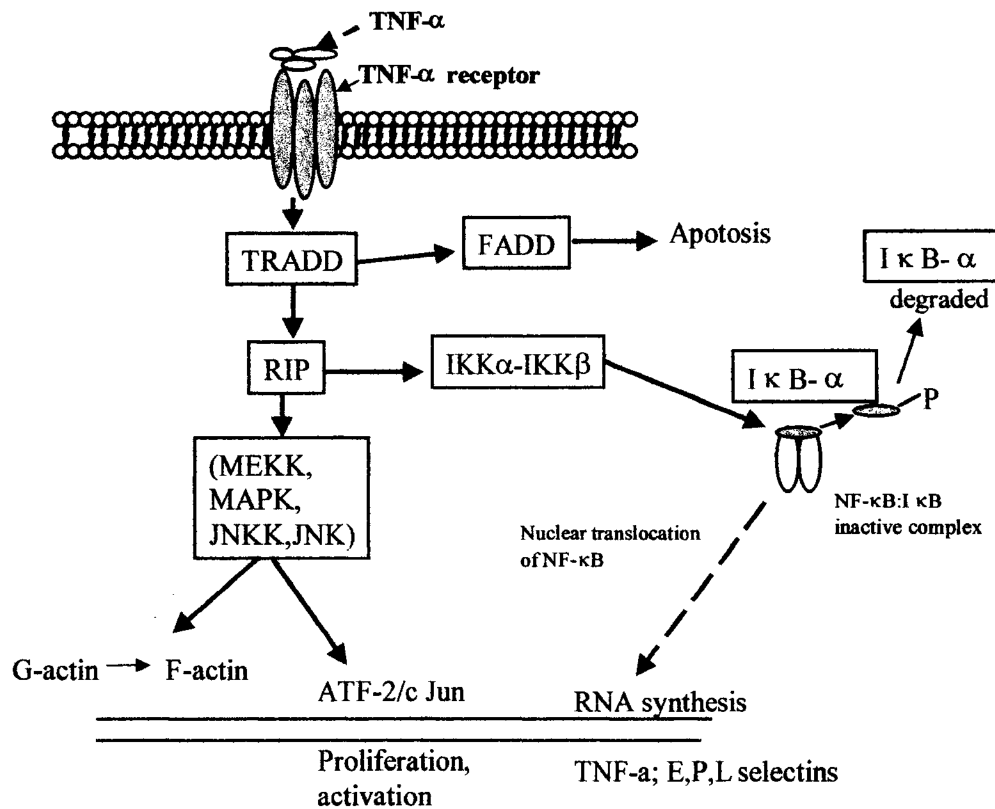

Figure 2.7: Endothelial cell activation by TNF-α. Activation of the TNF-receptor induces several pathways dependent on the receptor that is signaling, the cell type and the signal the cells receive. TNF-receptor-death-domain (TRADD) can activate the Fas-associated death domain (FADD) and apoptosis or the receptor interacting protein (RIP) which activates NF-κB or the mitogen activated protein kinase (MAPK) system. MEKK- mitogen extracellular signal related kinase-kinase-kinase; JNK-cJun amino (N)-terminal kinase.

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[49]. The elevated leukocyte adhesion correlates with rapid induction of mRNA transcripts for E-selectin and VCAM-1, but the P-selectin gene is not induced by TNF-α in humans, whereas the murine gene is responsive [49].

2.2.4.2 Signal transduction by TNF-α.

In the endothelium, TNF-α binding to tumor necrosis factor receptors (TNF-R) induces binding of TNF-R associated factors (TRAFs) to the receptor cytoplasmic domains and triggers activation of multiple transcription factors that mediate adhesion molecules expression and chemokine synthesis, as well as TNF-α production **(Figure 7)** [49]. However, the precise identity and nature of the interactions of the TRAFs with the receptor interacting protein (RIP) are unclear [49], but RIP is associated with activation of nuclear factor-Kb (NF-κB) and mitogen activated protein kinases **(Figure 7)**. NF-κB is a well-studied inducible transcription factor that performs an important function in the transcriptional regulation of genes. The relevant NF-κB protein for TNF-α-mediated activation is the p50/p65 heterodimer [42, 49]. NF-κB, normally resides in an inactive form in the cytosol complexed to an inhibitor IkBa. In response to TNF-α, IKBa is phosphorylated by kinases and subsequently ubiquinated, targeting IKBa for degradation by the proteasome, and NF-κB is released [42, 49]. NF-κB translocates to the nucleus where it binds to NF-κB recognition sites increasing mRNA for proteins associated with leukocyte adhesion (E-selectin, ICAM-1 and VCAM-1), activation (IL-8, MCP-1) and coagulation (PAI-1) **(Figure 7)** [42, 49].

2.2.4.3 TNF-α mediated effects on the endothelium.

TNF-α is increased in a variety of pathological conditions, including endotoxic shock, systemic inflammatory response and acute respiratory distress syndrome [17, 49]. Endothelial cells are the primary target of TNF-α during an inflammatory response [49].

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TNF-α exerts multiple biological effects, including induction of leukocyte adhesion molecules and proinflammatory cytokines, as well as fibrin deposition and modulation. Moreover, TNF-α increases vascular permeability, which is attributed to activation of p38 mitogen activated protein kinase (MAPK) signal cascade that increases the formation of F-actin filaments followed by cell contraction and formation of intracellular gaps [46, 56].

2.3 PULMONARY VASCULAR ENDOTHELIUM.

Vasoactive agents bind to their specific receptor and elicit a response through an intracellular signalling cascade that may activate other vasoactive peptides or alter the internal structure of the endothelial cells causing gap formations and increasing vascular permeability. Following is a brief review of the endothelial cytoskeleton and the processes that can cause gap formation and vascular leak. In addition, a summary of pulmonary endothelial cell phenotypes is discussed to give the reader some appreciation of endothelial cell heterogeneity and how it applies to the study of acute lung injury.

2.3.1 The cytoskeleton.

2.3.1.1 Overview

The endothelium acts as a primary barrier for lipid insoluble molecules between the vascular lumen and the interstitium [34, 65]. The cellular barrier is not constant, but changes according to the mechanical forces and chemical milieu acting on the endothelial cells. During inflammation, the barrier function changes reversibly, and in particular the endothelium in the venular segment of the microvasculature becomes leaky to macromolecules and particulate [34, 65]. Therefore, following is a summary on the

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transformation of actin filaments to stress fibers during endothelial cell activation, and two mechanisms by which gap formation forms; cell contraction or breakdown of the cell-cell junctional complex followed by passive recoil of actin filaments. Endothelial cells contain the actin protein in two different forms: in a filamentous form, called F-actin or stress fibers, and in a monomeric form called G-actin [4, 6]. Actin filaments shift between the monomeric and polymeric form playing a central role in cell shape and movement. In the vascular endothelium the actin filaments are found in approximately a 50:50 ratio [6]. Polymerization from G-actin to F-actin requires the presence of ATP, K^+ and Ca^{2+}, and the rate of conversion from G-actin to F-actin is limited by the monomers being assembled into dimers and trimers [4]. Once trimers are assembled the rate of polymerization is enhanced until an equilibrium phase is reached between the addition of new monomers being added and old ones dropping off. Once an equilibrium phase is reached, ATP is hydrolyzed to ADP, which protects the F-actin filament from rapid disassembly, a phenomenon called dynamic instability [4]. The formation of F-actin stress fibers allow cells to move, divide, and change shape, which are central roles in physiologic as well as pathophysiologic responses to various stimuli.

2.3.1.2 Signal transduction pathway.

Formation of stress fibers can be activated through the p38 mitogen activated protein kinase (MAPK) signaling cascade. Environmental stresses and inflammatory cytokines initiate the p38 MAPK signal transduction pathway by activating a Ras superfamily GTPase or an adapter protein [46, 56]. This input is fed into a three-tiered core-signaling module: mitogen activated kinase-kinase-kinase (MKKK), mitogen activated kinase-kinase (MKK) and p38 MAPK [46, 56]. Activation of p38 MAPK in turn activates a

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mitogen-activated protein kinase-activated protein (MAPKAP) activating heat shock protein-(HSP)-27. HSP-27 initiates polymerization of G-actin into stress fibers **(Figure 8)** [46, 56].

2.3.1.3 Gap formation.

Forces that cause gap formation and increase permeability in endothelial cells can be active or passive **(Figure 9)**. Active contraction of endothelial cells arises from tensional forces generated by contractile microfilaments that pull inward on the surface membrane and on the cells internal components, producing a membrane tension [6]. This in turn, can pull cells apart from one another, forming gaps, and increasing permeability. On the other hand, endothelial gaps may form without the need for active contraction when cell adhesion forces exceed the external and residual stresses, termed passive contraction [6]. Residual stress is defined as the remaining stress in a cell when all external loads have been removed. Residual stresses have been demonstrated by observing the rapid retraction of endothelial cells when detached from their basal surface (e.g.. trypsin) [6]. During steady state, the cell adhesion forces will balance or exceed the extrinsic (i.e. internal pressurization from blood) and residual forces. However, if the adhesion forces between cells or substrates or both are reduced, for example by F-actin rearrangement and local disruptions of adhesion molecules, then cells will retract from the imbalance between the adhesion and tensional force [6]. This phenomenon will likely occur only at specific regions at which the cellular adhesion has been disturbed **(Figure 10)**. In acute lung injury gap formation is most likely a combination of active and passive processes depending on the endothelial phenotype and the type of insult (e.g. inflammation and/or hypoxia) [6].

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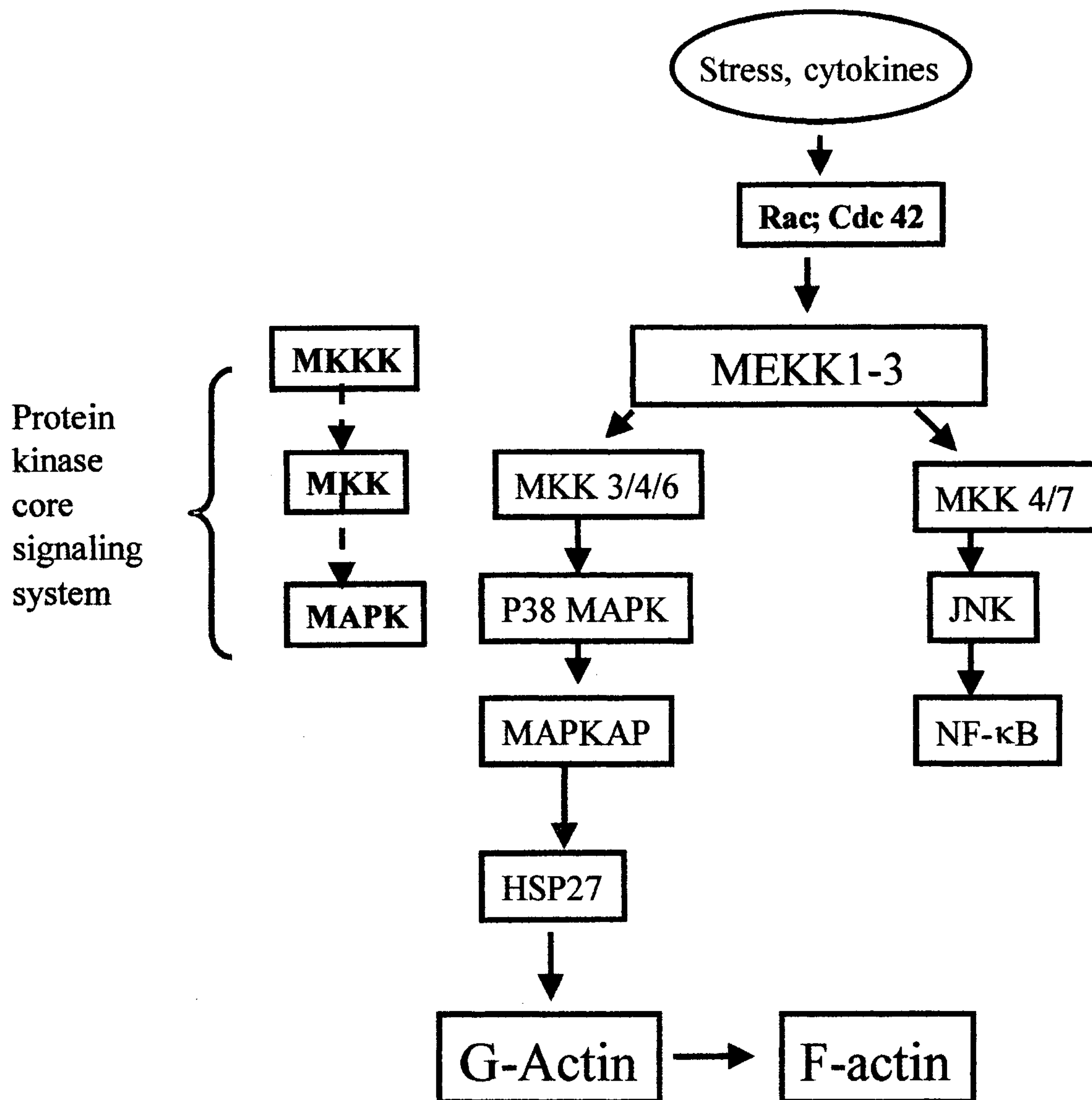

Figure 2.8: Three tiered mitogen activated protein kinase (MAPK) system. MEKK- mitogen extracellular signal related kinase-kinase-kinase; mitogen kinase kinase; MAPKAP-mitogen activated protein kinase-activated protein; HSP27-heat shock protein 27.

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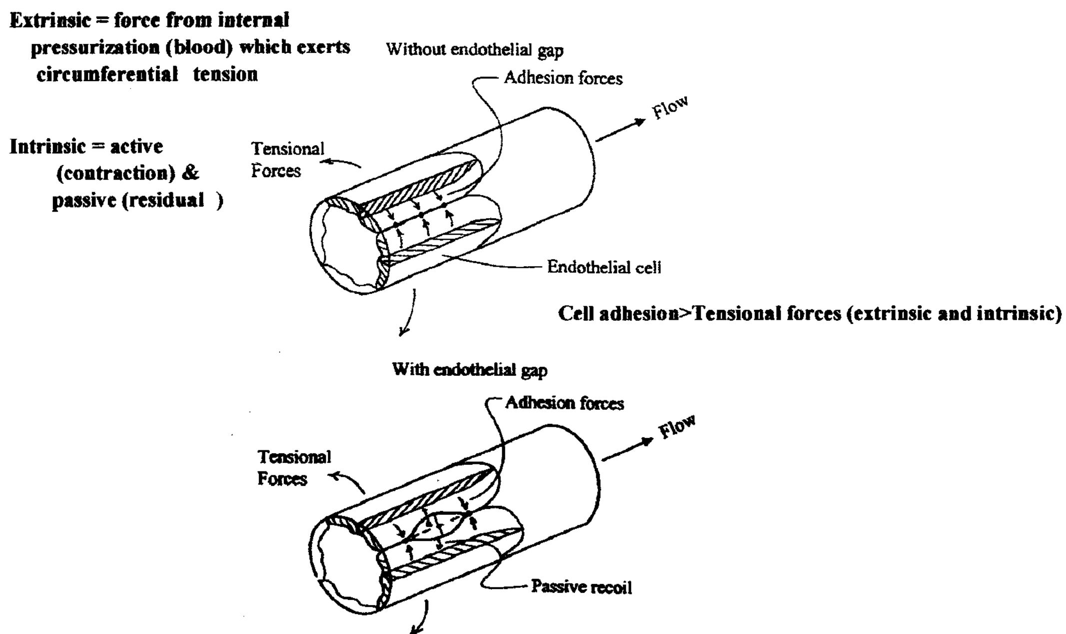

Figure 2.9: Adapted from **Baldwin and Thurston [6].** Forces exerted on endothelial cells. Cell adhesion forces must be equal or greater than the sum of tensional (pressurization of blood) forces, active and passive contraction. When tensional forces overcome adhesion forces gap formations occur.

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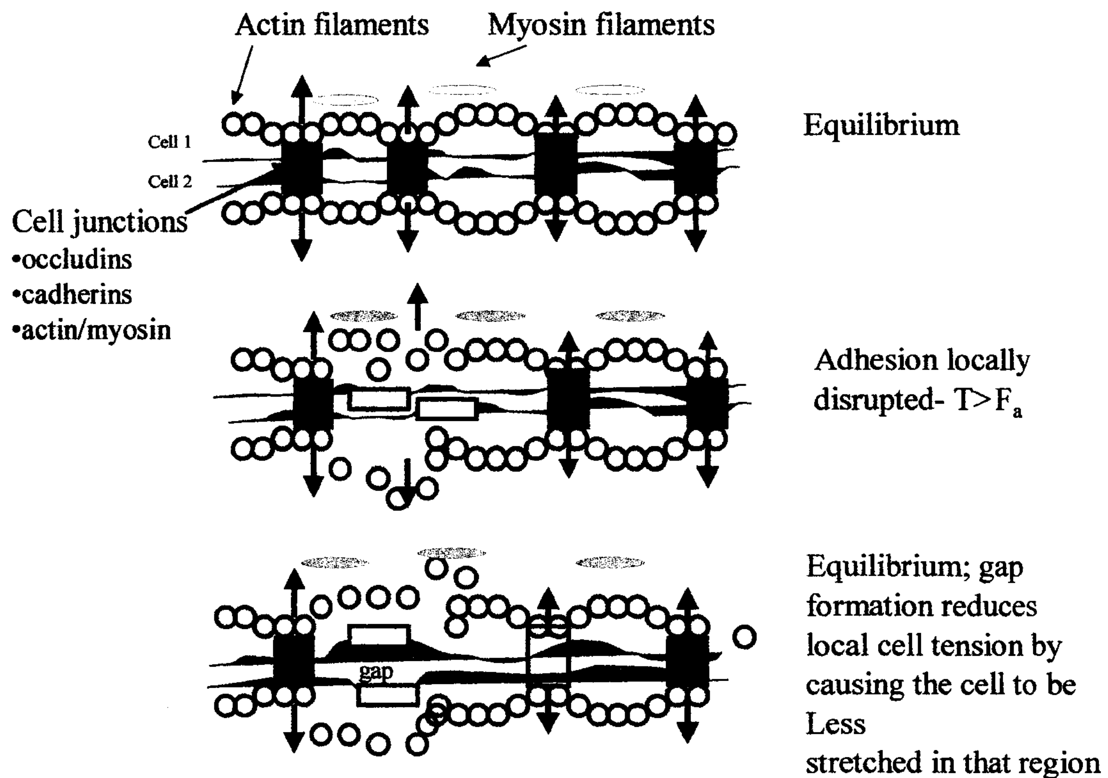

Figure 2.10: Changes in endothelial forces that cause gap formation from passive contraction. Changes in distribution of actin filaments, occludins or cadherins allow tensional force (T) to overcome adhesion forces. Two adjoining cells will separate until a new equilibrium is reached forming a gap.

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2.3.2 Endothelial cell heterogeneity.

2.3.2.1 Overview.

Endothelial cells function in a multitude of physiological processes including; control of cellular trafficking, the regulation of vasomotor tone, the maintenance of blood fluidity, and the growth of new blood vessels. However, it is only beginning to be recognized that endothelial cell phenotypes are differentially regulated [2, 34, 54, 65]. At a single point in time, structural and functional phenotypes vary between segments of the vascular tree, and at any given location the endothelial cell phenotypes may change from one moment to the next [2]. EC heterogeneity occurs between different organs, within the vascular loop of the same organ, and even between neighboring endothelial cells of a single blood vessel. Therefore, an overview on the differences between pulmonary microvascular and macrovascular endothelial cells will follow.

2.3.2.2 Embryogenesis

From a mechanistic standpoint there is evidence that *micro*vascular and *macro*vascular segments of the lung are derived from different origins during embryogenesis. *Micro*vascular endothelial cells are derived from blood islands by a process that involves vasculogenesis, whereas *macro*vascular endothelial cells originate from the pulmonary truncus by angiogenesis [2]. Although these genetic programs may be important in specifying *micro*vascular and *macro*vascular fates, terminal differentiation is likely to be critically dependent on cues from the microenvironmen [2].

2.3.2.3 Morphological and structural differences.

Structural differences exist between *micro*vascular and *macro*vascular endothelial cells. For example, within the extra-alveolar pulmonary microcirculation of the rat lung, the

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mean thickness of the endothelium in muscular vessels is greater than that of partially muscular or nonmuscular *micro*vessels [2, 34, 65]. Vessel densities are greater in alveolar capillary endothelium compared with endothelial cells from extra-alveolar *micro*vessels. Scanning electron microscopy studies of the pulmonary artery reveal a meshwork of irregular projections or microvilli, which increase the surface area of the alveolar endothelial cells. These structural differences are likely to be coupled to regional heterogeneity in function, and to reflect the unique demands of the environment [2, 34, 65].

2.3.2.4 Endothelial cell heterogeneity and acute lung injury.

If the structure and function of lung endothelial cells differ from other organs, it follows that the response of the pulmonary endothelium to a systemic stimulus such as sepsis or hypoxia will also differ between organs. However, in approaching the pathophysiology of acute lung injury, it is important to consider the multiple and complex interactions between the lung and other organ systems. Under normal conditions the endothelial cell phenotypes (and vulnerability to pathophysiological conditions) differ between organs and within a given organ, but in the clinical conditions associated with pulmonary edema, the lung endothelium is exposed to shifts in the microenvironment, both from the luminal and abluminal side [2]. Blood from the right ventricle may contain altered concentrations of /or activity of cytokines, hormones, acid/base-electrolytes and bacterial endotoxin (LPS) [2]. In addition, the blood may be hypoxic, hypo- or hyperthermic and delivered under lower than normal perfusion pressures [2]. Many of these properties are modulated by the health of other organ systems including the brain, kidney, and liver. However, a

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point to consider is that altered function of one organ may have downstream effects on other

organs. Furthermore, the abluminal side of the endothelium may be exposed to ventilator associated forces and/or changes in the properties of inhaled air [2]. Therefore, the local endothelial cell phenotypes conspire with the changes in systemic input (blood and air) to induce local pathophysiology, and once activated these site-specific pathways may engage in autocrine and paracrine loops, serving as a positive feed back loop in a vicious cycle [2].

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CHAPTER III

3 HIGH ALTITUDE-INDUCED PULMONARY VASCULAR LEAK IS REDUCED IN NEUTRAL ENDOPEPTIDASE GENE DELETED MICE

3.1 ABSTRACT

Neutral endopeptidase (NEP) is responsible for degradation of a variety of vasoactive peptides that play a role in lung fluid balance at high altitude (HA), and is well suited for studies of proteolytic control. NEP degrades atrial natriuretic peptide (ANP) and endothelin-1 (ET-1), two opposing peptides that play a role in the formation of high altitude pulmonary edema (HAPE). ANP is elevated in HAPE, and can abrogate pulmonary vascular leak *in vivo* and *in vitro.* ET-1 is a potent pulmonary vasoconstrictor, and recent evidence suggests it is increased in HAPE-prone mountaineers. We hypothesized that a decrease in NEP at high altitude would increase ANP and ET-1 concentrations, but ANP potency would mitigate the effects of ET-1, decreasing the development of HAPE. Plasma ANP and ET-1 concentrations, right ventricular pressure (P_{RV}) and indexes of lung injury were measured in wild type (NEP +/+) mice and mice in which the NEP gene was deleted (NEP -/-) on the same genetic background (C57BL/6). Mice were exposed to a simulated altitude (HA) of 22,000 ft (6728 m; P_B= 328 mm Hg) for 24 h. At HA lung wet weight-to-body weight increased in all animals, but was greatest in the NEP (+/+) mice. Vascular leak as measured by the Evans blue dye technique was increased only in the NEP (+/+)mice at HA. At HA P_{RV} was lowest in NEP (-/-) mice, but increased in both genotypes. Plasma ANP concentrations increased at HA, but plasma ET-1 concentrations were elevated only in the NEP (-/-) mice at HA. There were a negative correlations between plasma ANP concentration, lung wet weight-to-body weight ($r=-0.52$; $p=0.03$) and P_{RV} ($r=-0.62$; $p=0.0061$). We conclude that NEP (-

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/-) mice had increased ANP concentration and decreased pulmonary vascular pressure at HA, preventing high altitude-induced pulmonary vascular leak.

3.2 INTRODUCTION.

High altitude pulmonary edema (HAPE) is a life threatening form of noncardiogenic pulmonary edema that can develop after rapid ascent to elevations greater then 2500 m [3, 5, 41], and is characterized by exaggerated pulmonary hypertension [4, 11, 15, 39] and increased vascular permeability[8, 12]. Despite years of research, the pathogenesis of HAPE remains unclear [3, 8, 12, 15], due in part to our inability to unravel the complex web of interactions between vasoactive peptides during high altitude exposure. Peptide activity is controlled by synthesis and release and by the rate of degradation [31]. Neutral endopeptidase (NEP), also known as neprilysin [8, 10, 30, 31, 36], is a membrane bound, zinc metalloprotease that degrades a variety of biologically vasoactive peptides [27, 30-32]. Recently, mice bearing a targeted disruption in the NEP gene have further characterize NEP as a regulator of basal microvascular permeability, blood pressure and ventilatory response [10, 30, 31]. In addition, Carpenter and Stenmark [8] noted a decrease in NEP expression in weanling rats exposed to hypoxia which positively correlated to pulmonary vascular leak [8]. Therefore, NEP deficiency appears particularly well suited as a model for studies of the potency and proteolytic control between counteracting biologically active peptides and pulmonary vascular leak at high altitude.

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Atrial natriuretic peptide (ANP) and endothelin-1 (ET-1), which have opposing effects on the pulmonary vascular system during exposure to high altitude, are degraded by NEP [16, 27, 28, 36]. ANP, a cardiac hormone, maintains volume homeostasis through its vasorelaxant and natriuretic properties, and decreases pulmonary hypertension and vascular permeability during acute hypoxia *in vivo* and *in vitro* [1, 16, 19-24, 26, 28]. On the other hand, ET-1 is a potent vasoconstrictor and permeability agent, and may contribute to the exaggerated pulmonary hypertension associated with HAPE *in vivo* [6, 34, 8]. However, because ET-1 and its receptor expression are influenced by many diverse physical and biochemical mechanisms, the role of ET-1 in pathological states has been difficult to define [9, 18]. We are unaware of any studies that have examined the net effect of NEP inhibition on counteracting peptides ANP and ET-1 during high altitude exposure.

We hypothesized that a decrease in NEP at high altitude would increase circulating ANP and ET-1 concentrations, but ANP action would supersede the deleterious effects of ET-1, thus abrogating the development of HAPE. Our approach was to examine the effects of 24 h of hypoxic exposure on mice that had a targeted disruption of the NEP gene. We then determined plasma ANP and ET-1 concentrations, right ventricular pressure, lung water and pulmonary vascular permeability. The goal of this study was to determine the roles of opposing vasoactive agents in the development of pulmonary leak at high altitude.

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3.3 METHODS.

Animals. All protocols were reviewed and approved by the Colorado State University Animal Care and Use Committee prior to implementation. C57BL/6J wild-type (NEP +/+) mice, obtained from Jackson Laboratories at 8-12 wk of age were compared with sex- and age-matched NEP gene knockout mice (NEP-/-) bred into the same genetic background (C57BL/6J). All NEP +/+ mice were allowed to acclimate for at least 10 d before hypoxic exposure. A breeding pair of NEP -/- mice were kindly provided by Dr. Lu Hersch (Department of Molecular and Cellular Biochemistry, University of Kentucky) and were bred and raised in a barrier facility at Colorado State University(4920 ft; P_B=640 mm Hg). Sentinel animals housed in the same facility were periodically screened for infections by routine serology. All animals were allowed free access to food and water and were subjected to a 12-hour day-night light cycle.

DNA extraction and PCR confirmation of NEP gene deletion. DNA was extracted from the tails of offspring NEP -/- mice for confirmation of NEP gene deletion by PCR. Briefly, tail clippings were digested in proteinase K solution overnight at 50°C (0.1 M NaCl; 50 mM Tris pH 7.5; 1 mM EDTA 0.5% SDS and 200 ug/ml proteinase K). DNA was extracted using Tris saturated phenol, chloroform and isoamyl alcohol (50:48:2 % respectively). Samples were vortexed and chloroform/isoamyl was added to the aqueous phase and centrifuged (14,000 x g, 1 min). Samples were then precipitated with 95% ETOH. Deletion of the NEP gene was assessed by standard PCR technique. As previously described, a genomic fragment of the NEP gene was replaced by a neomycin resistant cassette, driven by a glucokinase promoter [31]. NEP and neomycin primers were designed from the published sequences of the murine NEP gene (Genbank

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accession number NM_008604) and the PGK neomycin resistant cassette (Genbank accession number AF_335419) with the PRIMER-1.0 primer design software program (SCI-ED, Durham, North Carolina). The following primers were used:

Forward NEP (1); 5' –AGTTGTTGTCTATGCTCCAG-3';

Reverse NEP (2) 5'–GAATGACCTCTTCCTACACA-3';

Forward NEO (3) 5'-TAGAAGGCGATGCGTGCGA-3';

Reverse NEO (4) 5'- TCTCCTGTACATCTCACCTTG-3'.

PCR was performed on mouse offspring DNA using the following combination of primers, cycling times and temperatures: (NEP 1 / NEP 2), (NEP 1 / NEO 4) or (NEO 3 / NEO 4); run on: 1 cycle 94° C /2 min; 30 cycles of 94° C /30 s;.42°-56° C (gradient) / 45 s; 72° C /1 min and 1 cycle 72° C /1 min. The PCR product was electrophoresed on an agarose gel with a 1000 bp ladder and stained with ethidium bromide for visualization. In addition, pLnBAZ, a plasmid containing the neomycin resistant gene was used as a positive control for the NEO (3-4) primers. DNA obtained from a mouse cell line PG13 was used as a positive control for NEP (1-2) primers and run in parallel with DNA to be genotyped. The mouse offspring DNA yielded a 550 bp PCR product with (NEP 1 / NEO 4) primers, and 470 bp PCR product with (NEO 3/ 4) and no band was apparent with (NEP 1 / 2) primers, which we accepted as confirmation of NEP gene deletion.

Exposure to high altitude. Animals were weighed prior to 24 h of exposure to a simulated altitude of 22,000 ft (6728 m; P_B= 328 mm Hg) in a hypobaric chamber. All high altitude procedures were conducted in the main chamber after gaining access through an independently controlled antechamber. In this manner, the high altitude animals were

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never exposed to ambient P_B during altitude exposure. Investigators collected all high altitude data at a simulated high altitude of 12,000 ft (3600 m; P_B = 483 mm Hg).

Right heart cathetrization. Mice were anesthetized with ketamine (100 mg/kg i.m) and xylazine (10 mg/kg i.m). A 25 g needle connected to polyvinyl tubing (PV 10, 0.28 mm ID) was introduced just below the sternum and guided into the right ventricle, while recording pressure on a Gilson Duograph recorder from a P23 Db transducer (Statham, Oxnard, CA) referenced at heart level. Right ventricular pressures (P_{RV}) were recorded for ~ 1-2 min under normoxic and high altitude (12,000 ft) conditions and the mean P_{RV} was obtained from the pressure tracings.

Blood and tissue collection. Midline thoracotomies were performed and animals exsanguinated via cardiac puncture. Blood was transferred into chilled vials containing EDTA (1 mg/ml) and aprotinin (500,000 IU/ml of blood). Plasma was separated by centrifugation (4°C; 14,000 x g; 10 min), removed and stored at -70°C until assayed. The heart and lungs were removed en bloc. The left lung was removed, weighed and oven dried (65°C, 48 h). The right lung was fixed in 10% formalin (24 h), embedded in paraffin and sectioned to 4 μm for histological analyses. Lung wet-weight-to dry weight ratios, lung wet-weight-to pre-high altitude body weight (mg/g) and lung wet-weight-to post-high altitude body weight (mg/g) ratios were calculated.

Histology analyses. Transverse sections from the right lung (3/lung) were prepared and stained with hemotoxylin and eosin (H&E). The slides were examined in a blinded

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fashion using the scale, (0=none; 1=mild; 2=moderate; 3=severe) to evaluate the following categories, (alveolar neutrophils; alveolar red blood cells; hemorrhage/blebs, thickened alveolar septa and interstitial pneumonia). The summation of categorical scores was used for comparison between groups.

Pulmonary vascular protein extravasation. To confirm our lung wet weight-to-dry weight /body weight ratios and histological findings, the protein leak from the pulmonary vasculature was assessed by measuring the accumulation of Evans blue dye in the lungs of additional groups of control and experimental animals (n=5/group) at low and high altitude (24 h). Evans blue dye (30 mg/kg) was injected via tail vein, and mice were euthanized after 10 min with ketamine (100 mg/kg; i.m) and xylazine (10 mg/kg; i.m.) . Animals were then transcardially perfused with phosphate buffered saline. Heart and lungs were removed en bloc, and extrapulmonary airways and tissues were removed. After weighing, Evans blue dye was extracted from the right lung by formamide (100%) incubation (18 h, 37°C). The left lung was weighed, oven dried (65°C, 48 h), and weighed again. Wet-to-dry weight ratio in the left lung was used to estimate the dry weight of the right lung. The extracted dye was quantified in a spectrophotmeter (Labsystems Multiscan, Helsinki, Finland;) by measuring the absorbance at 600 nm against standards of Evans blue dye dissolved in formamide. Evans blue dye extravasation is expressed as nanograms of Evans blue dye per milligram of dried tissue.

ANP and ET-1. Plasma peptides were extracted from mouse plasma as previously described [15]. Briefly, an equal amount of buffer A (1% trifluoracetic acid (TFA) in

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99% distilled water) was added to the plasma sample and loaded onto a pre-treated C18 sep column (Peninsula Laboratories, San Carlos, CA). The peptide was eluted with buffer B (60% acetonitrile; 1% TFA; in 39% distilled water), desiccated and resuspended in assay buffer for determination of ANP and ET-1 concentration. ANP and ET-1 concentration was determined by an enzyme immunoassay (EIA) (Peninsula Laboratories, San Carlos CA; ANP No. S-1132 and ET-1 No. S-1171), as previously described [34]. Samples were assayed in duplicate, quantified in a spectrophotmeter (450 nm) (Labsystems Multiskan, Thermo Electron Corp., Waltham, MA;) and expressed as ng/ml.

Statistical analyses. All results are expressed as means ± standard error (SEM). Statistical analyses were performed with the JMP (version 5) statistical package (SAS, Cary, North Carolina). Comparisons between groups were analyzed by unpaired Students t-test. Correlations between variables were determined by Pearson correlation coefficients. Differences were considered significant at $p < 0.05$.

3.4 RESULTS.

Body weight

The body weight of each mouse was determined before and after high altitude exposure and weight loss was calculated as a percentage of pre-high altitude weight. Although animals of both genotypes lost weight during exposure to high altitude, NEP -/- experienced a slightly greater weight loss than NEP +/+ ($p \leq 0.0001$ vs. low altitude; $p = 0.023$ NEP-/- vs. NEP +/+). To assess the effect of weight loss during high altitude on the indices of pulmonary edema, a separate set of NEP-/- (n=3) and NEP +/+ (n=5) mice were fasted at ambient altitude (food, 24 h; and water, 12 h) and weight loss was

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compared to that in high altitude animals. Wet lung weight-to-dry weights and wet lung weight-to- body weights were determined to evaluate the effect of dehydration. Body weight decreased in the fasted NEP -/- and NEP +/+ mice (10.6±0.6 and 8.4±0.8% respectively, $p \leq 0.001$ vs. satiated mice). Compared to the means of satiated mice, lung wet weight-to-body weight ratios were increased (7.3± 1.5%; $p = 0.05$).

Pulmonary edema.

To determine if inhibition of NEP abrogated the formation of HAPE we determined the lung wet weight-to-dry weight (W/D), lung wet weight-to-body weight (LW/BW) ratios and albumin lung leak in NEP knockout (NEP -/-) and NEP wild type (NEP +/+) mice at low altitude (4,920 ft; ≅ 1,500 m; $P_B \cong 640$ mm Hg) and high altitude (24 hours; 22,000 ft; ≅ 6,710 m ; $P_B \cong 328$ mm Hg). At low altitude there was no difference between W/D ratios in NEP -/- and NEP +/+ mice. Exposure to high altitude did not increase W/D ratios in NEP -/- or NEP +/+ animals **(Figure 1)**. However, LW/BW ratios were increased at high as compared to low altitude in both genotypes **(Figure 2)**, the greatest increase occurring in the NEP +/+ group **(Figure 3)**. Lung albumin leak, assessed by Evans blue dye extravasation, was increased only in the NEP +/+ mice at high altitude **(Figure 4)**.

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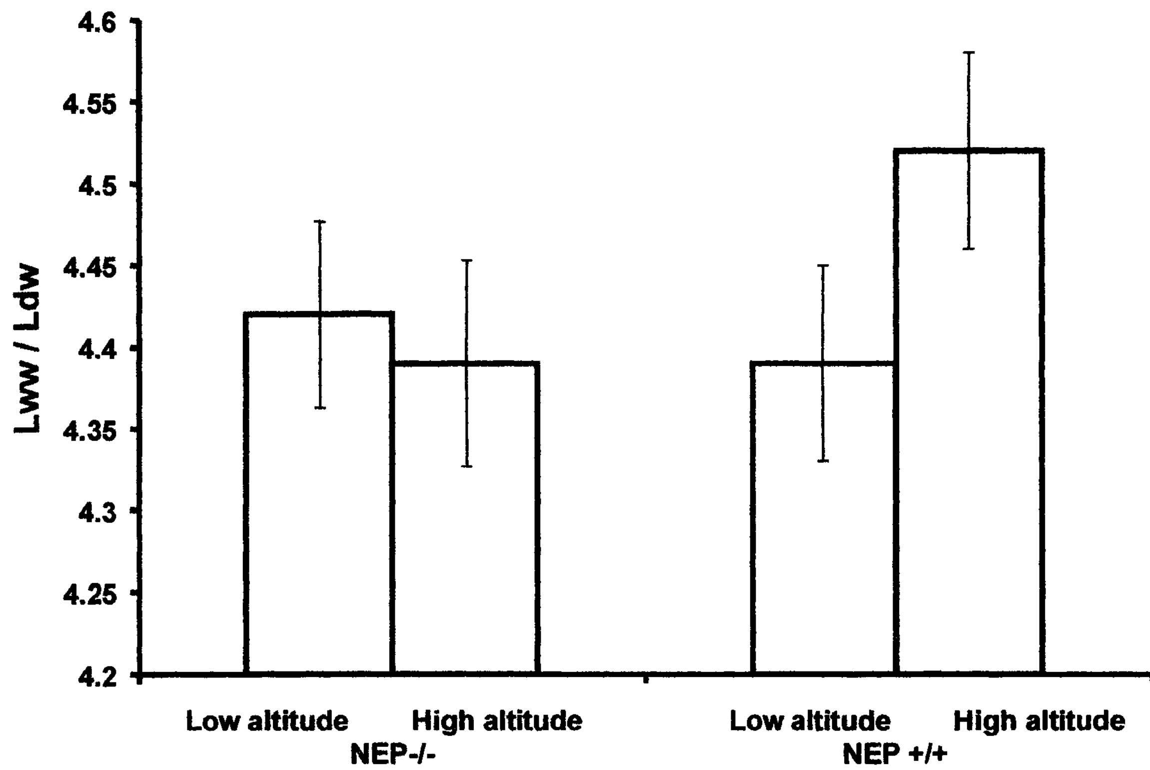

Figure 3.1: Lung wet weight-to-dry weight ratio between NEP -/- and NEP +/+ mice

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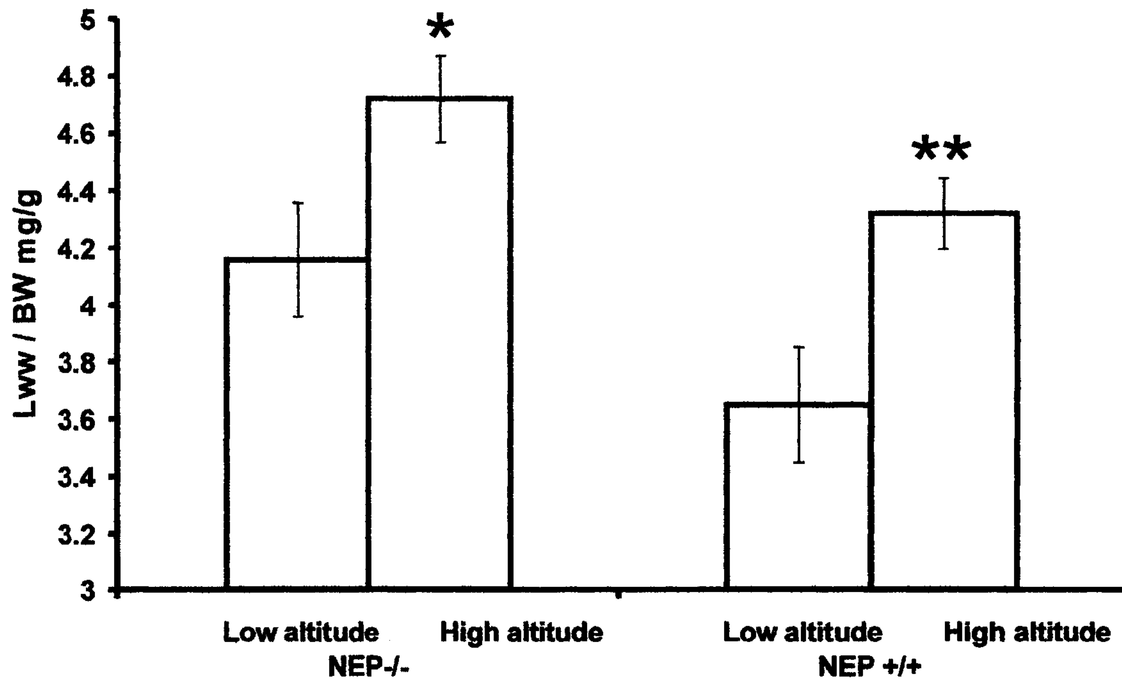

Figure 3.2: Lung wet weight-to-body weight‡ ratio of NEP -/- and NEP +/+ mice after 24 h of exposure to high altitude * P=0.04 vs. NEP-/- low altitude; **P=0.01 vs. NEP+/+ low altitude.

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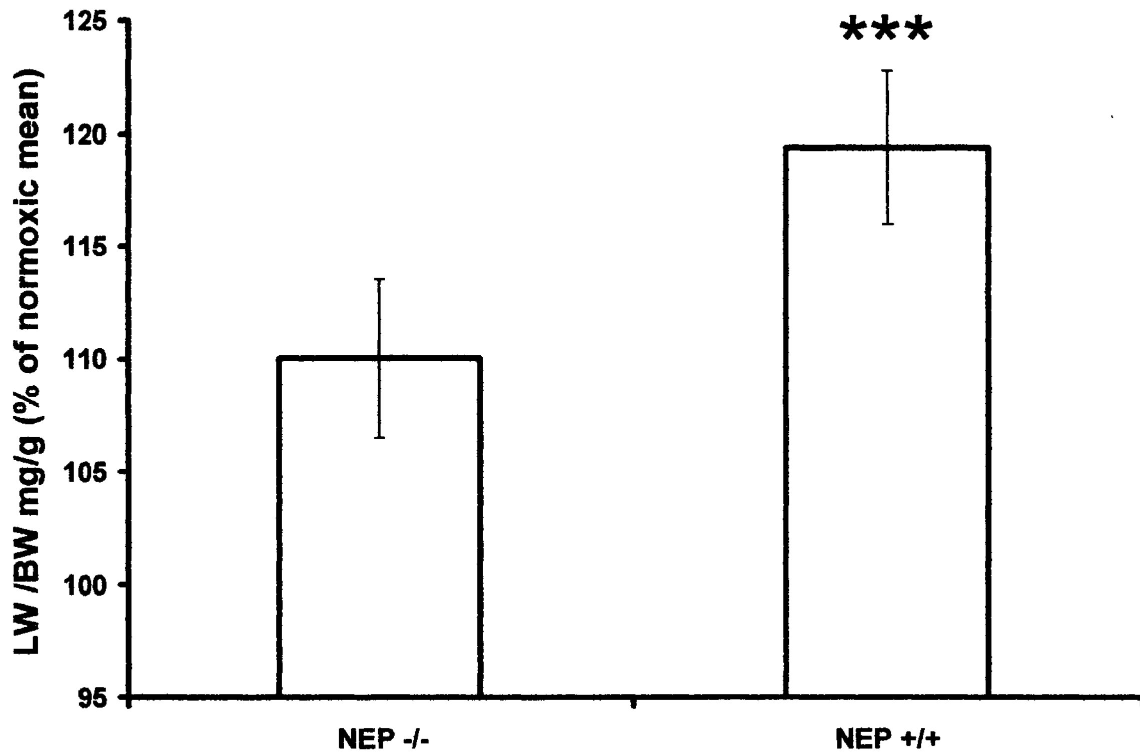

Figure 3.3: Lung wet weight-to-body weight ratio normalized to low altitude means. *** P =0.04 vs. NEP -/- mice.

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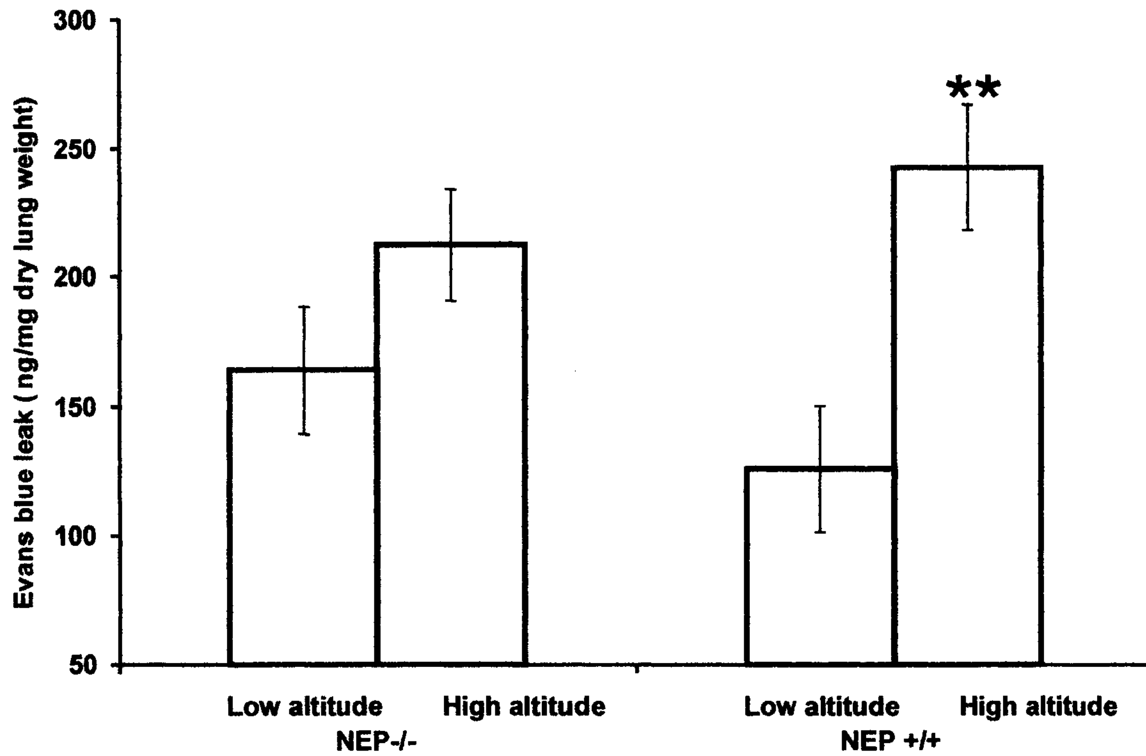

Figure 3.4. Evans blue dye leak in NEP -/- and NEP +/+ mice at low and high altitude. ** P=0.01 vs. NEP +/+ low altitude.

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Histology.

Lung structure was similar in NEP -/- and NEP +/+ animals. However, 72% of the high altitude animals had mild increases in alveolar neutrophils, septal thickening and scattered areas of hemorrhage compared to 23% of low altitude cohorts **(Figure 5).**

Pulmonary artery pressure

Pulmonary hypertension is associated with HAPE and ANP and ET-1 can alter vascular tone [1, 9, 18, 28, 37], therefore we determined mean right ventricular pressure (P_{RV}) as an indication of pulmonary artery pressure (PAP) in NEP -/- and NEP +/+ mice at low and high altitude. At low altitude, NEP -/- mice had lower P_{RV} compared to NEP +/+ mice ($p \leq 0.05$). P_{RV} increased in NEP -/- and NEP +/+ animals at high altitude **(Figure 6).**

Plasma ANP

Our group and others have reported that ANP inhibits pulmonary vascular leak *in vitro* and *in vivo* [14, 15, 38]. Because NEP degrades ANP [16, 28, 36], we measured plasma ANP concentration in NEP -/- and NEP +/+ mice at low and high altitude. There was no difference in plasma ANP concentration between animals of differing genotypes at low altitude. Plasma ANP concentration increased 3.1 and 2.7-fold in NEP -/- and NEP +/+ mice at high altitude **(Figure 7).**

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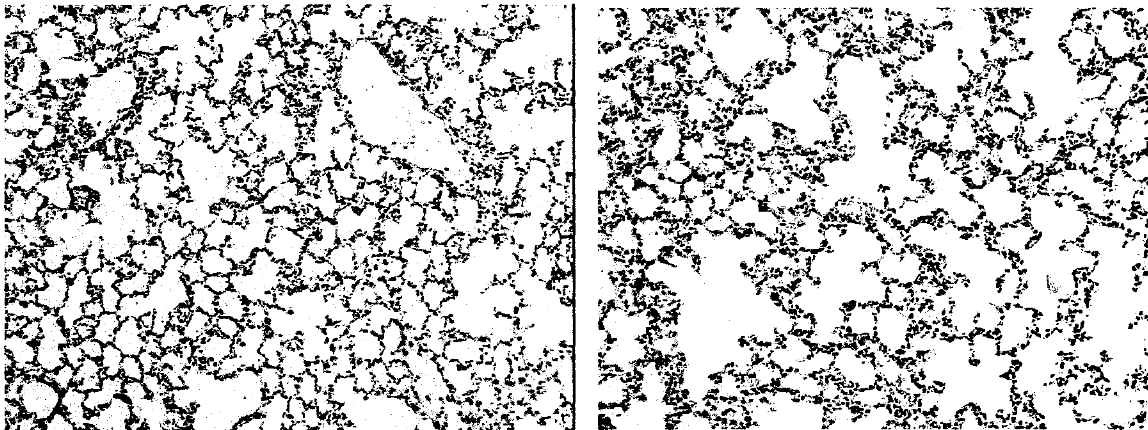

Figure 3.5. Typical photomicrograph of lung parenchyma of mice at low and high altitude. High altitude mice had alveolar septal thickening and patchy edema.

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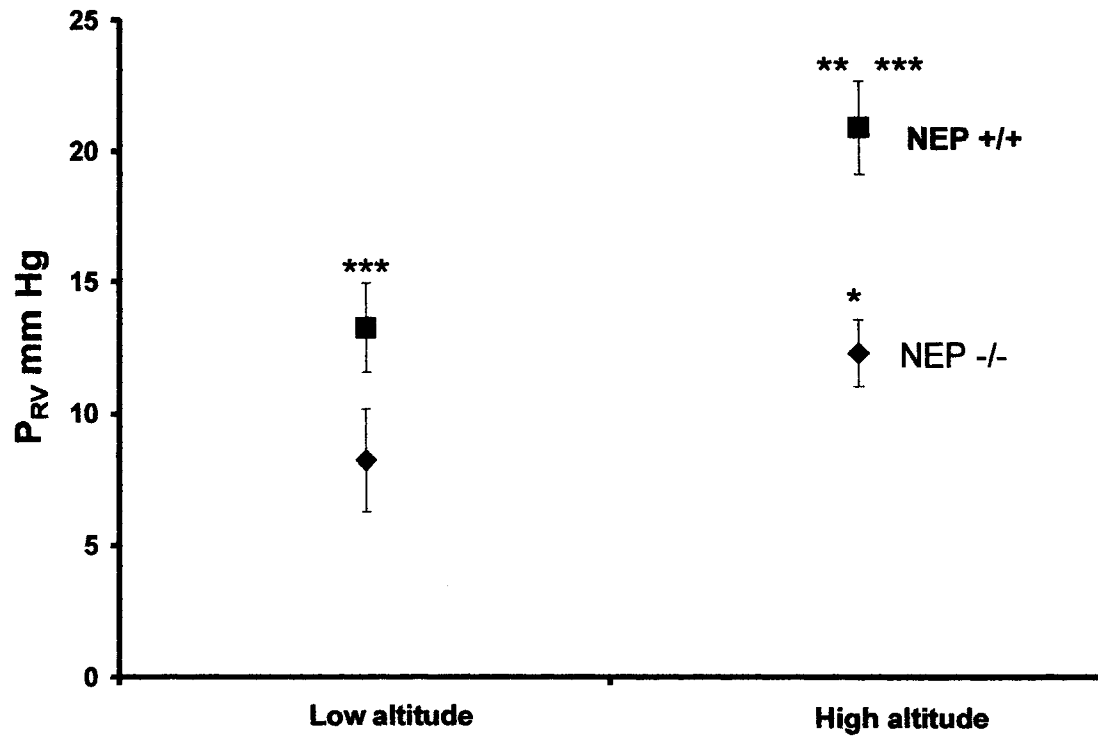

Figure 3.6. Right ventricular pressure (P_{rv}) in NEP -/- and NEP +/+ mice at low and high altitude. *P=0.02 vs. NEP -/- low altitude; **P0.002 vs. NEP +/+ low altitude. ***P≤0.01 NEP-/- vs. NEP +/+ mice.

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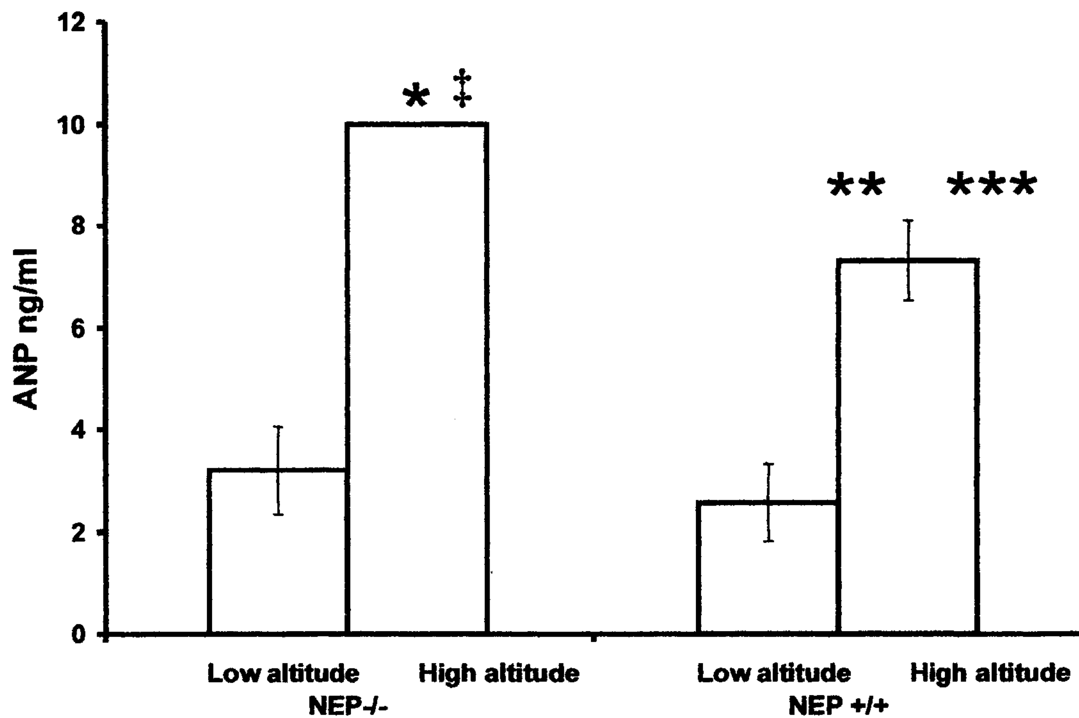

Figure 3.7. Plasma ANP levels in NEP -/- and NEP +/+ mice at high and low altitude. *P≤0.001 vs. NEP -/- low altitude; **P≤0.001 vs. NEP +/+ mice; ***P=0.02 vs. NEP -/- mice; ‡ Plasma ANP levels > 10 ng/ml maximum detectable range of assay.

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Plasma ET-1.

ET-1 is a potent hypoxia-induced vasoconstrictor that acts in opposition to ANP by causing vasoconstriction and increased pulmonary leak and is degraded by NEP [9, 25, 27]. We determined plasma ET-1 concentration in NEP -/- and NEP +/+ mice at low and high altitude. Low altitude plasma ET-1 concentrations were similar in NEP -/- and NEP +/+ mice, however plasma ET-1 concentration increased at high altitude in NEP -/-, but not NEP +/+ mice **(Figure 8)**.

Plasma ANP /ET-1 ratio

To investigate the relationship between NEP, ANP and ET-1 we determined plasma ANP/ET-1 ratios. Low altitude ANP/ET-1 ratios were similar between NEP -/- and NEP +/+ mice. At high altitude ANP/ET-1 ratios increased 2.5 and 2-fold in NEP -/- and NEP +/+ respectively **(Fig.9)**. NEP -/- mice may have had higher ANP/ET-1 ratios compared to NEP +/+ cohorts, however plasma ANP levels were above the maximum detectable range of 10 ng/ml at high altitude.

ANP, ET-1, P_{RV}, and LW/BW correlation's at high altitude

We determined correlations between indices of pulmonary hypertension and pulmonary edema, plasma ANP and ET-1 concentrations at high altitude. There was a significant correlation between the P_{RV} and the LW/BW ratio for each animal at high altitude **(Figure 10)**. Plasma ANP concentrations were inversely correlated with P_{RV} pressure **(Figure11)** and LW/BW ratio for each animal at high altitude **(Figure12)**. Plasma ET-1 concentration did not correlate with RV pressure or normalized LW/BW.

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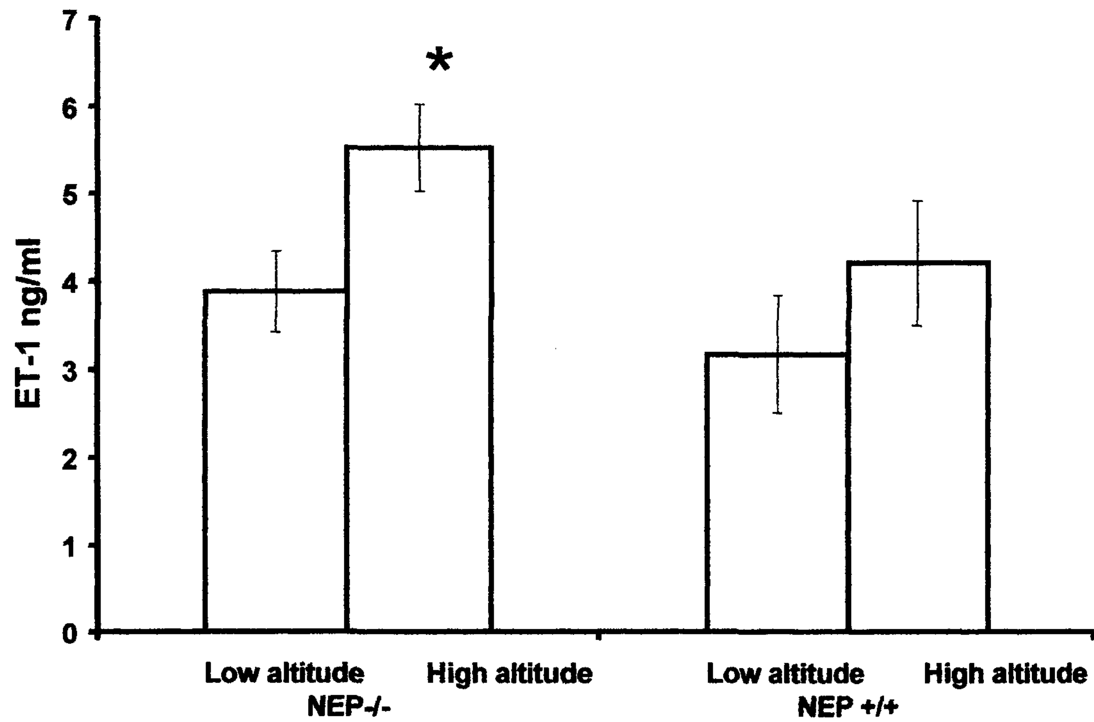

Figure 3.8. Plasma ET-1 concentration in NEP -/- and NEP +/+ mice at low and high altitude. *P=0.02 vs. NEP -/- low altitude.

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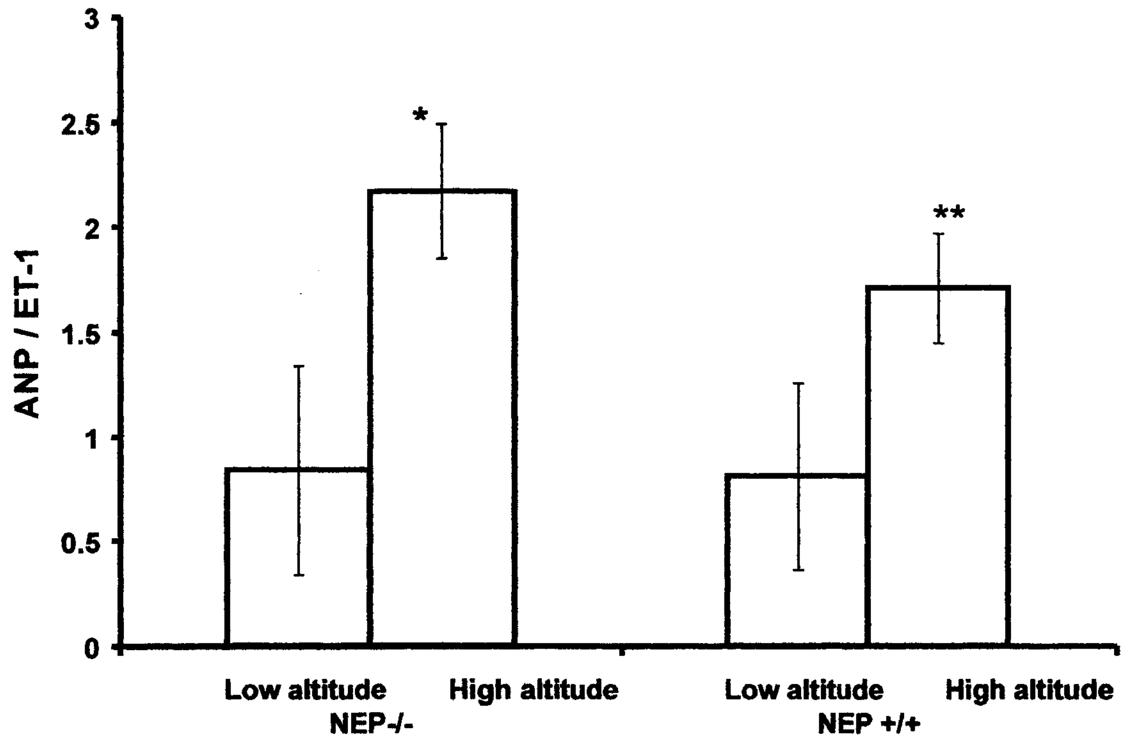

Figure 3.9: Plasma ANP/ ET-1 ratio. *P=0.004 vs. NEP -/- low altitude; P=0.04 vs. NEP+/+ low altitude.

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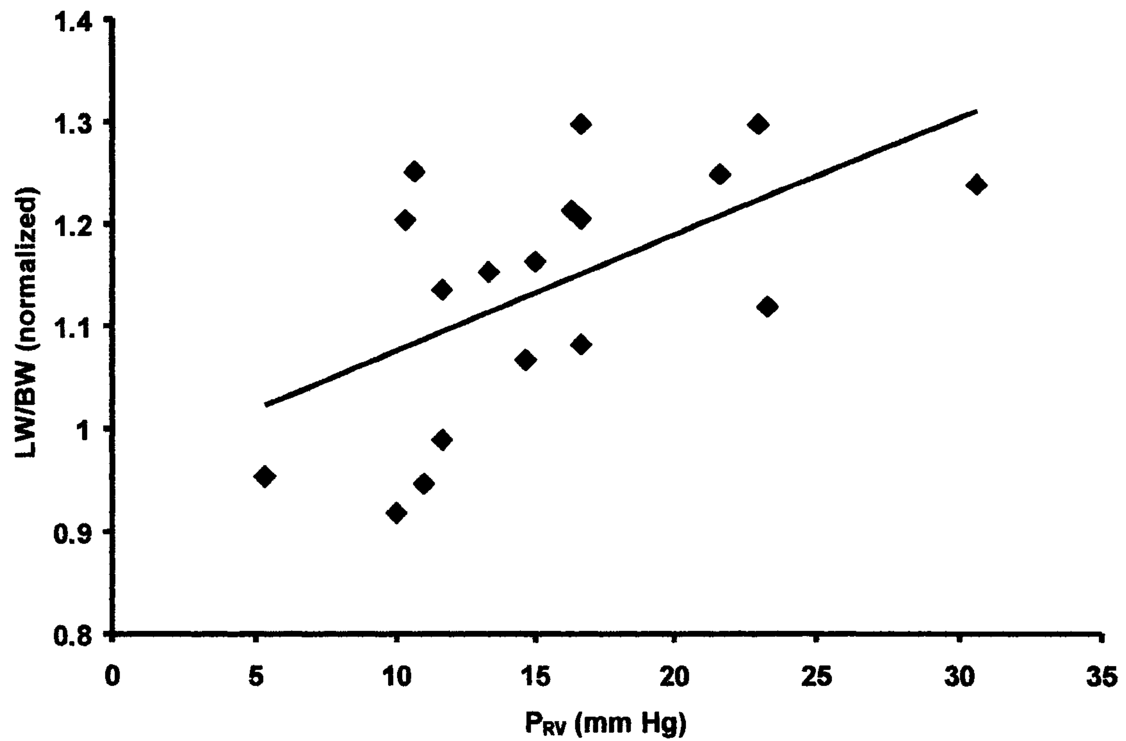

Figure 3.10: Scatter plot showing the relationship between the percent change in lung wet-weight-to-body weight ratios and right ventricular pressure (P_{RV}) in mice at high altitude (r=0.57; P=0.007).

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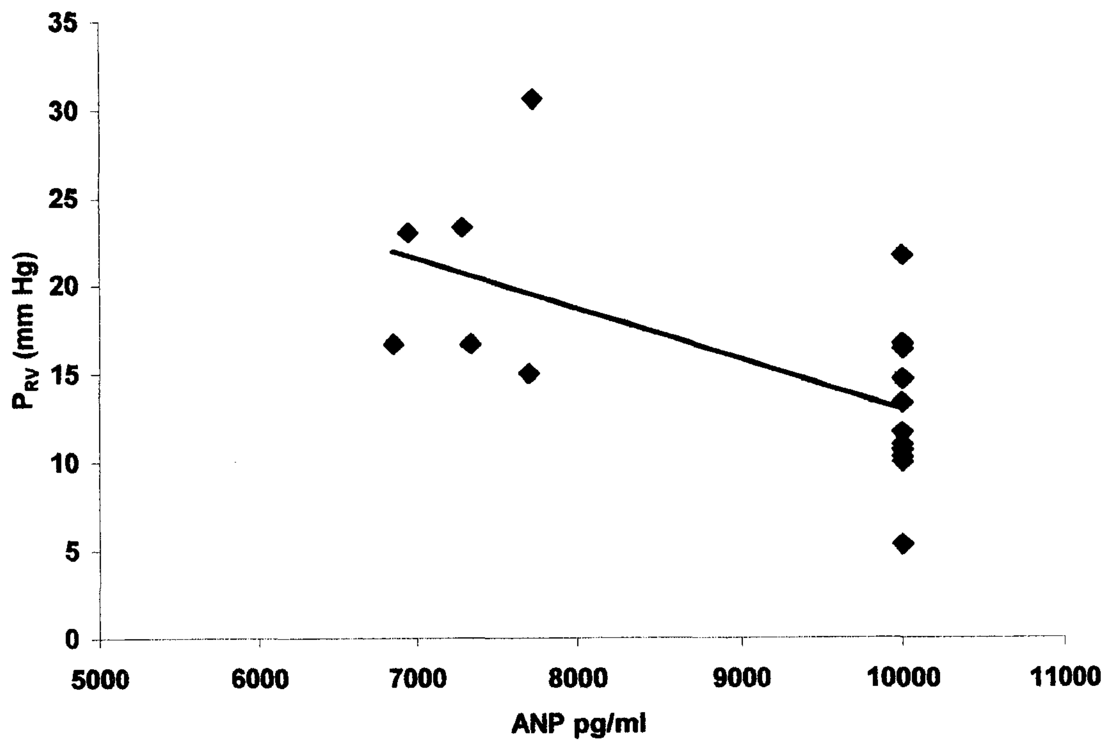

Figure 3.11: Scatter plot showing the relationship between right ventricular pressure (P_{RV}) and plasma ANP concentrations at high altitude (r=-0.62; P=0.0061). * NEP -/- mice at high altitude had ANP levels above the maximum detectable range of 10 ng/ml, the value used in Pearson correlation.

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3.5 DISCUSSION.

The present study demonstrates that targeted deletion of the NEP gene by homologous recombination reduces high altitude-induced pulmonary vascular leak in mice 10-12 weeks of age. Our data suggest that decreased pulmonary vascular pressure secondary to increased endogenous ANP may be responsible for lower pulmonary vascular leak in NEP -/- animals at high altitude. Our study focused on the contributions of two counteracting vasoactive agents degraded by NEP, ANP and ET-1, to the development of pulmonary leak at high altitude in mice. Circulating ANP and ET-1 concentrations were elevated at high altitude in NEP -/- mice compared to NEP +/+ mice, likely due to absence of NEP. Plasma ANP concentrations negatively correlated with right ventricular pressure and pulmonary vascular leak in high altitude exposed mice further suggesting a role for ANP in reducing altitude-induced pulmonary leak.

An inherent limitation of gene deletion animal models is that all vascular beds are affected, possibly altering responses to whole body stress such as high altitude [12]. However, the knockout model provides valuable insights that can be further explored in the non-transgenic models. Our data differ from previous reports that linked a down regulation of NEP mRNA to hypoxia-induced vascular leak in weanling rats exposed to normobaric hypoxia [8]. Because we studied adult mice rather than weanling animals,

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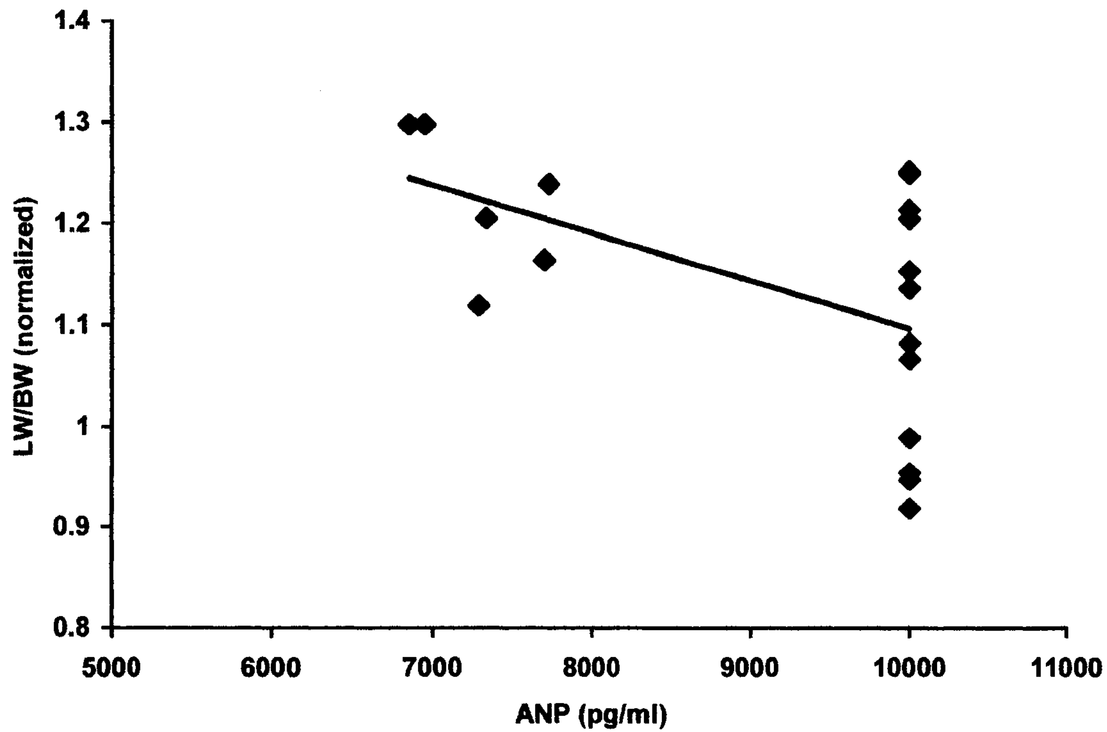

Figure 3.12: Scatter plot showing the relationship between the percent change in lung wet-weight-to-body weight‡ ratios and plasma ANP concentrations at high altitude ($r=-0.52$; $P=0.03$). * NEP -/- mice at high altitude had ANP levels above the maximum detectable range of 10 ng/ml, the value used in Pearson correlation. ‡ post high altitude body weight.

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comparisons between the two studies are difficult. Our results are consistent with previous animal models of acute hypoxia. While there was no gross alveolar flooding or increase in lung wet weight-to-dry weight ratios during hypoxia [5, 8, 15], there was evidence of early pulmonary edema, including increased albumin extravasation, alveolar neutrophil accumulation, and pulmonary hemorrhages at high altitude. The sensitivity of lung wet weight-to-dry weight ratios may have been impaired by alveolar protein extravasation [5, 8] or a loss of body weight. Our data from fasted mice indicating blunted wet weight-to-dry weight ratios, support the hypothesis that the 10% loss of body weight in the hypoxic animals may have reduced intracellular water and decreased sensitivity of lung wet weight/dry weight ratios.

Exposure to high altitude increased the lung wet weight-to-body weight ratios in animals of both genotypes, but normalizing the data to their normoxic group means indicated that the NEP -/- mice had a lower percent rise than NEP +/+ mice (10% vs. 19%). At high altitude NEP -/- mice also had lower lung W/D weight ratios and albumin extravasation, further suggesting they had less pulmonary vascular leak. The decreased body weight from the dehydration during 24 h of high altitude exposure could not fully account for the increase in lung wet weight-to-body weight ratios. Normalizing lung wet weight to pre-altitude body weight indicated a decrease in the lung wet-to-dry ratio in NEP -/- mice (-3.6%), but an increase in NEP +/+ mice (6%) relative to low altitude animals. Our data suggest that although NEP -/- mice may have a slightly higher permeability rate at low

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altitude, high altitude-induced pulmonary vascular leak is less than that of wild type animals.

Increased pulmonary artery pressure (PAP) due to hypoxic pulmonary vasoconstriction is a hallmark of high altitude pulmonary edema (HAPE) in humans, and there is much evidence to suggest it plays a role in the pathophysiology of this condition [3, 8, 11, 15, 35, 39]. Therefore, we measured right ventricular pressure as an indication of pulmonary vascular pressure at low and high altitude in NEP -/- and NEP +/+ mice. At low and high altitude right ventricular pressures (P_{RV}) were lower (~5 and 8 mm Hg respectively) in NEP -/- mice compared to NEP+/+ cohorts. Previous studies have shown that NEP -/- mice have mean arterial blood pressure approximately 20 mm Hg lower then NEP +/+ cohorts [30]. Our data suggest that NEP plays a similar role in maintaining blood pressure homeostasis in the pulmonary vascular system In addition, P_{RV} correlated with LW/BW ratio from the same animal. This is consistent with the concept that hydrostatic forces play an important part in the pathogenesis of HAPE [8, 11, 40]. Because NEP -/- mice had lower P_{RV}, the pulmonary vascular leak in these animals was likely due to increased permeability independent of pulmonary arterial pressure.

Consistent with earlier published reports, our data indicated that NEP -/- and NEP +/+ mice had similar basal plasma ANP concentrations [30]. However, during high altitude exposure plasma ANP concentrations were increased in mice of both genotypes, with the highest concentrations in NEP -/- mice. Data from previous studies indicated that hypoxia is a stimulant for ANP secretion *in vitro* and *in vivo* [2, 15, 17, 29,26]. ANP is degraded

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by NEP; [28, 42] therefore, at high altitude the higher ANP concentrations in NEP -/- mice compared to wild type cohorts may be accounted for by the absence of NEP. Furthermore, data indicating that ANP was inversely correlated with lung wet weight-to-body weight ratio and mean P_{RV} from the same animal suggest a role for ANP in controlling vascular leak by inhibiting hypoxic pulmonary vasoconstriction. Data from previous studies indicating that ANP may abrogate HAPE support our findings [15].

NEP degrades ET-1, a potent vasoactive and mitogenic peptide that is synthesized and released by endothelial cells [9, 18, 27, 36], and acute high altitude hypoxia is a potent stimulus of ET-1 [7, 35]. Plasma ET-1 concentrations are elevated in HAPE-susceptible subjects [35], and may contribute to HAPE by augmenting capillary hydrostatic pressure and/or by increasing microvascular permeability [9, 18, 35]. Similar to ANP, basal plasma ET-1 concentrations were equivalent between NEP -/- and NEP+/+ mice. However, high altitude exposure elevated plasma ET-1 concentration only in NEP -/- mice, possibly due to the absence of NEP degradation. Other studies have reported that hypoxia, has no effect, or decreases ET-1 release from endothelial cells [6, 7, 9]. Further research is needed to clarify the relationship between ET-1 and vascular leak associated with high altitude exposure [6].

To further understand how the net effect of counteracting vasoactive agents ANP and ET-1 alter vascular permeability in the development of HAPE, we investigated the ANP/ET-1 ratio in NEP-/- and NEP +/+ mice at low and high altitude. The basal ANP/ET-1 ratio was approximately one in both genotypes. As we hypothesized, at high altitude ANP

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increased by a 2:1 ratio compared to ET-1 in NEP +/+ mice and possibly to a much larger extent in NEP -/- mice. These data suggest that the absence of NEP allows the beneficial effects of ANP to override the deleterious effects of ET-1 (Fig. 13).

The natriuretic properties of ANP may have also played a role in preventing pulmonary edema in our HAPE model. ANP release induces diureses, natriuresis, and decreased blood volume and blood pressure, and at high altitude likely contributes to the severe dehydration [1, 2, 28, 33]. Our data further suggest that indices of pulmonary edema are less in dehydrated as compared to normally hydrated mice.

A full understanding of the pathogenesis of HAPE remains an unsolved [8, 12]. Although the incidence of this disease is relatively low, an estimated 38 million people each year are at risk while visiting the mountainous Western states [13]. NEP may be of particular interest in the study of high altitude physiology because of its influence on a wide variety of peptides that play a role in the adaptive or maladaptive process of high altitude exposure.

In mice, a decrease in NEP and subsequent rise in plasma ANP concentrations inhibits the development of pulmonary vascular leak and pulmonary hypertension at high altitude.

We thank Dr. Lu Hersch (Department of Molecular and Cellular Biochemistry, University of Kentucky) for providing us a breeding pair of NEP gene deficient mice, Dr. James DeMartini for hisotological evaluation and Dr. Ivan McMurtry from the University of Colorado Health Science Center (UCHSC) for reviewing this manuscript.

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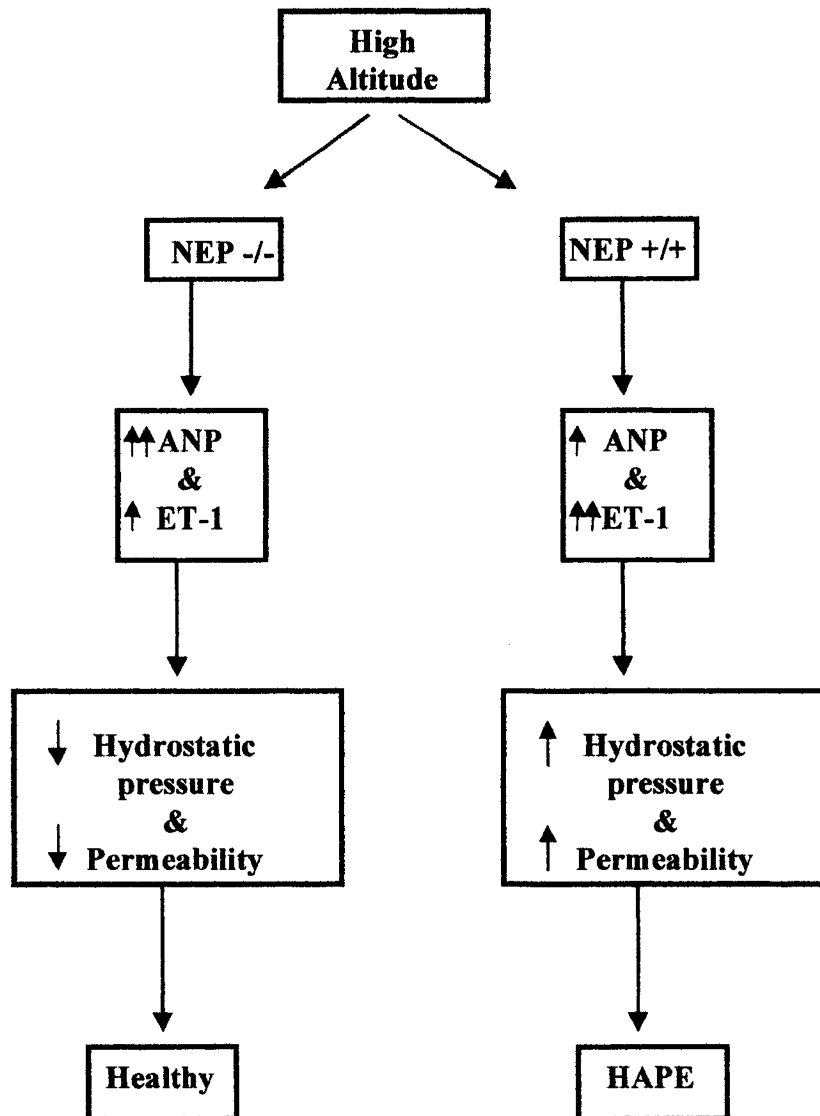

Figure 3.13: Schema of our proposed relationship between NEP, ANP and ET-1 and the development of high altitude exposed vascular leak. As NEP is decreased ANP concentration is sufficiently raised to override the deleterious effects of ET-1.

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CHAPTER IV

4 DIRECT ANP INHIBITION OF HYPOXIA-INDUCED INFLAMMATORY PATHWAYS IN PULMONARY MICROVASCULAR AND MACROVASCULAR ENDOTHELIAL MONOLAYERS

4.1 ABSTRACT

Atrial natriuretic peptide (ANP) has been shown to reduce hypoxia-induced pulmonary vascular leak in vivo, but no explanation of a mechanism has been offered other than its vasodilatory and natriuretic actions. Recently, data has shown that ANP can protect endothelial barrier functions in TNF-α-stimulated human umbilical vein endothelial cells (HUVEC). Therefore, we hypothesized that ANP actions would inhibit pulmonary vascular leak by inhibition of TNF-α secretion and F-actin formation. Bovine pulmonary microvascular (MVEC) and macrovascular (LEC) endothelial cell monolayers were stimulated with hypoxia, TNF-α or bacterial endotoxin (LPS) in the presence or absence of ANP, and albumin flux, NF-κB activation, TNF-α secretion, p38 mitogen activated protein kinase (MAPK) and F-actin (stress fiber) formation were assessed. In transwell cultures, ANP reduced hypoxia-induced permeability in MVEC, and TNF-α-induced permeability in MVEC and LEC. ANP inhibited the hypoxia- and LPS-induced increase in NF-κb activation and TNF-α synthesis in MVEC and LEC. Hypoxia decreased activation of p38 MAPK in MVEC, but increased activation of p38 MAPK and stress fiber formation in LEC. TNF-α had the opposite effect. ANP inhibited an activation of p38 MAPK in MVEC or LEC. These data indicate that in endothelial cell monolayers, hypoxia activates a signal cascade analogous to that initiated by inflammatory agents, and ANP has a direct cytoprotective affect on the pulmonary endothelium other then its

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vasodilatory and natriuretic properties. Furthermore, our data shows that MVEC and LEC respond differently to hypoxia, TNF-α-stimulation and ANP treatment.

4.2 INTRODUCTION.

Acute hypoxia or inflammatory agents increase vascular permeability and contribute to forms of noncardiogenic pulmonary edema such as high altitude pulmonary edema (HAPE) and acute respiratory distress syndrome [4, 7, 10, 11, 18, 44]. Despite extensive research into the effects of acute hypoxia or inflammation on the pulmonary circulation, the mechanisms underlying noncardiogenic pulmonary edema remain unclear [4, 13]. *In vivo* models of lung injury have revealed that atrial natriuretic peptide (ANP) can protect endothelial barrier function [15-17, 43], but apart from its vasodilatory and natriuretic actions, no other mechanisms have been identified to explain ANP's cytoprotective benefits. Recently, ANP has been shown to inhibit TNF-α synthesis in murine macrophages stimulated with bacterial endotoxin (LPS) [19]. ANP also inhibits NF-κB and p38 mitogen activated protein kinase (MAPK) pathways in TNF-α stimulated human umbilical vein endothelial cells (HUVEC) *in vitro* [25, 26]. ANP actions are highly cell type and organ-specific [31, 41, 45], and no experimental work has addressed ANP actions on cultured pulmonary *micro*vascular (MVEC) and *macro*vascular (LEC) endothelial cells exposed to acute hypoxia or inflammatory agents.

We hypothesized that ANP could preserve pulmonary endothelial barrier integrity during hypoxic or inflammatory stress by inhibiting the cytokines that initiate a signal cascade or the proteins within the signal transduction pathway that alter cell morphology, cause gap

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formation and increase permeability. Our approach was to examine the effect of ANP on TNF-α secretion, NF-κB activity and activation of p38 MAPK in cultured MVEC and LEC exposed to hypoxia, TNF-α or LPS. The goal was to determine if ANP protected cells from stress fiber formation, and preserved cell morphology, barrier function and EC permeability. Our study was designed to determine the effects of ANP on hypoxia, TNF-α and LPS-treated pulmonary EC and to further our knowledge of endothelial permeability in the pulmonary vasculature.

4.3 MATERIALS AND METHODS.

Materials. ANP was obtained from Calbiochem (King of Prussa, PA), collagenase II (CLS2) from Worthington Biomedical Corp (LakeWood, NJ), retinal derived growth factor was obtained from Vec Tec Inc.(Rensselaer, NY), nylon mesh from Sefar America Inc.(Depew, NY) and DiI-acetylated LDL from Biomedical Technologies Inc, (Stoughton, MA). FITC-phalloidin (Alexa Fluor 488 phalloidin) was purchased from Molecular Probes, (Eugene, OR). All other materials were purchased from Sigma (St. Louis, MO).

Culture and isolation of bovine pulmonary microvascular endothelial cells (MVEC). MVEC were isolated and cultured in our laboratory. Briefly, bovine lung tissue was harvested locally, transported (4°C) to our laboratory and processed within 4 h. Pleura was removed from the periphery of an external lobe and small pulmonary vessels (diameter ~200 μM) were dissected, minced and digested with collagenase (10 ml of 1000 U/ml collagenase II, 5% BSA; rocker, 37° C, 30 min). The digested tissue was then

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filtered (160 um nylon mesh) and centrifuged (200 x g, 5 min). The pellet was resuspended in culture media (RPMI containing 20% FBS, 70 ug/ml porcine intestinal heparin, 6 ug/ml retinal derived growth factor, 0.1% gentamicin and 0.1% fungizone), washed three times and resuspended in plating media: (3 parts RPMI + 1 part endothelial cell conditioned media). Cells were divided equally into 6 well gelatin-coated (0.1%) plates (~ 10^4 cells/well), and placed in a standard cell culture incubator (37° C, 5% CO_2). After 1week, cell populations exhibiting cobblestone morphology were removed and seeded onto new 6 cm gelatin-coated plates and grown to confluence. Cell populations appearing clear of contaminating fibroblast and smooth muscle cells were sorted by standard FACs analysis using uptake of 1,1′ -dioctadecly-3,3,3′,3′-tetramethylindocarbocyanine (DiI-acetylated LDL) to obtain MVEC populations > 98% pure [18, 37].

Culture and isolation of bovine pulmonary artery endothelial cells (LEC).

Endothelial cells were isolated from bovine conduit pulmonary arteries (diameter ~ 3 cm) obtained locally as previously described [37]. Briefly, vessels were cut longitudinally and the internal surface was digested with collagenase (0.1% collagenase type 1A, 30 min, 37° C). Endothelial cells were gently removed with a cell scraper and cultured in standard cell culture conditions (Media: D-valine MEM supplemented with 20% FBS, 70 ug/ml porcine intestinal heparin, 0.1% gentamicin and 0.1% amphoterocin B). Primary cultures were grown to confluence and populations appearing clear of contaminating fibroblasts and smooth muscle cells were sorted using FACs analysis as described above to obtain LEC populations > 98% pure [18, 37].

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Characterization of MVEC vs. LEC.

Lectin Binding: We performed an agglutination test with the lectin glycine max, which preferentially binds MVEC relative to LEC [27]. Briefly, Glycine max (1:1000) was added to confluent MVEC and LEC in 6 mm dishes and incubated for 10 min. The cells were trypsinized and pipetted to assure single cell suspension, then resuspended in PBS. A drop from each tube was applied to a glass microscope slide and viewed under a microscope. Glycine max preferentially bound to MVEC (data not shown).

Cell growth: MVEC have a faster rate of growth compared to LEC[27]. Therefore, to further characterize our endothelial cell phenotypes we determined the growth rate of our MVEC and LEC. Briefly, endothelial cells were seeded at $1X10^5$ cells per well in 6 cm culture dishes as described above (n=2), and counted every 24 h for 4 days after seeding. Cells were resuspended using trypsin and counted using a hemocytometer. Both LEC and MVEC exhibited a lag phase followed by exponential growth. MVEC displayed a faster rate of growth ($5.75 \pm 0.25 \times 10^5$ vs. $4.2 \pm 0.25 \times 10^5$/ at day 4 respectively).

Experimental design. For all experiments MVEC and LEC were used from the same passage (5-10) and grown to confluence over the same time frame, and cultured under standard cell culture conditions unless otherwise note. Unless noted, EC cell preparations were untreated or treated with ANP (-8,-7,-6 M) 30 min prior to EC stimulation with hypoxia, TNF-α or LPS.

In vitro hypoxia model. Cell preparations were placed into a special humidified hypoxic chamber equipped with a thermostat set at 37°C and an anti-chamber to pass supplies in

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and out. The anti-chamber ensured that once cells became hypoxic they were never exposed to a normoxic environment. The chamber utilized a positive pressure system and was supplied with a gas mixture of 3% O_2, 5% CO_2 and balance of nitrogen. All culture media used in permeability tests were allowed to equilibrate to 3% O_2 by placing in the hypoxic chamber ~ 2 h before the initiation of the test. All relevant permeability tests and monolayer fixation were conducted within the hypoxic chamber to avoid introducing normoxia during tissue preparation.

Permeability assay. MVEC and LEC cell preparations were either left untreated or stimulated with hypoxia or TNF-α (25 ng/ml), in the presence or absence of ANP. After 24 h, the permeability rate was determined. Briefly, MVEC and LEC were seeded (~ 100,000/insert) on gelatin coated (0.1%) polystyrene filters (Costar Transwell, No. 3470, 6.5-mm diameter, 0.4-um pore size; Costar, Cambridge, MA). Endothelial cells were grown to confluence on transwell inserts over 3 d. After cells were washed 3 times in serum-free medium, 50 uM FITC-labeled albumin suspended in serum free medium was added to the EC monolayers (100 ul). The insert was placed in a new well of a 24 well plate containing serum free medium (0.6 ml to ensure that the fluid volume on either side of the inserts were equalized to avoid a hydrostatic gradient that might alter the rate of albumin flux). The transfer rate of albumin across the monolayer was assessed by measuring the rise in concentration of FITC-albumin in the lower well after 60 min. FITC-albumin was quantified in a fluorescence spectrofluorophotometer (Mithras LB940; Berthold Tech.,Oak Ridge, TN). As previously described, albumin flux across the monolayer was expressed as: $F=(d[A]_2/dt \times V)/S$ (mol s^{-1} cm^{-2}) where V is volume in the abluminal well and $d[A]_2$ is rise of albumin concentration in the bottom well

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during the time interval dt and S is monolayer surface area [14]. To facilitate the comparison of data obtained from our study to those of others, the permeability coefficient (P, measured in cm/s) of the combined system of monolayer and filter support was calculated from F according to Fick's law of diffusion as follows: $P=F/([A]_1-[A]_2)$, where $[A]_1$ and $[A]_2$ are tracer concentrations in the luminal and abluminal compartments [14]. Data for mean permeability (P) are expressed either as percentage of a defined control situation (i.e. LEC under standard culture conditions) or as the actual P coefficient.

TNF-α secretion. MVEC and LEC monolayers were pretreated with ANP (-7 M), 30 min, then cells were stimulated with fo hypoxia 24 h or lipolysacheride (LPS; 1 ug/ml) for 6 h. Supernatants were collected and frozen at -70°C for TNF-α bioassay analyses.

TNF-α bioassay: This assay is based upon quantification of the cytotoxic activity of TNF-α on L929 cells in the presence of actinomycin D[19, 36]. The mitochondrial reduction of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was determined as an indicator of L929 cell viability [19, 36]. Briefly, supernatants described above were added to confluent L929 cells and incubated for 24 h at 37° C. MTT (5 mg/ml) was added to each well and incubated (4 h at 37° C). The formazan crystals were solublized in DMSO overnight, and absorbency was measured at 595 on a spectrophotometer (Labsystems Multiscan; Helsinki, Finland). TNF-α soluble receptor II (TNF-α sRII) was used to as a positive control. Serial dilutions (400-0.04 ng/ml) of TNF-α sRII indicated that the ED_{50} was 4 ng/ml in the presence of 500 pg of recombinant TNF-α. TNF-α sRII abolished the TNF-α activity in supernatants from

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hypoxic and LPS-stimulated cells at a concentration of 4 ng/ml, indicating that the assay was specific for TNF-α activity.

NF-κB and phospho (activated) p38 mitogen activated protein kinase: MVEC and LEC were treated as described above, however cells were stimulated with TNF-α (10 ng/ml; 4 h) rather than LPS. Nuclear extracts were prepared as previously described [38]. Briefly, cells were washed with ice cold PBS, scraped and resuspended in hypotonic buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1mM DTT; 0.5 mM PMSF, 15 min, on ice). Nonidet P-40 (10%, 25 ul) was added to the solution, vortexed and centrifuged (12,000 x *g* for 30 sec). Supernatant was removed and the nuclear pellet extracted with hypertonic buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT and 1 mM PMSF, shaking,4°C,15 min, and centrifuged at 12,000 x *g*). Supernatant was frozen (–70°C) prior to NF-κB analyses.

Cytosolic protein was prepared as previously described [26]. Briefly, MVEC and LEC were washed with ice cold PBS, scraped and resuspended in cell lysis buffer (1 ml; 50 mM B-glycerophosphate, 10mM HEPES,pH 7.4, 70 mM NaCl, 2 mM EDTA, 1% SDS,Assay Designs Inc. No.80-0943). Cells were incubated on ice (20 min), vortexed and frozen (–70°C) prior to activated p38 MAPK analyses.

Protein assay. Protein concentrations were determined by the method of Bradford. (Coomasie assay kit. No 23200; Pierce; Rockford, IL). Briefly, standard or unknown samples (5 ul) were added to Coomasie reagent (250 ul) in microplate wells and incubated for 10 m, room temperature. Absorbence was read by spectrophotmeter at 595 nm.

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NF-κB quantification. NF-κB activity was determined in nuclear extracts (50 ul, 200 ug/ml protein) by a commercially available enzyme linked immounassay kit (ELISA) (Oxford Biomedical Research, No. TF 01; Oxford, MI). This chemiluminescent ELISA employs an oligonucleotide containing the consensus binding sequence for NF-κB, bound to a 96-well ELISA plate. The DNA-bound NF-κB is selectively recognized by the primary antibody (p50 and p105specific), which in turn is detected by the secondary antibody alkaline phosphotase conjugate. Luminescence was measured at 540 nm in a chemiluminometer plate reader (Mithras LB940; Berthold Technologies, Oak Ridge, TN).

Activated (Phospho)-p38 MAPK quantification. The amount of activated p38 MAPK in cell lysates (100 ul, 100 ug/ml protein) was determined by a commercially available phospho-p38 MAPK enzyme immunometric assay kit (EIA) (Assay Designs Inc. No. 900-101; Ann Arbor, MI). The color generated by the enzyme reaction was read at 450 nm on a spectrophotometer, as previously described.

F-actin staining. MVEC and LEC were prepared on either 6 cm culture dishes or glass chamber slides for F-actin quantification and visualization respectively and treated as described above. Briefly, MVEC and LEC cells were fixed (4 % formaldehyde, 10 min, RT), permeabilized (5 min., 0.1% triton X-100), washed (PBS) and incubated (1% BSA, 30 min) before staining with FITC-phalloidin (6.6 uM, 20 minutes, 4° C, in the dark). Stained F-actin was visualized using a Nikon Eclipse E800 microscope equipped with epifluorescence illumination with a 100-400 fold-magnification.

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F-actin quantification. F-actin staining was performed as described above and the bound dye was extracted with methanol (1ml, 1 h, 4° C, in the dark). The extracted dye was measured in a chemilumenescence plate reader with excitation at 490 nm and emission 520 nm. Data are expressed as mean relative light units (RLU) and compared between groups.

Morphological investigation. Immediately following the permeability tests, transwells with EC were washed, fixed (3% glutaraldehyde, 1 h, RT) and stained (crystal violet,1 h, RT) for microscopy analyses. The stained inserts were removed from the transwell, placed on slides and visualized with a Nikon Eclipse microscope with 100-400-fold magnification to confirm the presence of an intact monolayer.

Statistical analysis. All experiments followed a randomized block design using cells from at least three different cell preparations. All assays were completed in duplicate or triplicate. Data are expressed as means ± standard error of measurement (SEM), and difference between groups was determined by one-way analysis of variance ANOVA using a statistical software package JMP (version-5) (SAS Institute Inc., Cary, NC). Statistical significance was set at $P \leq 0.05$.

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4.4 RESULTS.

Microvascular vs. Macrovascular Endothelial Cell Permeability:

Microvascular and macrovascular endothelial cells respond differently to various stimuli [1, 18, 33, 39], so we sought to determined the permeability of bovine pulmonary *micro*vascular endothelial cell (MVEC) and bovine pulmonary artery (*macro*vascular) endothelial cell (LEC) monolayers exposed to standard cell culture conditions (21%O_2; 5%CO_2; N2 balance, control), in response to hypoxia (3% O_2) or TNF-α (25 ng/ml) 24 hours. Hypoxia and TNF-α treatment increased endothelial permeability in MVEC and LEC **(Figure 1)**. However, MVEC had the greatest permeability in all conditions tested. When permeability rates were normalized to the control conditions within a similar cell type MVEC and LEC had similar relative changes in permeability **(Table 1)**.

ANP inhibits hypoxia- and TNF-α -induced endothelial monolayer permeability:

To test the hypothesis that ANP can prevent an increase in pulmonary vascular permeability, MVEC and LEC were pretreated with ANP (-8, -7,-6 M), then exposed to hypoxia or treated with TNF-α. ANP elicited a dose-dependent reduction in permeability in MVEC, during hypoxia and TNF-α treatment, and in LEC treated with TNF-α **(Figure 2, Table 1)**.

ANP inhibits endothelial secretion of TNF-α:

Because lung permeability may be enhanced by TNF-α at high altitude [12, 28, 29], and ANP inhibits synthesis and secretion of TNF-α [24], we determined TNF-α secretion from MVEC and LEC in response to hypoxia. LPS treatment was used as a positive

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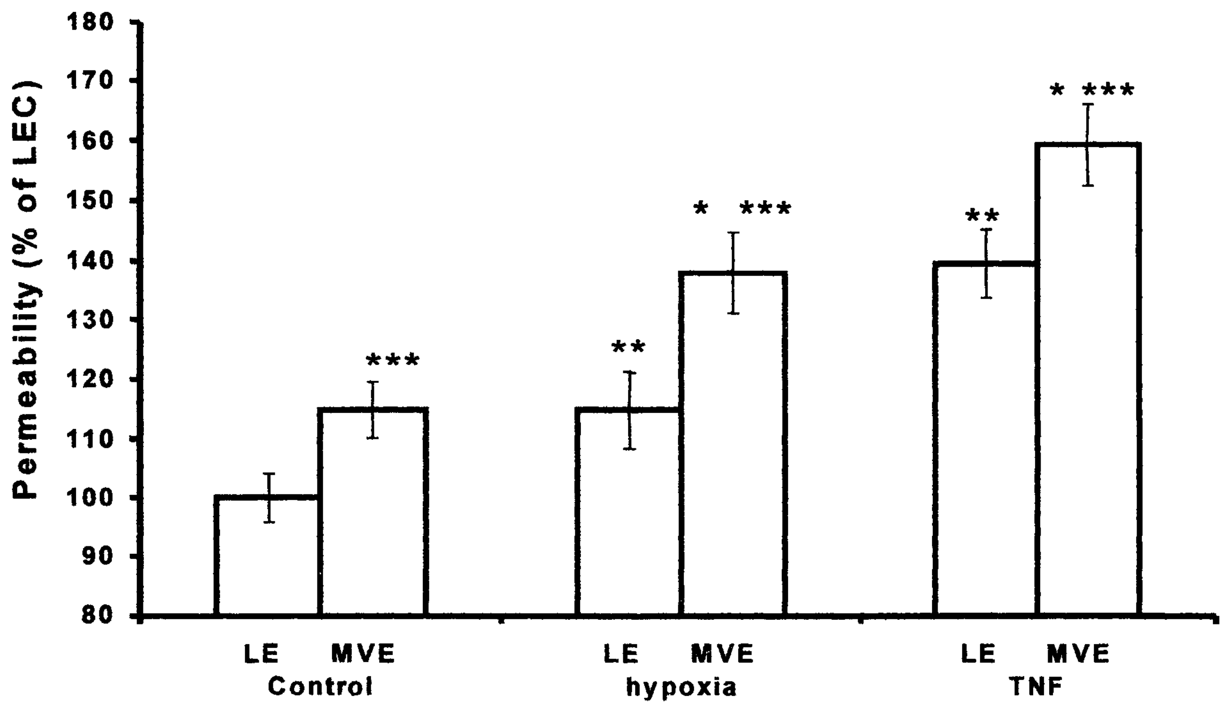

Figure 4.1: Permeability. Confluent MVEC and LEC in transwell chambers were unstimulated or stimulated with hypoxia (3% O_2, 5% CO_2 and 92%N_2) or TNF-α (25 ng/ml) for 24 hours. Values were normalized on permeability coeffecient in untreated cells, and permeability of control LE (normal cell culture conditions) was set at 100%. Data show mean ±SEM of 4 separate experiments done in triplicate (n=12). *$P \leq 0.005$ MVE vs. MVE control; **$P \leq 0.008$ LE vs. LE control; ***$P \leq 0.045$ MVE vs. LE control, hypoxia or TNF-α-stimulated.

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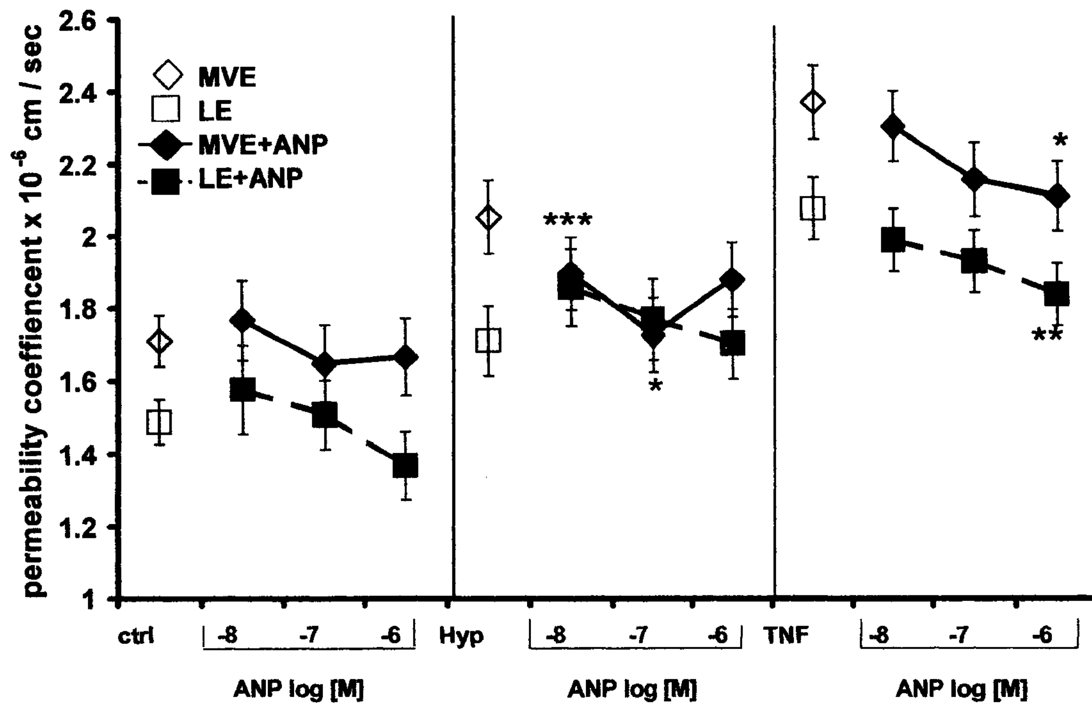

Figure 4.2: ANP effect on Permeability. Confluent MVEC and LEC in transwell chambers were un-stimulated or stimulated with hypoxia (3% O_2, 5% CO_2 and 92%N_2) or TNF-α(25 ng/ml) for 24 hours in the presence or absence of ANP[-8,-7,-6 M]. Data show mean ±SEM of 4 separate experiments done in triplicate (n=12). *P≤0.002 MVE vs. MVE hypoxia or TNF-α-stimulated; **P≤0.02 LE vs. LE TNF-α-stimulated; ***P≤0.02 ANP effect on MVE vs. LE.

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Table 4.1

Cell type & treatment	Permeability Coeff. (n=12)			TNF secretion (n=6)			Activated NF-kb (n=3)			Activated p38 (n=3)			F-actin formation (n=6)		
	Crtl (x 10^{-6} cm/sec)	hypoxia (x fold)	TNF (x fold)	Crtl pg/ml	Hypoxia (pg/ml)	LPS (pg/ml)	Ctrl pg/ug	Hypoxia (x fold)	TNF (x fold)	Ctrl pg/ug	Hypoxia (x fold)	TNF (x fold)	Ctrl RLU/100	Hypoxia (x fold)	TNF (x fold)
MVEC	1.71±0.07	1.2±.04[a]	1.4±0.03[a]	bd	117±17[a]	328±48[a]	2.8±0.5	4.0±0.4[a]	3.0±0.18[a]	444±3.6	0.62±0.07	1.5±0.03[a]	24.8±2.6	0.77±0.1	1.88±0.22[a]
ANP															
-8	1.77±0.11	1.1±0.05	1.35±0.05										20.3±2.7	0.8±0.14	0.72±0.14[a,b]
-7	1.65±0.10	1.0±0.06[b]	1.26±0.06[a,b]	bd	bd[b]	55±13[a,b]	1.55±1.0	2.0±0.55[a,b]	1.2±0.35[a,b]	301±11[a]	0.67±0.02	0.32±0.03[b]	24.4±5	0.69±0.13	0.54±0.09[a,b]
-6	1.66±0.1	1.09±0.06	1.23±0.06[a,b]										23±5.4	0.72±0.14	1±0.07[a,b]
LEC	1.49±.09	1.15±.06[c]	1.4±0.06[c]	bd	48±15[c]	237±55[c]	2.9±0.2	1.8±0.1[c]	3.0±0.4[c]	292±24	1.35±0.06[c]	1.1±0.1	18±4.8	1.8±0.18[c]	1.2±.14
ANP															
-8	1.58±0.12	1.24±0.07[c]	1.33±0.06[c]										15±3.8	1.22±0.2[d]	0.55±0.04[c,d]
-7	1.51±0.09	1.18±0.07[c]	1.29±0.06[c]	bd	bd[d]	35±5[c,d]	2.8±1.5	0.73±0.25[c,d]	0.42±0.35[c,d]	289±31	1.0±0.06[d]	1.17±0.2	17±2.7	1.38±0.21	0.46±0.04[c,d]
-6	1.37±0.09	1.14±0.06[c]	1.23±0.06[c,d]										15±1.17	1.18±0.22[d]	0.5±0.08[c,d]

Table 1. Summary table of ANP effect on MVEC and LEC permeability, TNF-α secretion, NF-κB, p38 MAPK and F-actin formation, under control, hypoxia (3%O2, 5%CO2, 92%N2; 24 hours) or TNF-α-stimulated (10-25 ng; 4-24 hours). Data represents mean±SEM of at least 3 independent experiments. bd=below detection; a P≤0.05 vs. MVEC control; b P≤0.05 MVEC-stimulated vs. MVEC-stimulated+ANP[M]; c P≤0.05 vs. LEC control; d P≤0.05 LEC-stimulated vs. LEC-stimulated+ANP[M].

control. Hypoxia-induced TNF-α secretion from MVEC and LEC ($P \leq 0.05$), but was 2 fold greater in MVEC vs. LEC ($P \leq 0.006$) (Table 1). LPS increased TNF-α secretion by the MVEC and LEC 3 and 5 fold more than hypoxia and was similar in MVEC and LEC (**Figure 3, Table 1**). ANP reduced hypoxia- and LPS-stimulated TNF-α secretion in MVEC and LEC (**Figure 3**).

ANP inhibits activated NF-κB.

NF-κB promotes TNF-α transcription during hypoxia and inflammation [32]. Therefore, to establish a mechanism for ANP inhibition of endothelial TNF-α secretion we determined the effect of ANP on the activity of transcription factor NF-κB. Hypoxia and TNF-α increased NF-κB activity in MVEC and LEC (**Table 1**). Hypoxia activated NF-κB 2-fold higher in MVEC vs. LEC ($P \leq 0.0005$) (**Figure 4**). ANP decreased hypoxia-and TNF-α-stimulated NF-κB activity in MVEC and LEC (**Figure 4**).

Differential activation of p38 MAPK in MVEC and LEC

Others have demonstrated that TNF-α can increase activation of p38 MAP kinase, which is known to alter cell morphology and increase endothelial permeability [30, 32]. Therefore, we sought to determine if hypoxia or TNF-α increased activation of p38 MAPK in MVEC and LEC, and if ANP could inhibit that activation. Hypoxia decreased activated p38 MAPK ($P \leq 0.05$) in MVEC but caused an increase in LEC ($P = 0.002$). Conversely, TNF-α-stimulation increased activation of p38 MAPK in MVEC, but not in LEC (**Table1**). Activation of p38 MAPK was *greater* in MVEC vs. LEC in standard cell

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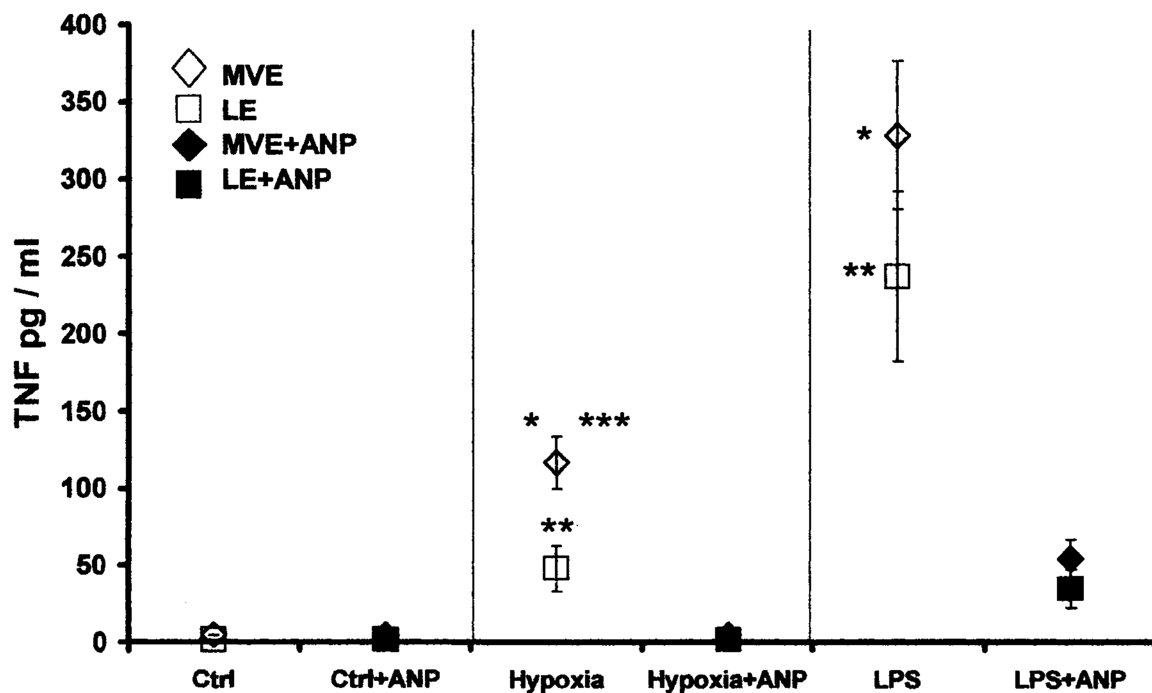

Figure 4.3: ANP inhibition of MVEC and LEC TNF-α secretion. MVEC and LEC were grown to confluence in a 6 cm culture dish and left un-stimulated, hypoxia (3% O_2, 5% CO_2 and 92%N_2; 24 hours) or LPS-stimulated (LPS 1μg /ml; 6 hours) in the presence or absence of ANP [-7 M]. Culture supernatants were assayed for TNF-α secretion using L929 cells in a bioassay. Data show mean ±SEM of 3 separate experiments done in duplicate (n=6) *$P \leq 0.001$ MVE vs. MVE control; **$P \leq 0.001$ LE vs. LE control; ***$P \leq 0.006$ MVE vs. LE hypoxia-stimulated.

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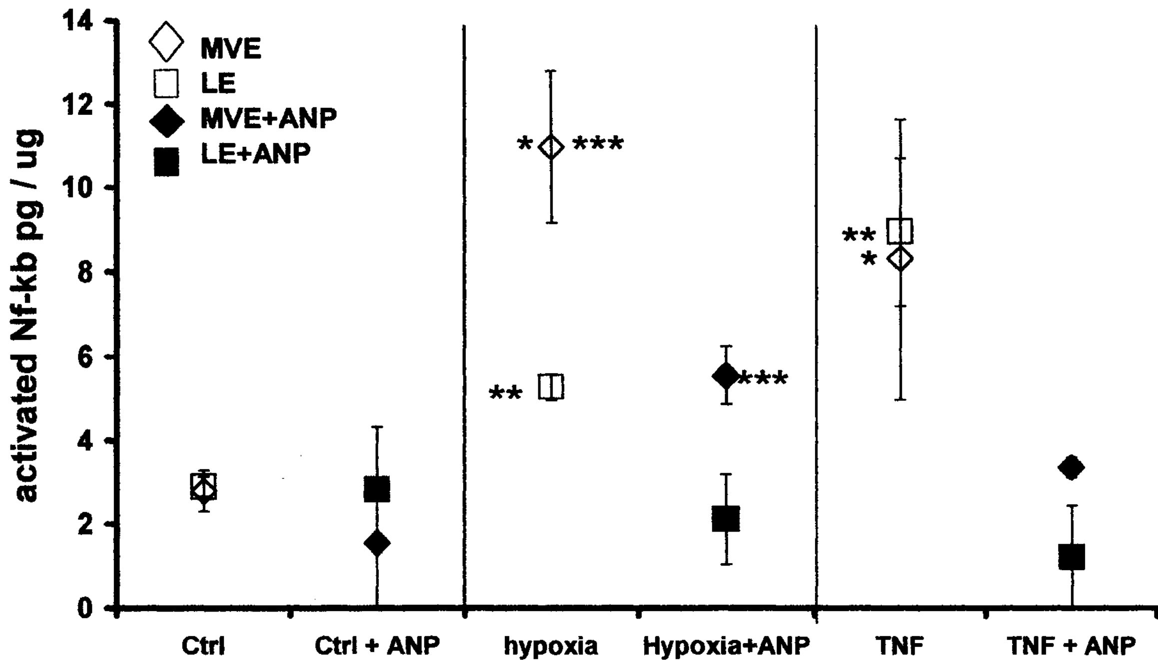

Figure 4.4: ANP inhibition of NF-κB activity. MVEC and LEC were grown to confluence in a 10 cm culture dishes and left un-stimulated, hypoxia (3% O_2, 5% CO_2 and 92%N_2; 24 hours) or TNF-α-stumulated (10 ng/ml; 4 hours) in the presence or absence of ANP [-7 M]. Nuclear protein was assayed for activated NF-κB using a chemiluminescence based sandwhich type ELISA. Data show mean ±SEM of 3 separate experiments assayed in duplicate (n=3) *$P \leq 0.0015$ MVE vs. MVE control; **$P \leq 0.03$ LE vs. LE control; ***$P \leq 0.0015$ MVE vs. LE hypoxia or TNF-α-stimulated.

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culture conditions and with TNF-α-treatment, but during hypoxia, activation of p38 MAPK was *less* in MVEC vs. LEC **(Figure 5).** In standard culture conditions, ANP decreased activation of p38 MAPK in MVEC, but had no effect on LEC **(Figure 5).** ANP had no effect on activation of p38 in hypoxia-stimulated MVEC, but inhibited activation of p38 MAPK in LEC. ANP treatment reduced activation of p38 MAPK in TNF-α-stimulated MVEC, but had no effect on LEC **(Table 1).**

Differential actin polymerization in MVEC and LEC.

Activation of p38 MAPK increases endothelial permeability by stimulating a signal cascade that causes polymerization of G-actin into F-actin [2, 25, 30, 35] also known as stress fibers. Therefore, we sought to determine if hypoxia or TNF-α increased stress fiber formation in MVEC and LEC and if ANP could inhibit that activation. Hypoxia did not affect stress fiber formation in MVEC, but increased stress fibers in LEC **(Figure 6).** TNF-α stimulation increased stress fiber formation approximately 2-fold in MVEC, but no change was evident in LEC ANP reduced stress fiber formation in hypoxic LEC, and TNF-α -stimulated MVEC **(Figure 7).**

Morphological differences between MVEC and LEC

MVEC and LEC presented typical cobblestone morphology. Hypoxia-stimulated MVEC and LEV started to retract from one another and form intercellular gaps **(Figure 8-9).** TNF-α-stimulated MVEC and LEC showed more pronounced retraction and formation of intercellular gaps with a noticeable change in architecture from their typical cobblestone

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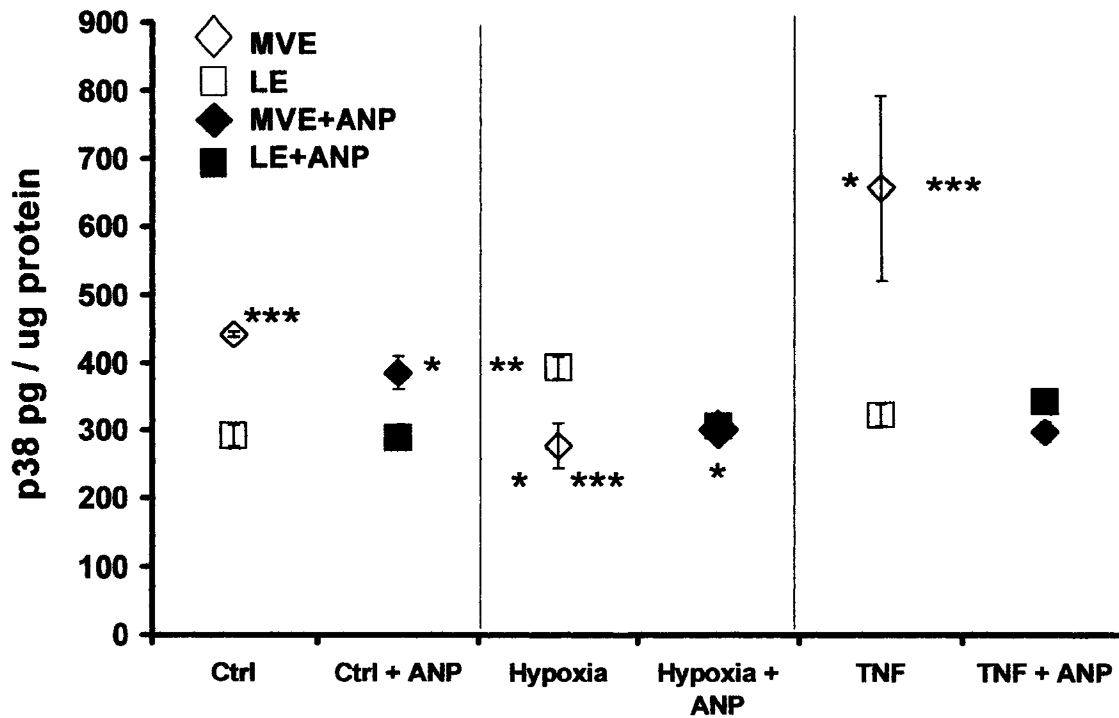

Figure 4.5: ANP inhibition of activated p-38 MAPK. MVEC and LEC were grown to confluence in a 10 cm culture dishes and left un-stimulated, hypoxia (3% O_2, 5% CO_2 and 92%N_2; 24 hours) or TNF-α-stimulated (10 ng/ml; 4 hours) in the presence or absence of ANP [-7 M]. Cytosolic protein was assayed for phospho-p38 MAPK using an enzyme immunometric assay kit. Data show mean ±SEM of 3 separate experiments assayed in duplicate (n=3) *$P \leq 0.05$ MVE vs. MVE control; **$P \leq 0.002$ LE vs. LE control; ***$P \leq 0.001$ MVE vs. LE.

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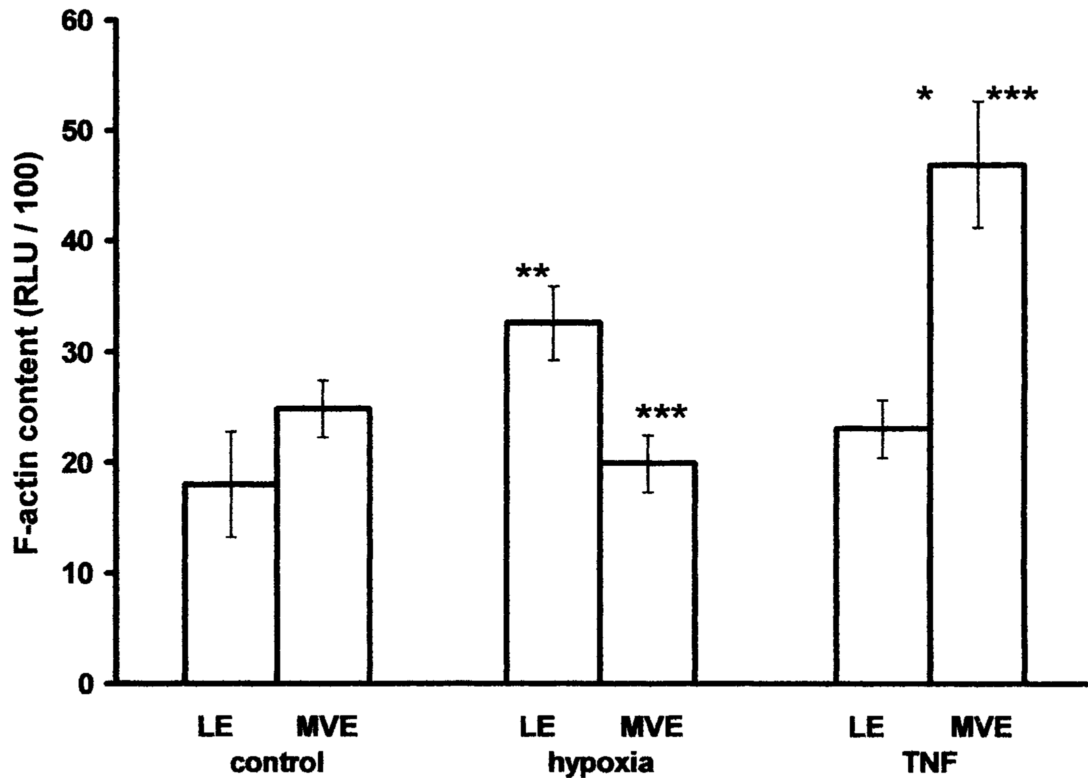

Figure 4.6: F-actin formation. MVEC and LEC in were grown to confluence in 6 cm culture dishes and unstimulated, hypoxia (3% O_2, 5% CO_2 and 92%N_2; 24 hours) or TNF-α-stimulated (10 ng/ml; 24 hours). F-actin was quantified by staining with FITC-phalloidin followed by fluorescence photometry. Data are express as relative light units (RLU/100). Data show mean ±SEM of 3 separate experiments done in duplicate (n=6). *$P \leq 0.05$ MVE vs. MVE control; **$P \leq 0.05$ LE v LE control; ***$P \leq 0.05$ MVE vs. LE control, hypoxia or TNF-α-induced.

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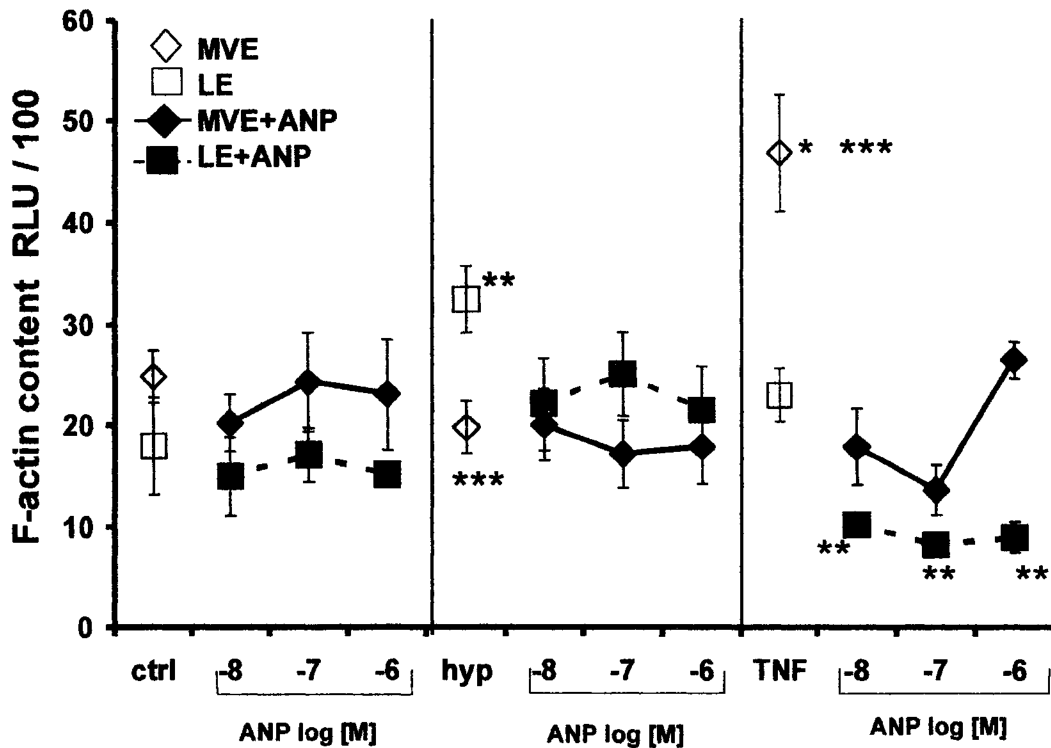

Figure 4.7: ANP inhibition on F-actin formation. MVEC and LEC in were grown to confluence in 6 cm culture dishes and unstimulated, hypoxia (3% O2, 5% CO2 and 92%N2; 24 hours) or TNF-α-stimulated (10 ng/ml; 24 hours) in the presence or absence of ANP [-8,-7,-6 M]. F-actin was quantified by staining with FITC-phalloidin followed by fluorescence photometry. Data are express as relative light units (RLU/100). Data show mean ±SEM of 3 separate experiments done in duplicate (n=6). *P≤0.05 MVE vs. MVE control; **P≤0.05 LE vs. LE control; ***P≤0.05 MVE vs. LE hypoxia or TNF-α-stimulated.

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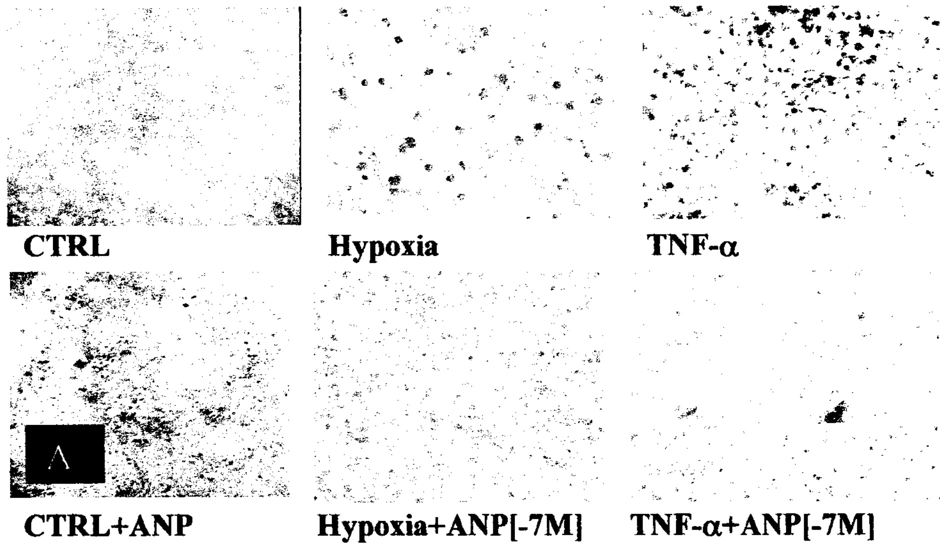
MVE



LE

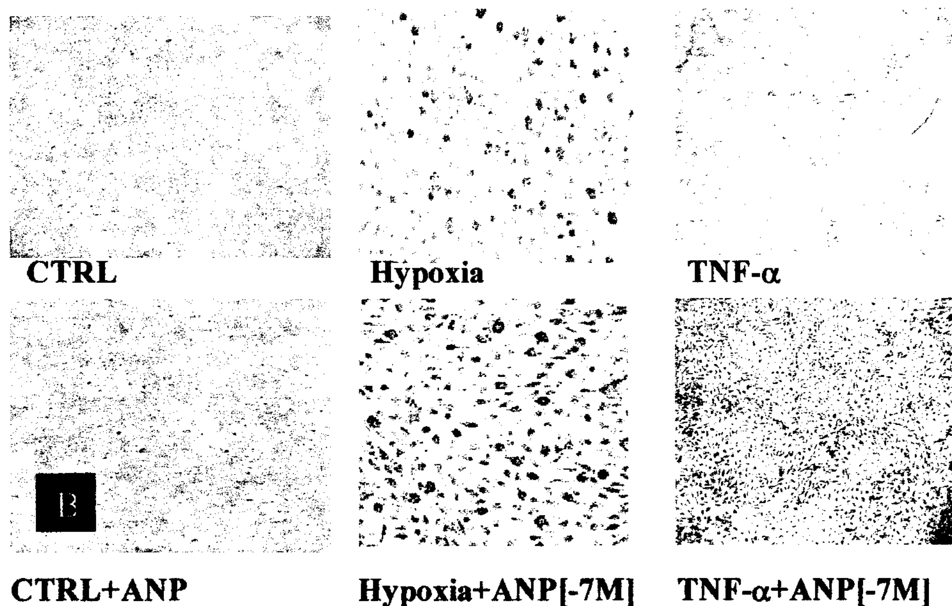


Figure 4.8. Morphology. ANP inhibits hypoxic (3%O2, 5%CO2 and N2; 24 hours) and TNF-α-stimulated (25 ng/ml; 24 hours) morphological changes in MVEC and MVEC/LEC respectively. Confluent MVEC and LEC in transwell chambers were cultured in control, hypoxia or TNF-α-stimulated conditions in the presence or absence of ANP[-8,-7,-6 M]. Cells were stained with crystal violate immediately after the experiment. Pictures show representative photographs out of 4 independent experiments ANP[-7M]. Original magnification 10x. **A**, MVEC; **B**, LEC.

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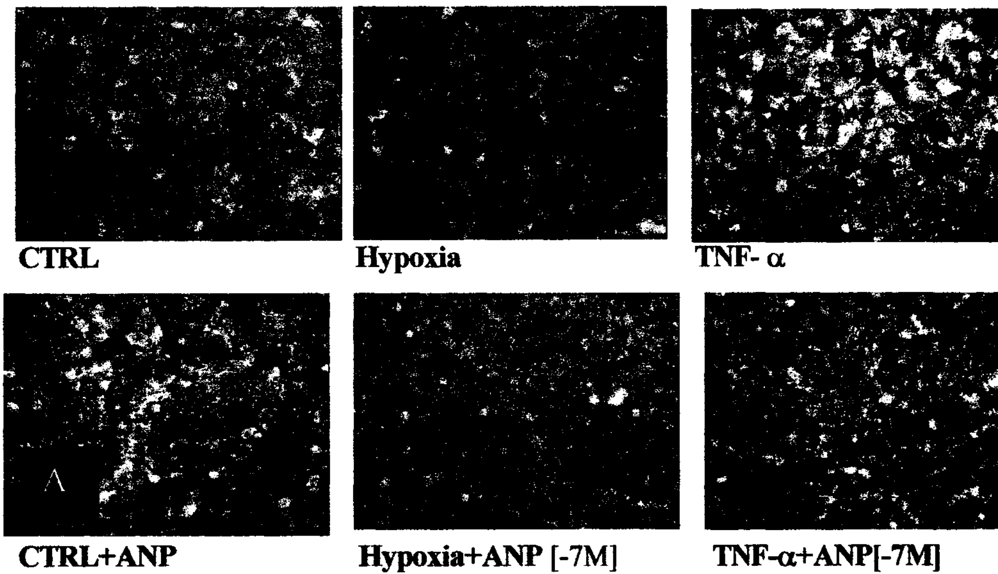

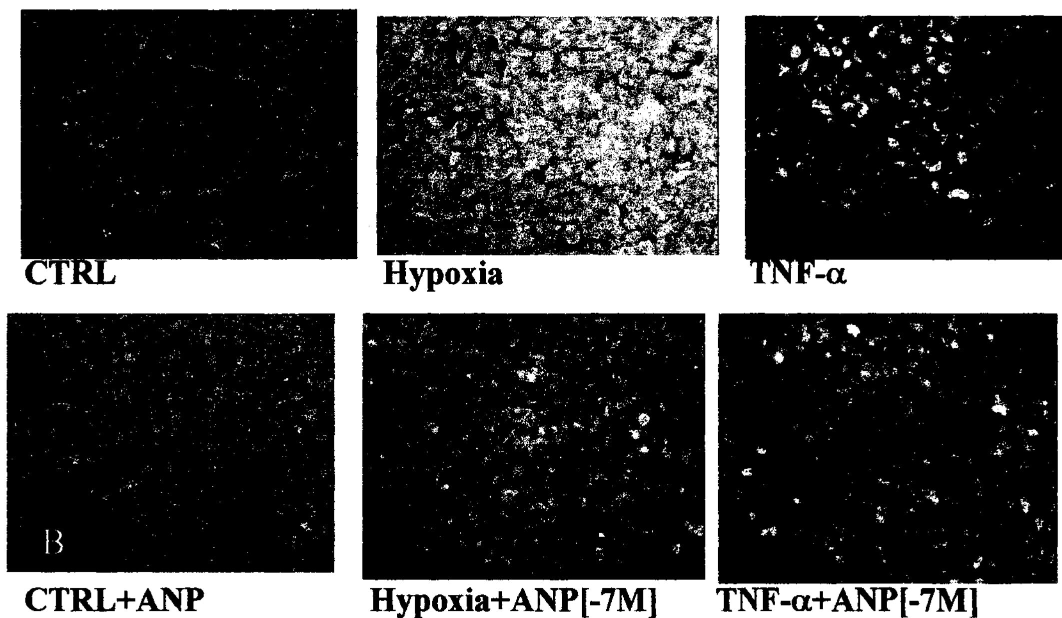

Figure 4.9. ANP reduces F-actin formation in hypoxic and TNF-α-stimulated LEC and MVEC respectively. Confluent MVEC and LEC in 8 well chamber slides were cultured in control, hypoxia (3%O2, 5%CO2 and N2; 24 hours) or TNF-α-stimulated (25 ng/ml; 24 hours) conditions in the presence or absence of ANP[-7 M]. Cells were stained with FITC-phalloidin. Pictures show representative photographs out of 3 independent experiments ANP[-7M]. Original magnification 20x. A, MVEC; B, LEC.

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appearance to more elongated cells **(Figures 8-9)**. ANP abrogated hypoxia and TNF-α-stimulated changes in MVEC and MVEC/LEC respectively **(Figures 8-9)**.

Compared to quiescent MVEC, stress fibers appeared to be slightly less in hypoxia-stimulated MVEC in the central region and peripheral band around the margin of the cell. However, hypoxic LEC had more stress fibers transversing the central region of the cell and a thickening of the dense peripheral band around the margin of the cell. TNF-α-stimulated MVEC appeared to show increased central region stress fibers in a dense meshwork of thickened filament bands compared to unstimulated cells, but TNF-α-stimulated LEC showed only a small change in central region and peripheral band actin filaments when compared to quiescent cells. ANP reduced hypoxia and TNF-α-stimulated changes in stress fiber formation in LEC and MVEC respectively **(Figures 8-9)**.

4.5 DISCUSSION.

Our data provides evidence of a previously undescribed mechanism for an hypoxia-induced increase in pulmonary vascular permeability: enhanced NF-κB activity, TNF-α secretion, activation of 38 MAPK, activation of stress fiber formation and ultimately altered morphology leading to increased permeability. These are the first data to provide evidence that during hypoxia, ANP not only had vasodilatory and natriuretic properties, but may also have directly reduced pulmonary endothelial leak. Our data also indicated that microvascular and macrovascular pulmonary endothelium responded differently to hypoxia and ANP treatment.

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Acute hypoxia increases cardiac output [12, 13, 46], causes pulmonary hypertension [4, 16] and releases a web of vasoactive agents resulting in increased pulmonary vascular permeability [3,13]. Caution must be applied when interpreting data from our *in vitro* experiments on barrier function properties in EC because they fail to take into account shear stress, hydrostatic pressure and other fluid flow properties that are present *in vivo*. Cell culture preparations are only exposed to a few of the myriad of interacting vasoactive peptides that tip the balance in favor of pulmonary edema, and therefore may yield different results than *in vivo* models. It has also been noted that preparation of MVEC and LEC cultures may alter phenotypes from that occurring *in vivo* [18]. To reduce the risk of inducing phenotypic changes, we used cells from a low passage number (5-10), and the same passage within each experiment, and cells were seeded and grown to monolayers within the same time frame. Although MVEC appear distinct from LEC in culture, MVEC cannot be identified as derived from arteriole, capillary or venous vessels.

Our data suggesting that hypoxia increased permeability, activated NF-κB, increased secretion of TNF-α, activated p38 activation and changed F-actin concentration in MVEC and LEC are consistent with other studies investigating endothelium activated by hypoxia or TNF-α in other organs such as the brain, liver and umbilical cord [8, 9, 20, 25, 26]. However, as far as we know this is the first study exploring the relationship between the effects of hypoxia and inflammatory mediators in cultured pulmonary endothelial cells. Our data further indicates that when MVEC and LEC become hypoxic, a pathway is activated analogous to the inflammatory response induced by TNF-α or

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bacterial endotoxin (LPS). Similar data has been previously reported in TNF-α stimulated HUVEC, but not in pulmonary endothelium [19, 20, 24-26].

MVEC had a higher permeability rate compared to LEC in all conditions. Data from previous studies provided evidence that under standard culture conditions MVEC form a more restrictive barrier to macromolecules (MW > 77 kilodaltons) and have a lower permeability rate compared to LEC [18, 39]. The differing results may be due to differences between the transwell matrices on which cells were cultured [34]. Also, we used FITC-labeled-albumin, while others used dextran as a permeability tracer [18]. MVEC have a transcytotic pathway selective for albumin [42], which may not be inherent in LEC. Although it has been reported that MVEC form a tighter barrier compared to LEC [18, 39], our data suggest this may not be true for all molecules. Further investigations testing the permeability rates of a variety of compounds passing through MVEC and LEC monolayers are needed to clarify this issue.

Our data indicating differential regulation of p38 MAPK activation and F-actin arrangement between hypoxic and TNF-α-stimulated MVEC and LEC are consistent with previous studies demonstrating that *micro* and *macro* vascular endothelial cells respond differently to various stimuli [1, 5, 18]. Our data suggest that during hypoxia, activation of p38 MAPK and F-actin formation was decreased or unchanged in MVEC, but increased in LEC. In contrast, these results were reversed with TNF-α treatment. Decreased or unchanged p38 MAPK and F-actin formation may be a reflection of decreased endothelial cell vitality. However, this was not supported by our microscopy analyses, but an absolute measure of cell vitality such as an apoptosis assay was not

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performed. During hypoxia calcium is a required upstream activator of p38 MAPK [6]. We did not test intracellular calcium and therefore we cannot determine if it played a role in our model. Our data further indicate that hypoxia-stimulated MVEC did not change F-actin concentration but increased permeability. These data are supported by previously published data indicating that brain microvascular endothelial cells increased permeability while F-actin concentration remained unchanged but distribution was altered [8, 9].

In a series of recently published papers, data indicated that ANP inhibited production of pro-inflammatory agents from immune cells (macrophages) and prevented deleterious effects on HUVEC vascular endothelial cells *in vitro* [19-26]. Similarly, our data indicated that ANP inhibited vascular permeability, TNF-α secretion, NF-κB activity, and activation of p38 MAPK in hypoxia-and-TNF-α-stimulated MVEC and LEC. Ours is the first study to investigate the cytoprotective effect of ANP on hypoxia-stimulated pulmonary *micro* and *macro* endothelial cells *in vitro*.

There are some notable differences between ANP action on MVEC vs. LEC. During hypoxia, ANP reduced permeability in MVEC, but not LEC. However, ANP had no effect on the activation of p38 MAPK and subsequent F-actin formation in MVEC, but inhibited this pathway in LEC. These data suggest that during hypoxia ANP can preserve MVEC barrier function by means other then inhibition of p38 MAPK activation. ANP reduced permeability in TNF-α-.but not hypoxia-stimulated LEC. It has been reported that C type natriuretic peptide receptor (NPR-C) is down-regulated in the lung by hypoxia [40] *in vivo*. However, our study design did not allow us to determine if there

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was a connection between the inability of ANP to reduce the leak in hypoxic LEC and the NPR-C receptor.

Although ANP had differential effects on MVEC and LEC, our results are consistent with animal models in which ANP ameliorated high altitude pulmonary edema (HAPE) [16]. HAPE is a rare noncardiogenic pulmonary edema [12] that may be caused by increased hydrostatic pressure, inflammatory agents or a combination of the two [44]. Our data provide unique insights into the mechanisms controlling pulmonary endothelial leak during acute hypoxia, unassociated with changes in vascular pressure, that are impossible to determine in the intact lung. Data indicating that hypoxia alters barrier function in pulmonary endothelial monolayers suggest that there may be a permeability component to HAPE independent of fluid dynamics. Our data further suggest that hypoxia can activate the same pathways as inflammatory agents such as TNF-α and the bacterial endotoxin LPS **(Figure 10).** We propose that acute hypoxia and inflammatory stimuli may share some common pathways leading to enhanced pulmonary endothelial permeability and pulmonary edema.

Our data suggest that inflammatory mediators may play a role in the hypoxia-induced increase in pulmonary endothelial permeability, and that ANP may have an important

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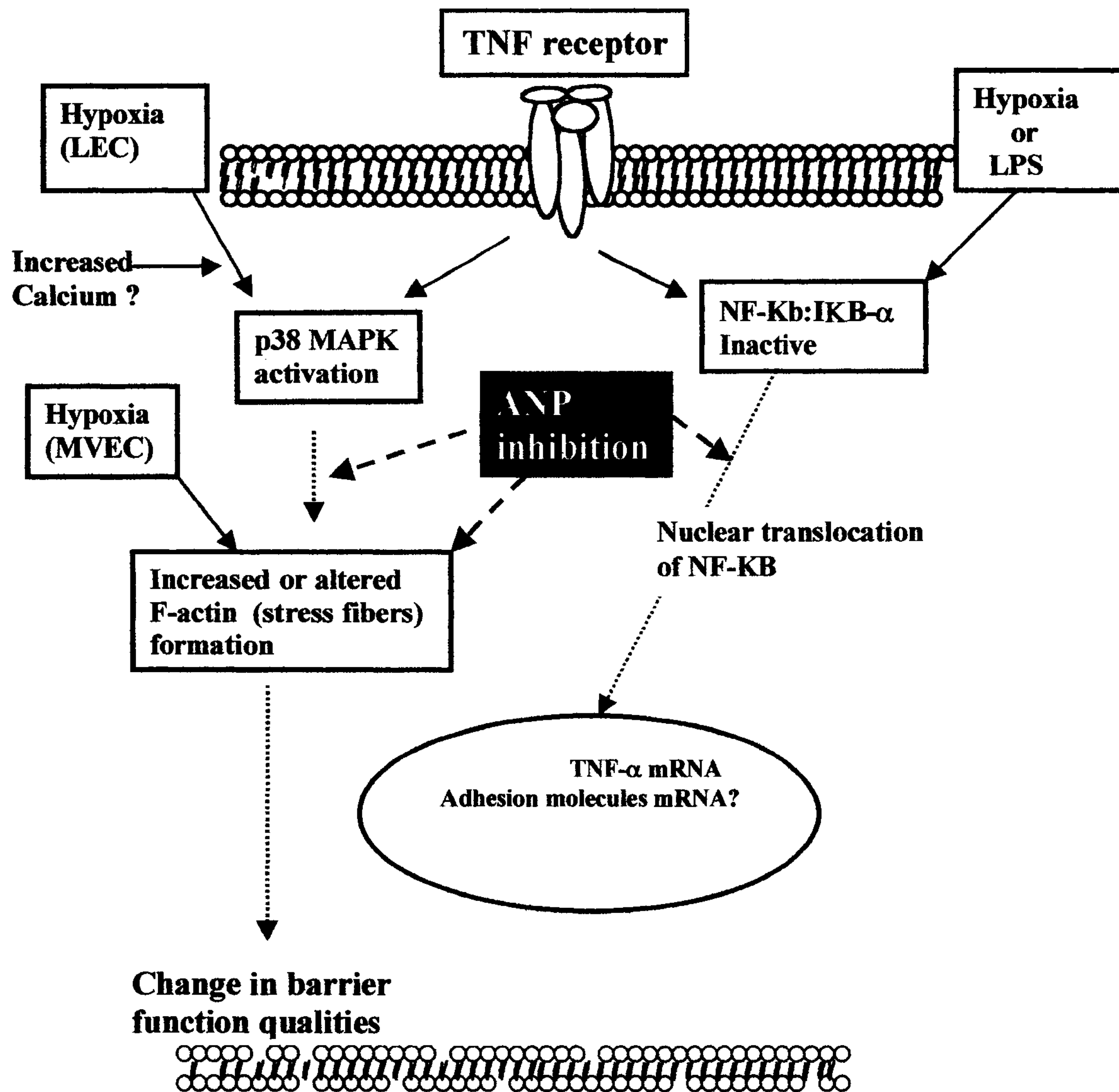

Figure 4.10. Overall schema proposing where ANP exerts its effect on MVEC and LEC cultured under control, hypoxia, TNF-α and LPS conditions.

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role in inhibiting hypoxia-inflammatory mediated permeability independent of its vasodilatory and natriuretic actions.

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CHAPTER V

5 SUMMARY AND SPECULATIONS

The goal of this study was to investigate whether ANP could inhibit the formation of high altitude pulmonary edema by superceding the effects of ET-1 and protecting the pulmonary endothelium by means other than its vasodilatory and natriuretic properties.

To this end, we utilized a genetically altered strain of mice lacking the gene to express neutral endopeptidase (a cell surface protease responsible for degrading ANP and ET-1) and bovine pulmonary endothelial cells cultured from the microvasculature and the main pulmonary artery. the following specific hypotheses were addressed: 1) Neutral endopetidase gene knockout mice (NEP -/-) exposed to high altitude will have an increased plasma ANP/ ET-1 ratio as compared to control mice and this will negatively correlate with pulmonary vasoconstriction and edema; 2) ANP will inhibit permeability in hypoxia- or TNF-α-stimulated pulmonary *micro*vascular and *macro*vascular endothelial cells; and, 3) ANP will inhibit NF-κB activation, TNF-α secretion, activation of p38 mitogen activated protein kinase in hypoxia- or TNF-α-stimulated pulmonary *micro*vascular and *macro*vascular endothelial cells. Following is a summary of results for each specific aim.

5.1 Summary of Specific aim 1

As described in chapter 2, we exposed wild type (NEP +/+) mice versus mice in which the NEP gene was deleted (NEP -/-) on the same genetic background to a simulated high altitude of 22,000 ft (6728 m; P_B= 328 mm Hg) for 24 h. Plasma ANP and ET-1 concentrations, right ventricular pressure (P_{RV}) and indexes of lung injury were assessed (table 1). Our data suggest that endogenous ANP concentration increases at high altitude

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in NEP (-/-) mice, reducing pulmonary vascular pressure and high altitude-induced pulmonary vascular leak.

Change from low altitude						
Variable	WW/BW	Lung Permeability	P_{RV}	ANP	ET-1	ANP/ET-1
NEP -/-	+	No change	+	+	+	+
NEP +/+	++	++	++	+	No change	+

Table 5.1. + = significant ($p < 0.05$) increase compared to low altitude cohorts. ++ = significant ($p < 0.05$) compared to NEP (-/-) mice. WW/BW, ratio of wet lung weight to body weight; PRV, right ventricular pressure; ANP, atrial natriuretic peptide; ET-1, endothelin-1.

5.2 Summary of specific aim 2:

As described in chapter 3, pulmonary microvascular (MVEC) and pulmonary artery (LEC) were isolated from bovine lungs and stimulated with hypoxia or TNF-α in the presence or absence of ANP and the albumin permeability was assessed. Our data indicated that in transwell cultures ANP reduced hypoxia and TNF-α-induced permeability in MVEC and TNF-α-induced permeability in LEC **(Figure. 1; Table 2).**

Cell type & TX	Permeability			TNF secretion			Activated NF-Kb			Activated p38 MAPK			F-actin formatioin		
	CRTL	HYP	TNF	CRTL	HYP	TNF	CRTL	HYP	TNF	CRTL	HYP	TNF	CRTL	HYP	TNF
MVEC	na	↑	↑	na	↑	↑	na	↑	↑	na	↓	↑	na	↔	↑
MVEC + ANP	↔	↓	↓	↔	↓	↓	↔	↓	↓	↓	↔	↓	↔	↔	↓
LEC	na	↑	↑	na	↑	↑	na	↑	↑	na	↑	↔	na	↑	↔
LEC + ANP	↔	↔	↓	↔	↓	↓	↔	↓	↓	↔	↑	↔	↔	↑	↓

Table 5.2. Summary table of effects on pulmonary endothelial cells. MVEC=*micro*vascular endothelial cells; LEC=*macro*vascular endothelial cells. ↑ or ↓ arrow indicates a significant change vs. control. ↔ indicates no change vs. control.

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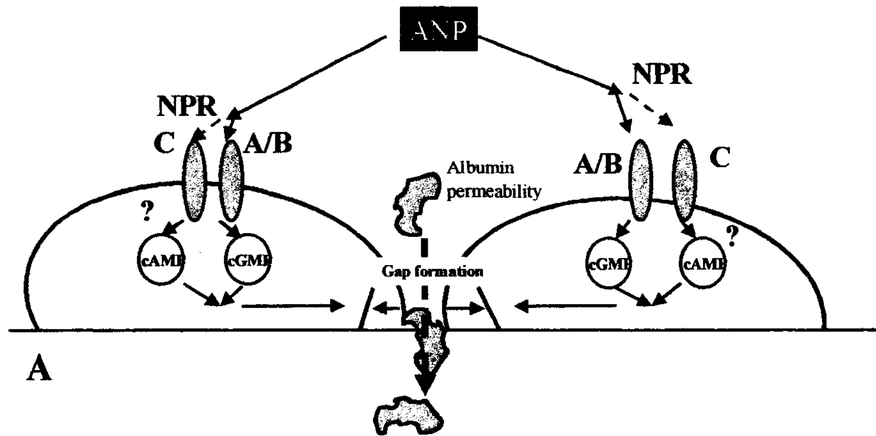
*Micro*endothelial cells (MVEC)



*Macro*endothelial cells (LEC)

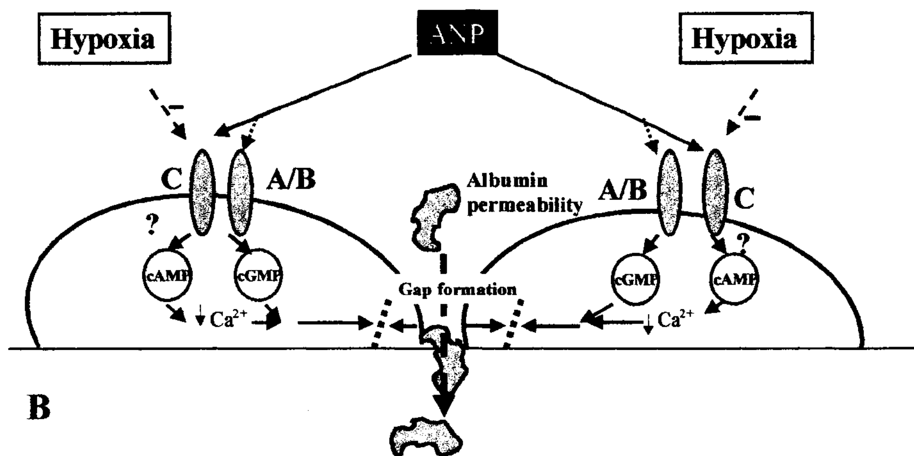


Figure 5.1: Schematic of proposed ANP inhibition of gap formation. A) ANP may predominantly act through the natriuretic peptid A and B receptors in *micro*vascular endothelial cells inhibiting hypoxia- and TNF-α-stimulated leak. B) ANP may predominantly act through the natriuretic peptide C receptor decreasing intracellular calcium stores in *macro*vascular endothelial cells. The C receptor may be down regulated during acute hypoxic exposure preventing ANP inhibition of vascular leak.

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5.3 Summary of specific aim 3:

As described in chapter 3, pulmonary microvascular (MVEC) and pulmonary artery (LEC) were isolated from bovine lungs and stimulated with hypoxia, TNF-α or bacterial endotoxin (LPS) in the presence or absence of ANP and NF-κB activity, TNF-α secretion, activation of p38 mitogen activated protein kinase (MAPK) and F-actin formation was assessed. Our results indicated that hypoxia decreased activation of p38 MAPK in MVEC, but increased activation of p38 MAPK and stress fiber formation in LEC **(Figure 2)**. However, this data was reversed with administration of TNF-α **(Figure 2)**. ANP inhibited activation of p38 MAPK in either MVEC or LEC. These data suggest that hypoxia-stimulated EC may activate signal cascades analogous to those initiated by inflammatory agents, and ANP has a direct cytoprotective effect on the pulmonary endothelium other then its vasodilatory and natriuretic properties **(Figures 1-2)**. Furthermore, our data shows that MVEC and LEC respond differently to hypoxia, TNF-α-stimulation and ANP treatment. Data indicate that ANP decreases TNF-α secretion and inhibits NF-Kb activation in hypoxia-induced bovine pulmonary microvascular endothelial cells (MVEC).

5.4 Conclusion:

Investigating the role of ANP in ameliorating high altitude pulmonary edema our three specific aims produced: 1) Data describing interactions between ANP, ET-1 and neutral endopeptidase (NEP) in mice lacking the NEP gene and wild type cohorts at low and high altitude; 2) Suggesting a new mechanism by which ANP acts to prevent

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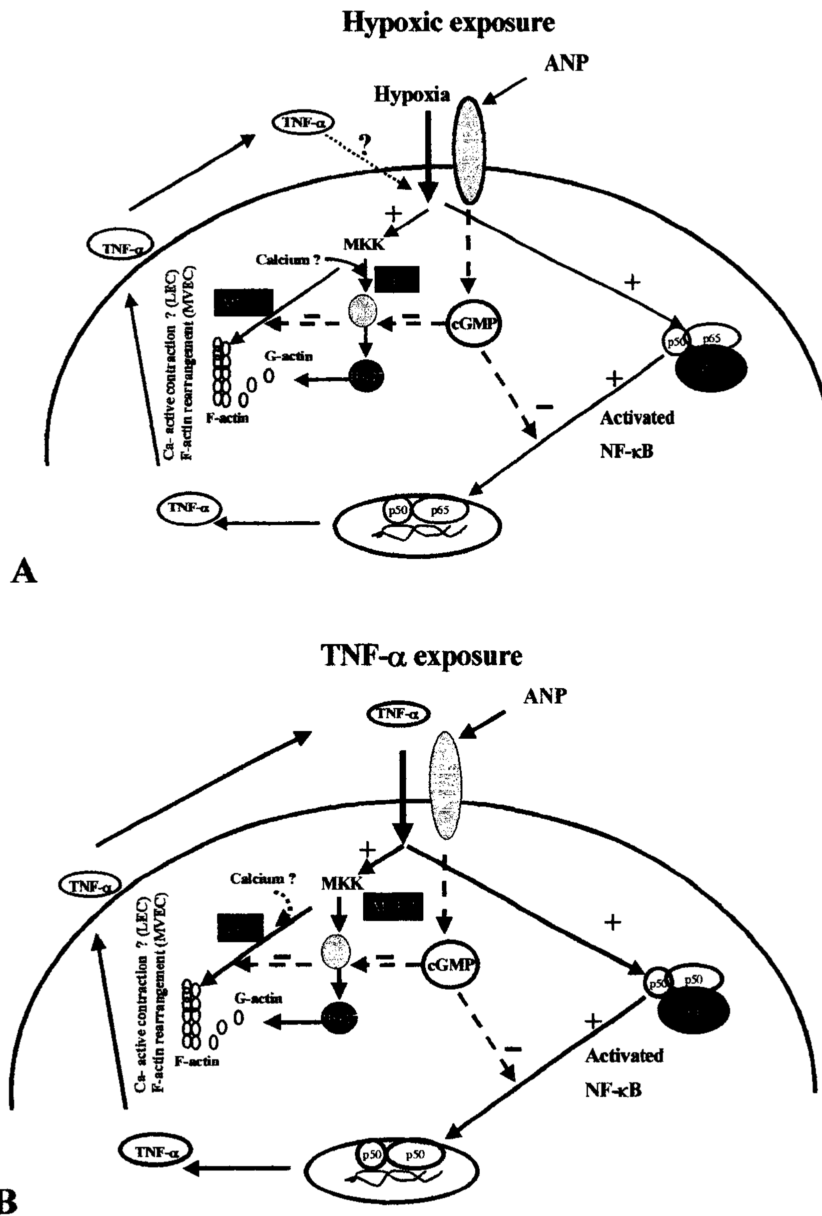

Figure 5.2: Schematic of proposed intracellular actions of ANP inhibition of gap formation. **A)** *Hypoxia* activates a mitogen extracellular kinase-kinase-kinase which increases activation of p38 MAPK (calcium may play a role) and F-actin formation inducing gap formation *macro*vascular (LEC), but alters F-actin in *micro*vascular (MVEC) endothelial cells. In addition, hypoxia activates NF-KB which increases TNF-α synthesis/secretion. TNF-α may act in an autocrine or paracrine fashion. ANP inhibits p38 MAPK and NF-Kb most likely through the cGMP pathway. **B)** TNF-α increases p38 MAPK and F-actin formation in *micro*vascular, but is unchanged in LEC. TNF-α activates NF-Kb which increases TNF-α synthesis/secretion. TNF-α may act in an autocrine or paracrine fashion exacerbating pulmonary vascular leak.

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pulmonary vascular leak at high altitude and during inflammation; 3) Data suggesting a pathway by which acute hypoxia may induce pulmonary vascular leak; and 4) Data indicating unique differences between *micro*vascular and *macro*vascular endothelial cells stimulated by hypoxia or TNF-α and treated with ANP.

5.5 Speculations and future research:

The data provided herein suggest a broader role for ANP in maintaining pulmonary vascular homeostasis as well as unique differences between pulmonary microvascular endothelial cells versus endothelial cells from the pulmonary artery. However, our data indicates many unresolved questions with regard to high altitude-induced pulmonary vascular leak that need to be addressed. First, our data indicated no increase in circulating ET-1 concentrations in wild type mice exposed to high altitude. However, this is in contrast to data that suggest that ET-1 is elevated in HAPE . This may reflect the differences in experimental design, but further research needs to address the contribution and mechanism by which ET-1 acts during high altitude exposure and the formation of HAPE. Second, we demonstrated that ANP inhibited permeability in hypoxia-stimulated MVEC, but had no effect on hypoxia-stimulated LEC. This is the first data to suggest that ANP has a differential effect between pulmonary endothelial cell phenotypes that are hypoxia-stimulated. We speculate that this failure to inhibit permeability in LEC may be a manifestation of a down regulation of the natriuretic peptide C receptor **(Figure 1)**. However, further research is needed to understand the interactions between hypoxia, natriuretic peptide receptors and ANP actions. Finally, our data indicated that the activation of p38 MAPK and F-actin formation were reversed in hypoxia and TNF-α-stimulated MVEC and LEC. This unique paradox needs to be addressed to understand the

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differences in hypoxia- versus inflammatory-induced pulmonary vascular leak in MVEC and LEC. We speculate that intra-cellular stores of calcium may be responsible for this differential signaling (Fig. 2), but further research is needed to understand the relationship between calcium, p38 MAPK, F-actin formation and pulmonary vascular leak.

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6 APPENDICES

6.1 Pulmonary microvascular endothelial cell line

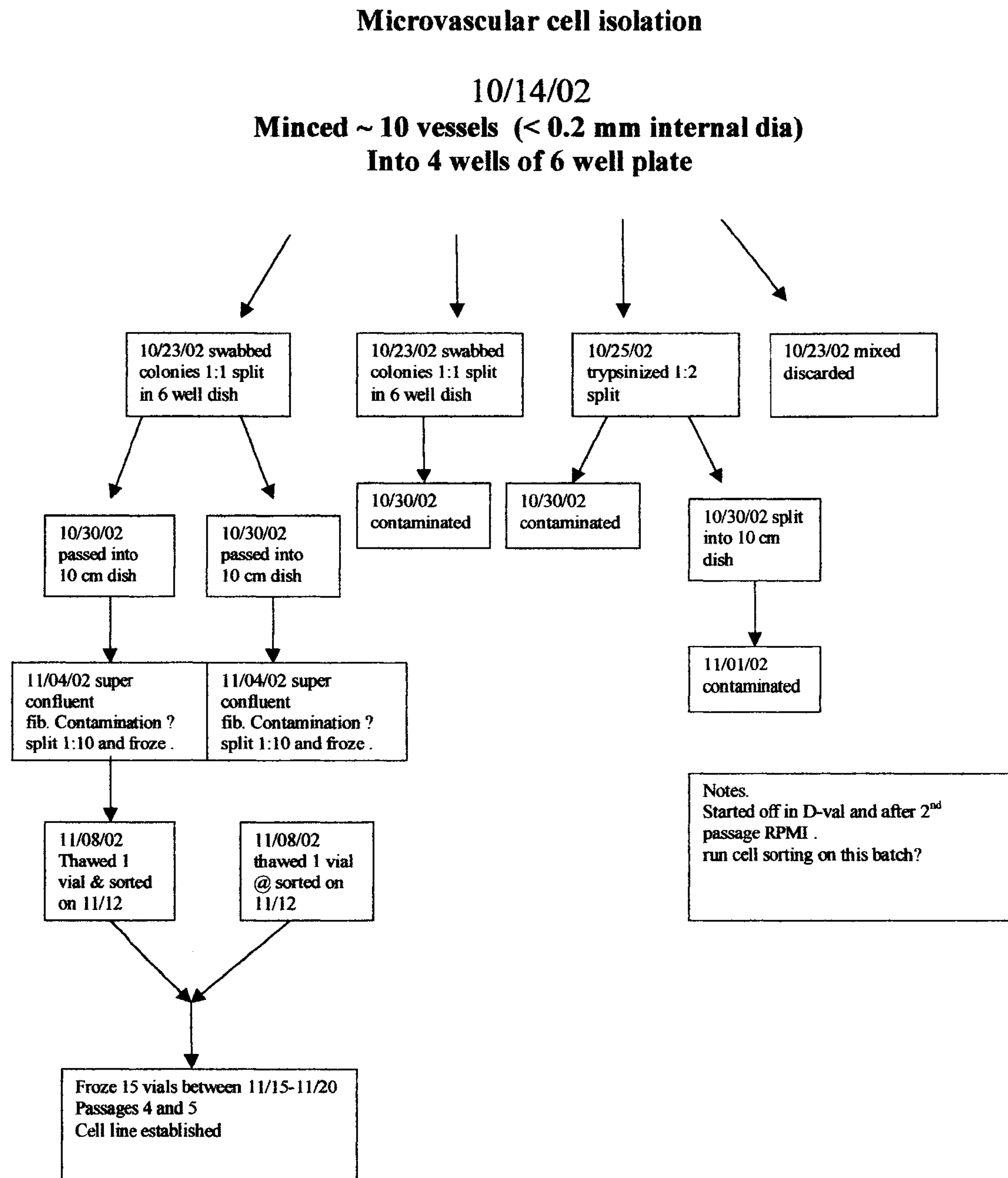

Figure 6.1: Flow chart of microvascular (MVEC) cell isolation.

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Microvascular cell isolation

10/22/02
Minced 6 vessels (< 0.2 mm internal dia)
Into 4 wells of 6 well plate

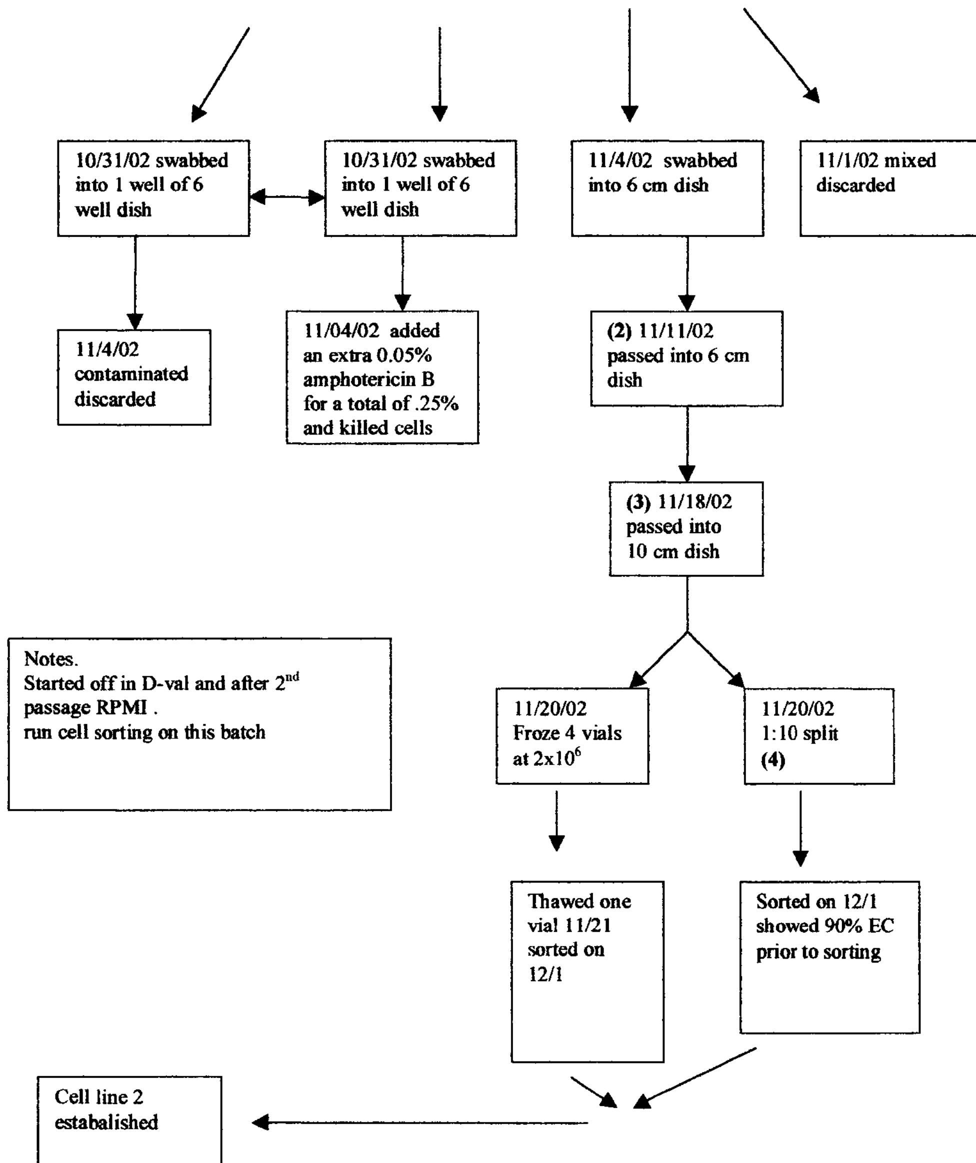

Figure 6.2: Flow chart of "cell line 2" microvascular MVEC cell isolation.

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6.2 Macrovascular endothelial cell line (LEC).

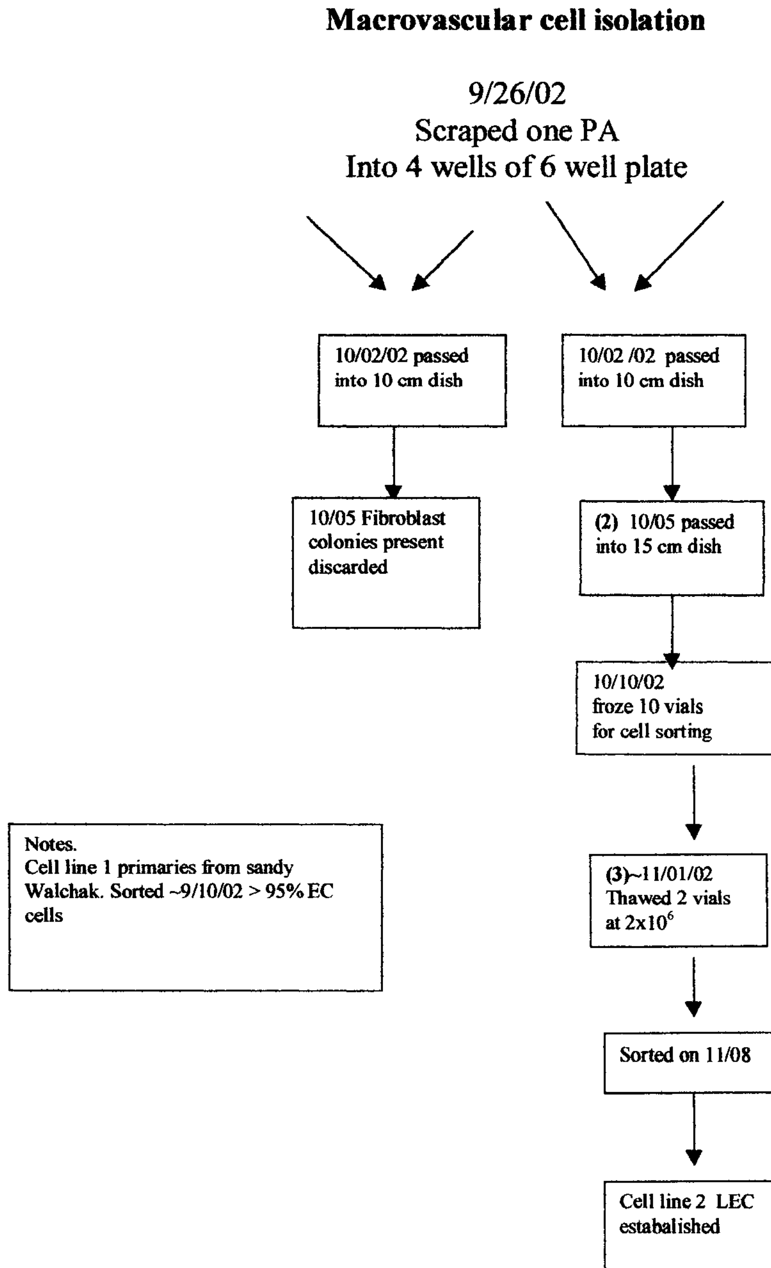

Figure 6.3: Flow chart of macrovascular (LEC) cell isolation.

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