STUDIES ON THE EFFECTS OF PHARMACOLOGICAL AGENTS ON ENDOTOXIN INDUCED PULMONARY INJURY

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a severe form of acute lung injury, characterized by inflammation and increased capillary permeability, associated with a constellation of clinical, radiological and physiological abnormalities. The incidence of ARDS is uncertain but has been estimated to be as high as 75 cases per 100,000 hospitalized patients per year. The overall mortality of patients with ARDS remains at 40 to 60 percent. Many factors predispose to ARDS, with sepsis caused by gram negative bacteria being one of the most important. Experimentally, ARDS can be mimicked by the injection of bacterial endotoxin.

Previous studies in our laboratory showed that pretreatment with pentoxifylline (a methylated xanthine), bepafant (a platelet activating factor antagonist) and nicardipine (a calcium channel blocker), 15 minutes before the administration of endotoxin, reduced the mortality and manifestations of disseminated intravascular coagulation caused by endotoxin in rats. The objective of the present study was to determine whether these drugs would also protect the rat lung against the deleterious effects of endotoxin. Anesthetized rats were given endotoxin (10 mg/kg) intravenously. One hour later, the lungs were removed and perfused with a buffered salt solution containing 4% Ficoll and aerated with air and 5% CO_2. Pulmonary arterial pressure, capillary pressure, capillary permeability, and arterial and venous segmental resistances were significantly higher in lungs obtained from endotoxin-treated animals than in lungs from saline control rats. Endotoxin also caused an increase in lung weight, lung water content and the outflow of

lung filtrate as compared to saline-treated controls. Pretreatment in vivo with nicardipine and bepafant, 15 minutes before the administration of endotoxin, significantly reduced the endotoxin-induced increases in capillary permeability and filtrate outflow but did not significantly affect the other parameters of measurement. Pretreatment with pentoxifylline differed from other two drugs in that the methylated xanthine significantly reduced the endotoxin-induced increases in all of the hemodynamic parameters as well as the increase in capillary permeability and filtrate outflow.

Studies were also made on the effect of endotoxin on the pulmonary leukocyte count in rats. In these experiments, lungs were removed for histological examination one hour after the intravenous administration of endotoxin (10 mg/kg). Leukocyte numbers were significantly increased in the endotoxin group as compared to the saline group. Pretreatment with nicardipine and bepafant but not pentoxifylline significantly reduced the endotoxin-induced increase in pulmonary leukocyte count.

The present results thus showed that the three drugs can protect against endotoxin-induced lung injury, in addition to preventing disseminated intravascular coagulation and death caused by the lipopolysaccharide. Pulmonary migration/sequestration of leucocytes and production/release of autacoids and cytokines from leucocytes are thought to play important roles in the lung injury caused by endotoxin. The results with nicardipine and bepafant suggest that these agents may act at least in part by inhibiting the pulmonary migration/sequestration of leucocytes.

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LIST OF ABBREVIATIONS

π_p	Colloid Osmotic Pressure of Plasma
π_t	Colloid Osmotic Pressure of Tissue Fluid
σ_d	Osmotic Reflection Coefficient
ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
BAL	Bronchoalveolar Lavage
BW	Body Weight
CCP	Complement Control Proteins
CD	Cluster of Differentiation
DIC	Disseminated Intravascular Coagulation
EGF-R	Epidermal Growth Factor Receptor
ETX	Endotoxin
FIO_2	Inspired Oxygen Concentration
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon Gamma
IL	Interleukin
J_v	Fluid Flux Across Capillary Membrane
K_f	Filtration Coefficient
LBP	LPS-Binding Protein

LFA-1α	Lymphocyte Function-associated Antigen-1α
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
LW	Lung Weight
MPO	Myeloperoxidase
NAC	N-acetylcysteine
PAF	Platelet Activating Factor
PaO_2	Arterial Oxygen tension
PAP	Pulmonary Arterial Pressure
Pc	Capillary Pressure
PDE	Phosphodiesterase
PDGF	Platelet Derived Growth Factor
PEEP	Positive End-Expiratory Pressure
PGE_1	Prostaglandin E_1
PMNs	Polymorphonuclear Cells
P_t	Interstitial Fluid Hydrostatic Pressure
PVP	Pulmonary Venous Pressure
Ra	Arterial Resistance
Rt	Total Vascular Resistance
Rv	Venous Resistance
TGF-β	Transforming Growth Factor-β
TNF	Tumor Necrosis Factor

CHAPTER 1

INTRODUCTION

1.1 Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) was first described in 1967 by Ashbaugh and colleagues [1] as syndrome of acute respiratory failure associated with sepsis, trauma, and drug overdose that invariably led to multiple organ failure. A recent consensus conference on ARDS sponsored by American Thoracic Society and European Respiratory Society defined ARDS (Table 1) [2] as "The most severe form of acute lung injury, which is a syndrome of inflammation and increased permeability that is associated with constellation of clinical, radiological and physiological abnormalities that can not be explained by, but may exist with, left atrial or pulmonary capillary hypertension." Another definition proposed by Kollef and Schuster [3] describes ARDS as a set of clinical manifestations due to severe acute lung injury precipitated by direct or indirect lung injury, characterized pathologically by diffuse alveolar damage, and pathophysiologically by the development of noncardiogenic pulmonary edema due to increased microvascular permeability.

The incidence of ARDS is unknown but has been estimated to be as high as 75 cases per 100,000 hospitalized patients per year [4]. The overall mortality of patients with ARDS remains 40% to 60% [2, 5]. Most of the patients who die with ARDS do so in the first 2 weeks of the illness, usually due to sepsis and multi-organ failure. On the other

Table 1. Recommended Criteria for Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) by American Thoracic Society and European Respiratory Society

	Timing	Oxygenation	Chest Radiograph	Pulmonary Artery Wedge Pressure
ALI Criteria	Acute onset	$PaO_2/FIO_2 \leq 300$ mm Hg (regardless of PEEP level)	Bilateral Infiltrates seen on frontal chest radiograph	≤ 18 mm Hg when measured or no clinical evidence of left atrial hypertension
ARDS Criteria	Acute onset	$PaO_2/FIO_2 \leq 200$ mm Hg (regardless of PEEP level)	Bilateral Infiltrates seen on frontal chest radiograph	≤ 18 mm Hg when measured or no clinical evidence of left atrial hypertension

PaO_2 = arterial oxygen tension; FIO_2 = inspired oxygen concentration; PEEP = positive end-expiratory pressure.

hand, it has been shown that outcome is better in patients who are younger than 60 years of age and those with sepsis as the risk factor for ARDS [6].

Many factors predispose to ARDS (Table 2). These are categorized as causing direct injury to the lung, or factors that indirectly lead to lung injury. Certain factors like sepsis, near drowning, aspiration of gastric contents, multiple traumas, and multiple transfusions are more likely than others to lead to ARDS [2, 7, 8]. Also, the risk for the development of ARDS rises with the increase in the number of potential risk factors [8].

The recognized pathologic lesion in acute lung injury and ARDS is diffuse alveolar damage, the appearance of which is determined by the duration and extent of lung injury and repair. The earliest lesion is described as exudative and typically occurs in the first week after injury, with findings of widespread but patchy areas of alveolitis with interstitial edema and hemorrhage. Hyaline membrane formation occurs as a result of precipitated plasma proteins and fibrin accumulation. Subsequently, Type II pneumocyte hyperplasia occurs, with local areas of Type I cell destruction and exposure of basement membranes. There is an early interstitial infiltration of polymorphonuclear leukocytes and mononuclear cells. Although the alveolar capillary barrier may be most affected, large endothelial gaps are not seen [9 - 11].

A proliferative or organizing stage follows after 7 to 10 days, manifest by fibrin deposition in the alveoli and increased infiltration of inflammatory cells and fibroblasts. Type II pneumocytes proliferate, resulting in almost cuboidal alveolar walls. The air-blood interface may be dramatically altered by the interstitial and cellular changes and

Table 2. Risk Factors for Development of Acute Respiratory Distress syndrome

Direct Lung Injury
Aspiration
Pneumonia
Pulmonary contusion
Toxic inhalation
Near drowning
Indirect Lung Injury
Sepsis
Multiple trauma
Fat embolism
Disseminated intravascular coagulation
Prolonged hypotension
Acute pancreatitis
Cardiopulmonary bypass
Multiple transfusions
Extensive burn
Drug overdose
Multisystem disease (e.g. vasculitis, acute hepatic failure)

the vascular bed may be disrupted. A final stage of fibrotic changes of alveolar ducts, alveoli, and the interstitium can result in almost complete alveolar obliteration [10].

After the initiating aggression, the widespread and acute inflammatory response begins with localization of active polymorphonuclear cells to the pulmonary microvasculature. These inflammatory cells actively participate in the process of phagocytosis and opsonization of foreign bodies and cellular debris. Subsequent degranulation and release of reactive oxygen species and other enzymes occur, and through interactions with endothelial cells, cytokines, and complement components, the ensuing local inflammation results in endothelial cell injury. This endothelial injury produces changes in the permeability, release, and metabolism of vasoactive and inflammatory substances and alterations in hemofluidity and hemodynamics, with resultant abnormalities in gas exchange (Figure 1).

Pulmonary endothelial cells face both the environmental surface and the vascular compartment, and thus are highly vulnerable to injury. Endothelial cells are actively involved in ARDS development by changes in production and release of vasoactive substances, including serotonin, norepinephrine, bradykinin, prostaglandins, endothelins, and cytokines [12, 13].

One of the early, major steps involved in neutrophil and monocyte migration is the modulation and display of adhesion molecules. Adhesion molecules play an early and central role in the inflammatory response by promoting adhesion of neutrophils and monocytes to the endothelium [9, 12]. The selectins are a family of molecules with three single-chain glycoproteins designated by the prefixes E, P, and L for endothelial, platelet,

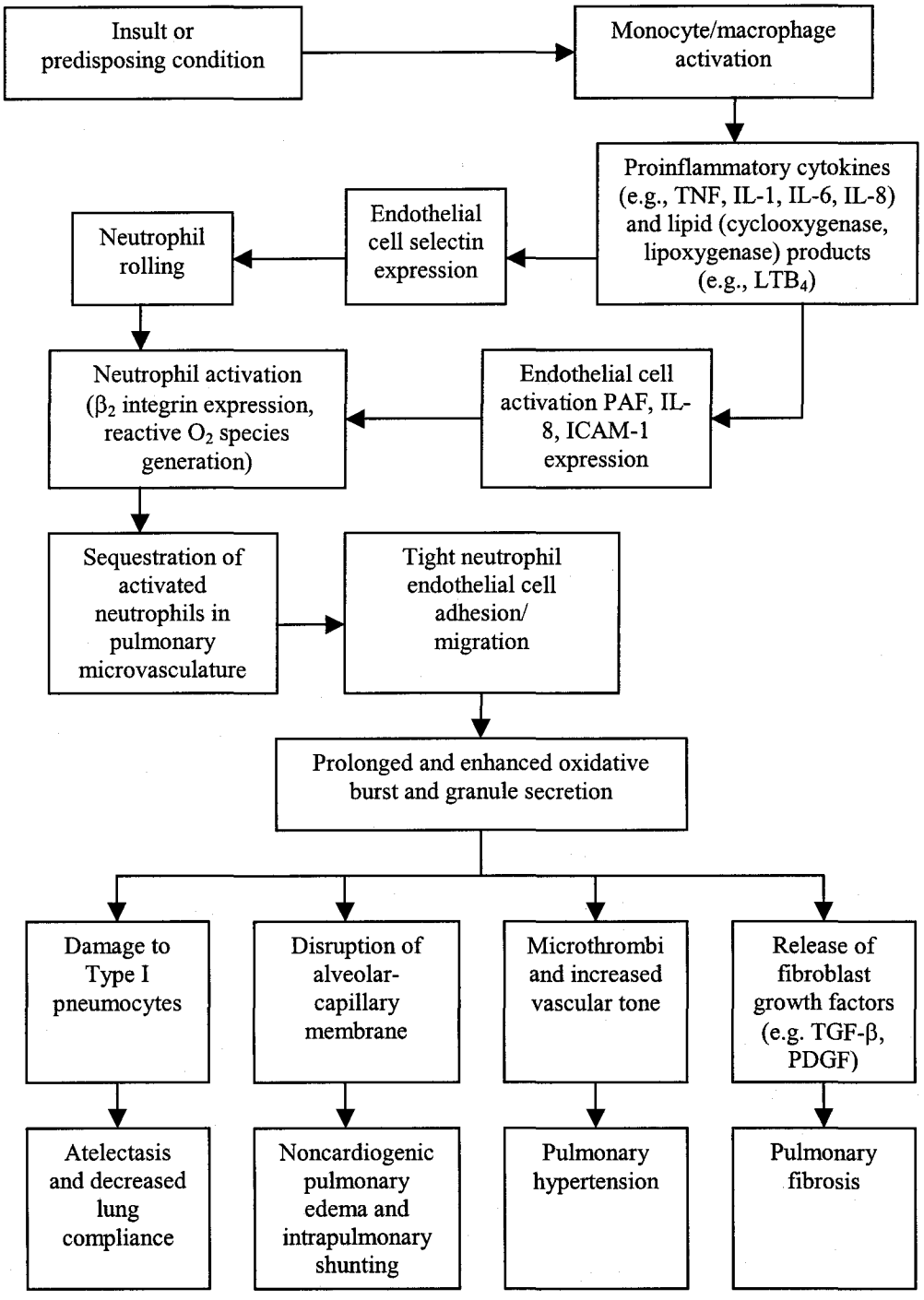


Figure 1. The steps detailed describe a likely mechanism that leads to acute lung injury. TNF = tumor necrosis factor; IL = interleukin; LTB_4 = leukotriene B_4; PAF = platelet-activation factor; ICAM-1 = intercellular adhesion molecule-1; O_2 = oxygen; TGF-β = transforming growth factor-β; PDGF = platelet derived growth factor.

and leukocyte, respectively. Both E- and P-selectins are expressed on endothelial surfaces (Figure 2) [13].

E-selectin protein is translated and is expressed following exposure to TNF-α, IL-1, and bacterial lipopolysaccharide (LPS). Four to six hours are required after transcription to increase expression, with subsequent induction of neutrophil adhesion by promoting cell rolling through interactions with neutrophil carbohydrate ligands, sialyl Lewis^x (SLe^x) and others (e.g., SLe^a) [13]. P-selectin is upregulated rapidly following endothelial cell exposure to proinflammatory mediators because it is localized in cytoplasmic granules.

Intercellular adhesion molecule-1 (ICAM-1) is expressed constitutively on endothelial surfaces. Following cytokine, leukotriene B_4 (LTB_4), or LPS exposure, transcription occurs for up to 24 hours [13, 14]. Intercellular adhesion molecule-1 and closely related ICAM-2, located on endothelial cells, are ligands for β_2-integrins on the neutrophil surface and arrest neutrophil rolling by initiating adhesion and subsequent migration [13, 15 - 17]. ICAM-1 interacts with lymphocyte function-associated antigen-1α (LFA-1α), which is also known as CD11α. In an acid aspiration model of acute lung injury, administration of monoclonal antibodies to block the ICAM-1/LFA-1α pathway resulted in attenuation of leukocyte numbers recovered in bronchoalveolar lavage (BAL) fluid and subsequent injury, indicating the importance of this pathway to the inflammatory response in ARDS [18]. Elevated plasma levels of E- and P-selectins and ICAM-1 were measured early in critically ill patients with sepsis and those with established acute respiratory failure, and have prognostic significance [13, 19, 20].

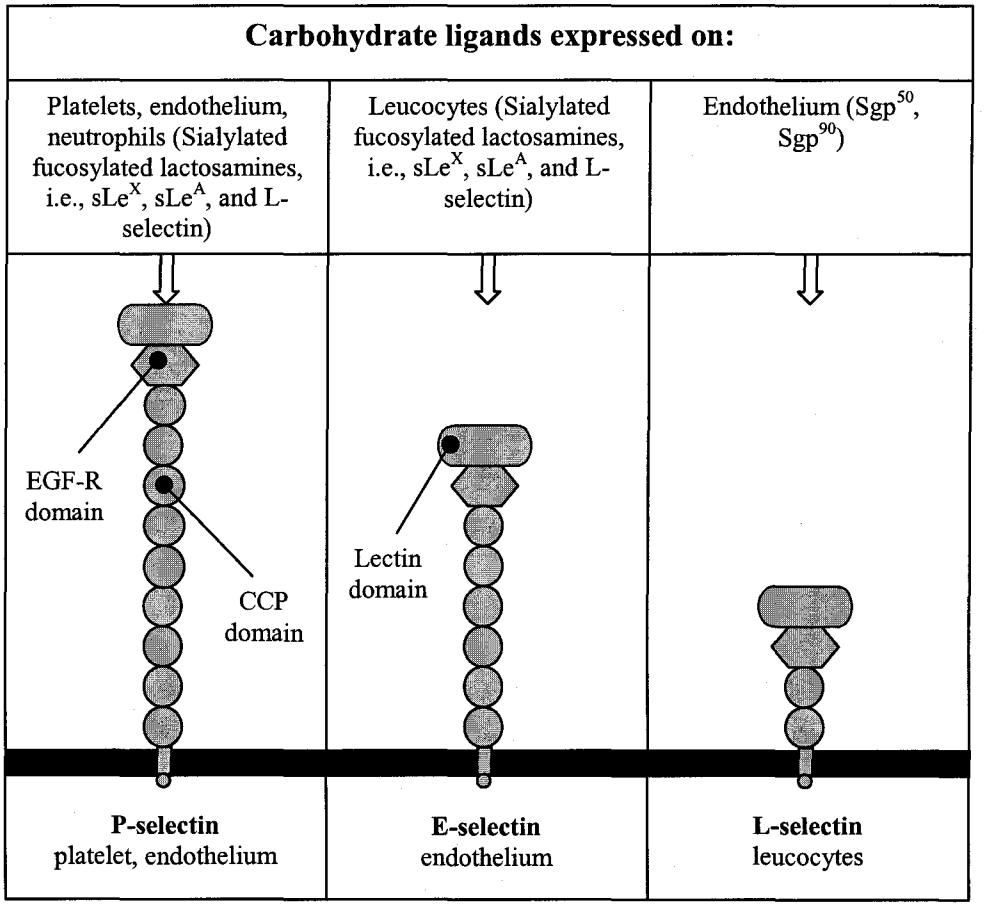


Figure 2. The structures of three selectins are shown. They have terminal lectin domains that bind carbohydrates on the cells listed. EGF-R: epidermal growth factor receptor; CCP: complement control proteins (factor H).

The endothelial cell-neutrophil interactions facilitated by proinflammatory mediators are complex. Normal endothelium and neutrophils possess negatively charged surface particles, causing repulsion during random contact. To overcome shear and repulsive forces, as neutrophils course through the circulation at rates of up to 2.5 mm/second [21], vascular P- and E-selectins expressed after proinflammatory insult interact with polymorphonuclear cells (PMNs) to begin rolling adhesion [13, 22]. Endothelial-expressed carbohydrate ligands (Gly-CAM-1) interact with L-selectin on the neutrophil, and P- and E-selectin interact with SLe^x on the neutrophil surface. Studies have shown that both anti-L-selectin and anti-SLe^x antibodies are required to attenuate neutrophil rolling [19, 23]. Further endothelial activation with synthesis and release of mediators such as platelet activation factor (PAF) and interleukin-8 (IL-8) promote rolling [13]. Finally, integrin-ICAM-1 binding occurs, and activated PMNs adhere tightly and flatten, forming a microenvironment protected from circulating inhibitors that favors local endothelial injury [13].

Neutrophils play a prominent role in the pathogenesis of ARDS, through changes in local accumulation, activation state (local production of injurious compounds), and interactions with the production of cytokines. Neutrophils are not required for lung injury, however, as ARDS develops in neutropenic patients after chemotherapy or radiation [12, 24, 25]. Neutrophil influx is known to occur before lung damage occurs [26]. In patients with ARDS following sepsis, a recent study found that BAL neutrophil counts were higher in the first 2 weeks post-injury and correlated inversely with survival, indicating that persistent alveolitis is associated with a worse prognosis [27].

Neutrophil adherence to the endothelium is an early event facilitated by selectin and β-integrins. L-selectin is expressed on most circulating PMNs in high-density microvillous-like processes. Once activated, L-selectin is shed and retains biologic activity in plasma [28]. Carbohydrate determinants (SLe^x and others) on neutrophils and monocytes are recognized by all three selectins. Removal of L-selectin from PMN surfaces still permits binding of E-selectin to PMNs [29]. Sialyl $Lewis^x$ has a higher affinity for E- and P-selectins and only when anti-L-selectin and anti-SLe^x antibodies are combined is PMN-endothelial binding completely inhibited [13, 30]. The counter receptor for L-selectin on the endothelium is unknown, but is thought to be Gly-CAM-1 [13, 31].

The β_2 integrins are molecules consisting of noncovalently linked α and β subunits expressed on leukocytes [13] that mediate firm adherence to the endothelium, chemotaxis, and phagocytosis [32]. β subunits are composed of a common structure designated CD18; α subunits vary in size and structure and are designated CD11a (LFA-1), CD11b (designated MAC-1, MO-1), and CD11c (known as p150) [13]. Endothelial surface ICAM-1 is the major ligand for β_2 integrins. Lymphocyte function-associated antigen-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) are the primary β_2 integrins that PMNs employ for adhesion following upregulation by inflammatory cytokines [13, 33]. Elevated basal expression of the β_2 integrin CD11b/CD18 in both whole blood and in BAL fluid has been shown in patients with ARDS. This overexpression of adhesion molecules may contribute to ongoing lung injury in ARDS [34]; indeed, CD11b/CD18 has been shown in experimental models to play a key role in lung injury [34 - 37]. In a study of ARDS patients, both whole-blood and alveolar PMNs were activated under basal

conditions, as shown by decreased CD62L (caused by the shedding of L-selectin after activation) and increased CD11b expression. The degree of PMN activation correlated with the degree of lung injury [34].

Not only are the numbers of alveolar and interstitial neutrophils increased; the activation state of the neutrophils is altered in ARDS as well. This was reported earlier [37, 38], and a study using flow cytometry to examine the activation status of circulating neutrophils found that a subpopulation of "primed" neutrophils with an increased capacity to release hydrogen peroxide was present in ARDS patients [39]. Neutrophils possess many functional receptors and adhesion molecules that are involved in activation. Interleukin-1, IL-8, and TNF-α are potent activators of neutrophils and have been found in increased concentration in plasma and BAL fluid of ARDS patients [40 - 42]. Endotoxin (LPS) from gram-negative bacteria is a potent activator via the LPS-binding protein complex (LBP), which is designated CD14 [43~47]. Complement fragment C5a and IL-8 are potent chemotactic stimuli that also induce degranulation [45]. The lipoxygenase pathway product, LTB_4, and PAF induce activation [45]. Granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and interferon gamma (IFN-γ) are weak activators of PMNs.

Neutrophil causes lung damage through the release of proteases and generation of reactive oxygen species. Activated neutrophils show increased adhesiveness and subsequent irreversible binding to stromal cells with subsequent degranulation and production of hydrogen peroxide, hydroxyl radicals, and superoxide anions. Elastase is the major protease released from primary granules by neutrophils in ARDS and can

degrade elastin, other substrates including collagen (Types I, II, III, IV), fibrinogen, fibronectin, and proteoglycans [45, 48]. Lung neutrophils in those with ARDS contain increased quantities of elastase; increased concentrations of elastase found in BAL fluid correlate with severity of lung injury [12, 49, 50].

Neutrophils also contain metalloproteinases (MMP), including collagenase (MMP-1), gelatinase (MMP-2), and stromelysin (MMP-3) [51]. Collagenase is stored in small secondary granules, some of which are lost during migration. Excessive collagenolytic activity of collagen types I, II, and III is mediated by this enzyme and has been shown to occur in patients with ARDS [12]. Gelatinase is a subfamily of two members named gelatinase A and B; these enzymes degrade type IV collagen, a major component of basement membranes, denatured collagens, and elastin, and may play a significant role in ARDS. Increased gelatinase A and B activity has been documented in the BAL fluid of early ARDS patients [12].

Large amounts of oxygen metabolites, such as hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and superoxide (O_2^-), are generated by an activated neutrophil surface and NADPH, and contribute to tissue injury in ARDS [52, 53]. These oxidants are difficult to measure directly, but the myeloperoxidase (MPO) in neutrophil primary granules, which catalyzes the release of chloramine, halides, and hypochlorous acid, is elevated in the BAL of patients with ARDS [12, 54]. Further evidence for a role of excessive oxidant activity in the cellular injury in ARDS is the change in measured antioxidants in BAL fluid. In a study of patients with ARDS, total antioxidant levels were found to be increased [55], whereas glutathione-specific antioxidant activity was markedly reduced [56].

N-acetylcysteine (NAC), a known antioxidant that may restore intracellular stores of glutathione, has been shown to prevent deterioration of lung mechanics in endotoxemic sheep and prevent capillary leak induced by IL-1 in rats [1, 57, 58]. In humans with ARDS, NAC reduced ventilator days in one study [59]. In another study of severe ARDS, intravenous NAC administration increased intracellular (neutrophil and other cells) glutathione stores and decreased H_2O_2 production without a real change in plasma elastase concentrations (thus without altering neutrophil degranulation), possibly explaining its beneficial effects by the decreased neutrophil oxidant injury [58].

The increased understanding of the pathophysiology of ARDS that has been achieved over the last decade has led to several new pharmacological approaches for the prevention and management of ARDS. Based on in vitro information and animal models, many of these strategies are quite compelling. Nevertheless, to date, no specific pharmacologic approach for the prevention or treatment of ARDS has been validated conclusively in clinical trials. Active basic and clinical investigations are continuing (Table 3) [60].

1.2 Endotoxin

Gram-negative sepsis is a frequent cause of ARDS and experimentally, the syndrome can be mimicked by the injection of bacterial endotoxin. The mechanism by which sepsis and endotoxin produce ARDS has not been settled.

Endotoxin or lipopolysaccharide (LPS) is a glycolipid that constitutes the major portion of the outermost membrane of Gram-negative bacteria [61]. LPS is an extremely

Table 3. Proposed Systemic Pharmacological Therapies for ARDS

Drug	Mechanisms of Action	Clinical Results
Corticosteroids	1) Inhibition of arachidonic acid metabolites 2) Inhibition of complement-induced neutrophil aggregation 3) Suppression of cytokine release from macrophages 4) Suppression of platelet-activating factor and nitric oxide production 5) Modification of fibrogenesis	The result of several large multi-center clinical investigations suggested that corticosteroids could not prevent the development of ARDS or improve its mortality rate. But animal models of ARDS demonstrate that prolonged corticosteroid administration is effective.
Cytokine Antagonists (Monoclonal Antibodies, Receptor Antagonists)	Inhibition of inflammatory cytokine action	Clinical trials testing the effect of antagonism of these cytokines (with monoclonal antibodies, receptor antagonists, and so forth) have not revealed any consistent benefit in ARDS patients.
Ketoconazole	1) Inhibition of thromboxane synthetase 2) Inhibition of procoagulant activity by macrophages 3) Blockade of 5-lipoxygenase	Clinical trials suggest that ketoconazole may be effective in preventing the onset of ARDS in high-risk individuals, but not in the treatment of established ALI or ARDS.
N-acetylcysteine, Procysteine	Repletion of glutathione store (antioxidant activity)	The use of N-acetylcysteine and Procysteine as antioxidant therapy in ARDS has met with modest and somewhat conflicting results in trials of ARDS patients. Further investigation will be necessary to clarify the usefulness of this approach.
Prostaglandin E_1 (PGE_1)	1) Pulmonary vasodilation 2) Inhibition of mediator release from granulocytes	In animal models of ARDS, PGE_1 reduces pulmonary hypertension, lessens the accumulation of pulmonary edema, improves gas exchange, and helps to reduce the release of damaging mediators (such as oxygen radicals and leukotreine B_4) from activated granulocytes. However these beneficial results apparently were not confirmed in a large multicenter randomized study.
Pentoxifylline Lisophylline	Inhibition of TNF release by macrophages	Being currently evaluated by the NIH ARDS Treatment Network in a randomized and double-blind fashion
Anti-adhesion Molecules	Inhibition of leukocyte adherence to endothelium	Clinical trials testing this approach are being planned.

potent toxin: macrophages can be activated at concentrations of LPS as low as 1 pg/ml. The lipid A portion of LPS is conserved among Gram-negative bacteria and appears to be responsible for the biological toxicity of LPS [61, 62]. Three cloned molecules expressed on the surface of monocytes and macrophages are known to bind the lipid A moiety of LPS. These include CD14, the macrophage scavenger receptor (SR), and the β_2 leukocyte integrins (CD11a/CD18, CD11b/CD18, and CD11c/CD18). Both CD14 and β_2 integrins are capable of initiating signals to cell, resulting in phagocyte activation, bacterial internalization (phagocytosis, endocytosis), and the activation of bactericidal defenses. The scavenger receptor does not appear to function as a signaling receptor for LPS. Lastly, CD14 and the β_2 leukocyte integrins interact with LPS in conjunction with blood-borne proteins that enhance their signaling capabilities. Both soluble CD14 (sCD14) and LPS-binding protein (LBP) are present in blood and are known to enhance to effects of LPS.

The earliest cell-mediated events following endotoxin release appear to involve the transfer of LPS to the GPI-linked protein CD14. LPS is an amphiphilic membrane phospholipid that forms aggregates in aqueous environments such as tissue culture medium or blood. Spontaneous diffusion of LPS monomers from these aggregates to CD14 occurs at a very slow rate [63]. The plasma protein LBP [64], however, dramatically accelerates binding of LPS monomers from aggregates to CD14 [63], thereby enhancing the sensitivity of cells to LPS [63, 65 - 67]. Additional studies have shown that LBP can transfer LPS to lipoproteins [68]. After transfer to lipoprotein particles, LPS is functionally neutralized [69 - 72]. Thus, LBP may serve to both enhance and neutralize the biological

activities of LPS. LBP is an acute-phase reactant [64], predominantly derived from the liver, and plasma levels rise dramatically after inflammatory challenge [73 - 75]. LBP can also be synthesized in the lung and local production of LBP might account for many of its biological effects [76]. No role for LBP has ever been established with respect to the CD11/CD18 integrins, which may also participate in LPS signaling [77, 78].

Figure 3 summarizes the interactions of LPS that are known to be important for LPS-initiated activation of a wide variety of cell types. Among those cells that respond to LPS, there are two groups: those that express membrane-bound CD14 (mCD14) and those that do not. Until recently, only myeloid cells such as monocytes, macrophages, and neutrophils were known to express mCD14 [79], but recent evidence shows that mesangial cells express mCD14 ex vivo and other cells such as gingival fibroblasts may also express mCD14 [80 - 82]. Some cells do not express mCD14 but respond to LPS in the presence of the soluble plasma form of CD14 (sCD14). These include endothelial and epithelial cells [83], smooth-muscle cells [84], astrocytes [85], and dendritic cells [86]. It is interesting that in both pathways, LPS alone is less active as an agonist than is an LPS-protein complex (LPS-LBP for mCD14 bearing cells [66, 88] and LPS-sCD14 for the non-mCD14 bearing cells) [83]. However irrespective of whether the cells express mCD14, after interacting with the cells the LPS may partition between two paths, one leading to internalization and the other leading to cellular activation [87 - 89].

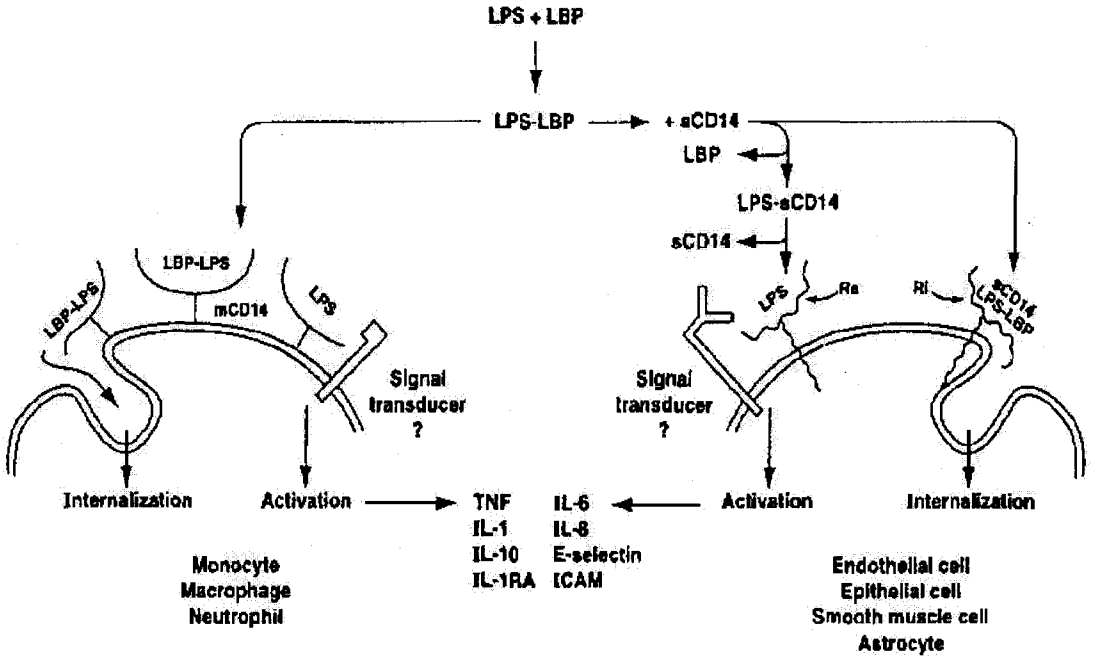


Figure 3. An overview of the association of lipopolysaccharide (LPS) with cells mediated by LPS binding protein (LBP) and CD14. Ra and Ri refer to postulated molecules mediating activation and internalization, respectively. (Adapted from Tobias PS, et al. [90])

1.3 Objectives

The results of previous studies in this laboratory showed that calcium channel blockers (such as nicardipine) were highly effective in reducing endotoxin-induced mortality and suggested that the protective action of these agents was at least partly related to prevention of endotoxin-induced disseminated intravascular coagulation (DIC). Studies in our laboratory have also shown that PAF antagonists (such as bepafant), ibuprofen (a cyclo-oxygenase inhibitor) and pentoxifylline exerted similar beneficial effects on endotoxin-induced DIC and mortality in rats. The objectives of the present investigation are to determine if these drugs can effectively protect the rat lung from the deleterious effect of endotoxin and to try to explore the possible role of PMN on endotoxin induced lung injury. Our working hypothesis is that these agents may prevent endotoxin induced ARDS and that the beneficial effect may be related to preventing PMN migration to lungs and/or to modulating release of mediators from PMNs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation of the Animals

Male Wistar rats obtained from Simonsen weighing 300 – 350g were used. After their arrival, the animals were kept in a room with a twelve-hour light and a twelve-hour dark cycle. All of the rats were allowed free access to food and water. All procedures used were in accordance with the institutional guidelines for animal studies. *E. coli* endotoxin was given intravenously via a tail vein into unanesthetized rats at a dose of 10 mg/kg. Nicardipine (1 mg/kg), bepafant (1 mg/kg) and pentoxifylline (50 mg/kg) were given 15 minutes before the administration of endotoxin. The control groups were treated with an equivalent volume of normal saline instead of the agents described above.

2.2 Isolated Perfused Rat Lung

After injecting the chemicals and drugs described above, and waiting for 60 minutes, the rats were injected intraperitoneally with pentobarbital sodium (60 mg/kg). Surgery was started about 10 minutes later. A tracheal cannula was inserted and the lungs were ventilated with a humidified gas mixture containing 5% CO_2 in room air at a frequency of 50 cycles/min, a tidal volume of 2 ml, and an end-expiratory pressure of 2 cm H_2O. After opening the chest, heparin sodium (1 unit/g body weight) was injected into the right ventricle. The pulmonary artery was cannulated and perfused with a physiological salt solution containing (in mM) 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 22.6

$NaHCO_3$, 1.18 KH_2PO_4, 1.6 $CaCl_2$, 5.5 glucose, 50 sucrose and 4 g/100 ml Ficoll (MW 70,000, Sigma, St. Louis, MO). A wide-bore cannula was inserted into the left ventricle to collect and drain the effluent perfusate into a reservoir, which was maintained at 37°C in a water bath. The first 50-ml of perfusate was discarded. The heart and lungs were then removed *en bloc* and placed on a double-level weighing pan, mounted on a Grass FT-03-C force displacement transducer for detecting the change of lung weight and for collecting accumulated fluid. The preparation was suspended in a humidified chamber and kept at a constant temperature (Figure 4). All fluid drained from the lung was collected in the lower pan. The preparation was perfused at a constant rate of 3 ml/min/100g body weight by means of a roller pump. All cannulae were fixed in positions that had least effect on the weight of the lungs. The pulmonary arterial pressure and the pulmonary venous pressure (as represented by left atrial pressure) were continuously monitored using physiological pressure transducers. Recordings were made using a Beckman Dynograph.

2.3 Determination of Segmental Resistance

The total vascular resistance (Rt) was computed as the ratio of the difference between pulmonary arterial pressure (PAP) and pulmonary venous pressure (PVP) to the perfusion flow rate. Arterial resistance (Ra) is defined as the resistance contributed by vascular segments upstream to the microvasculature, whereas venous resistance (Rv) represents the resistance contributed by venous segments. The fractional arterial

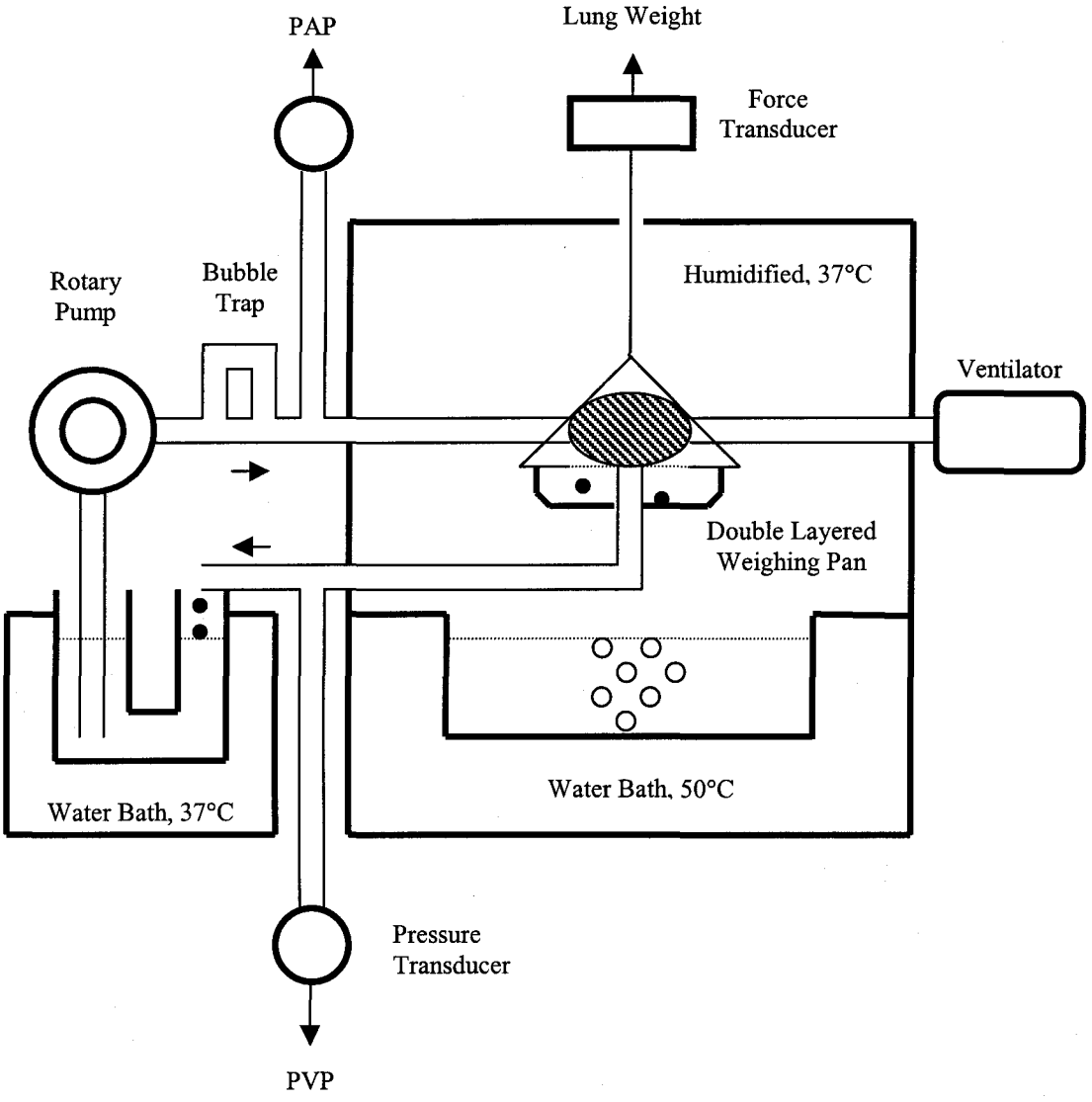


Figure 4. Schematic representation of the perfusing system for isolated rat lung. Lung was mounted on a weight pan that is connected to a force transducer for measuring the lung weight continuously. The lungs were ventilated with humidified air and were perfused with physiological salt solution. The arterial (PAP) and venous (PVP) pressures were monitored continuously (Adapted from Kunlun Huang [91]).

resistance (Ra/Rt) and fractional venous resistance (Rv/Rt) are calculated as followed (where, Q = 3 ml/min/100g):

$$Rt = \frac{(PAP - PVP)}{Q} \quad (1)$$

$$\frac{Ra}{Rt} = \frac{(PAP - Pc)}{(PAP - PVP)} \quad (2)$$

$$\frac{Rv}{Rt} = \frac{(Pc - PVP)}{(PAP - PVP)} \quad (3)$$

2.4 Measurement of Filtration Coefficient (K_f)

The fluid flux (J_v) caused by an imbalanced Starling force across the capillary wall can be described by the modified Starling equation:

$$J_v = K_f [(P_c - P_t) - \sigma_d (\pi_p - \pi_t)] \quad (4)$$

Where K_f is the filtration coefficient describing the volume-flow characteristics of the capillary wall and is an index of the microvascular permeability. P_c is the microvascular fluid hydrostatic pressure, P_t is the interstitial fluid hydrostatic pressure, and σ_d refers to the osmotic reflection coefficient that is an indicator of the ability of oncotic force to oppose the hydrostatic force across the capillary wall. The colloid osmotic pressure of the plasma and the tissue fluid are represented by π_p and π_t, respectively.

We determined K_f by using the gravimetric method described by Drake et al [92]. When isolated lung reaches an isogravimetric state, the PVP was rapidly elevated by diverting the venous flow to a 10-cm vertical tube for 10 minutes. This hydrostatic challenge resulted in an elevation of lung weight that is composed of a rapidly rising

component and a slow component. The rapid component, ended at the third minute after the onset of the hydrostatic challenge, represents the expansion of pulmonary vessels, whereas the slow component is most likely due to fluid filtration into the interstitial tissue of the lung. The log of the rate of weight gain ($\Delta W/\Delta t$) was plotted as a function of time. The initial rate of fluid filtration can be estimated by extrapolating the slow component to zero time (Figure 5). The value of y-intercept was then divided by the change in microvascular pressure (ΔPc), which is the driving pressure causing the fluid shift and the increase of lung weight,

$$K_f = \frac{(\Delta W / \Delta t)_{t=0}}{\Delta Pc} \qquad (5)$$

K_f is expressed in units of ml per minute per centimeter of water per 100 gram of lung (ml/min/cmH_2O/100g). We estimate the initial lung weight (LW) of the rat from its body weight (BW) by the equation [93]:

$$LW = 0.05BW + 0.43 \qquad (6)$$

During the hydrostatic challenge, Pc can not be measured directly with the venous occlusion method; hence, it is calculated by the equation of Gaar et al [94].

$$Pc = PVP + 0.44(PAP - PVP) \qquad (7)$$

2.5 Histological Examination

After injecting the chemicals and drugs described above into rats, and waiting for 60 minutes, the rats were injected intraperitoneally with pentobarbital sodium (60 mg/kg body weight). Surgery was started about 10 minutes later. The lungs were instilled via a tracheal cannula with 10% formalin at a pressure of 20 cmH_2O for 5 minutes. After

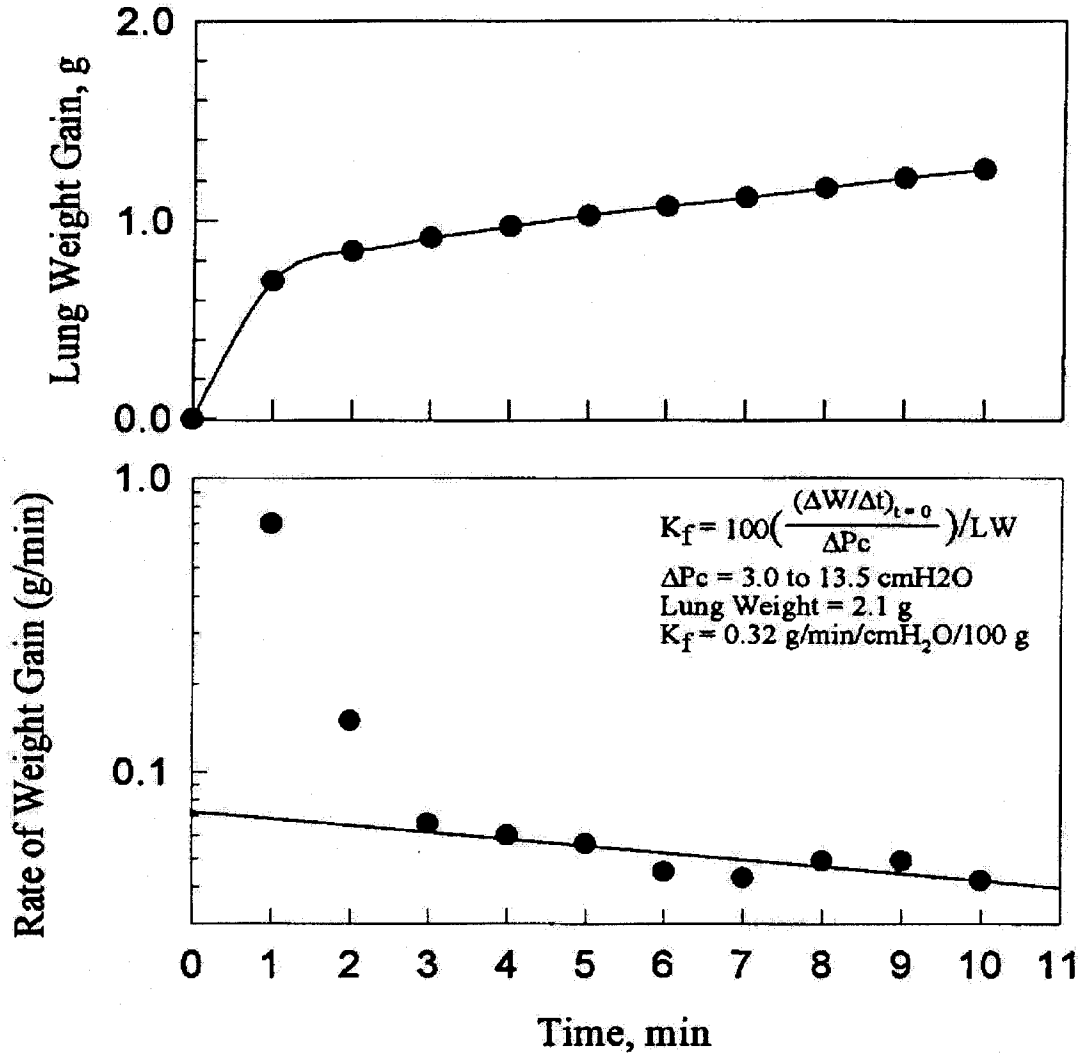


Figure 5. An example of estimated filtration coefficient (K_f) in isolated perfused rat lung by using a gravimetric method. The upper panel is the total weight gain including the fluid cumulated in the weight pan. The lower panel is a semilogarithmic plot in which the rate of weight gain ($\Delta w/\Delta t$) was plotting as a function of time. Extrapolated values of the rate of weight gain after 3rd min were divided by initial driving force (ΔPc, 10.5 cmH_2O) and normalized by 100 g lung weight to yield a K_f of 0.32 g/min/cmH_2O/100g lung weight (Adapted from Kunlun Huang [91])

instillation, heart and lungs were removed *en bloc* and were kept in 10% formalin for about 24 hours. A mid-horizontal slice from the left lung was processed for light microscopic examination. The sections were stained with hematoxylin and eosin. The total number of leucocytes was counted in ten randomly selected high power fields (380 x).

2.6 Experimental protocol for isolated perfused rat lung experiments (Figure 6)

Rat lungs were isolated and prepared for continuous recording of PAP, PVP and weight change as described above. Capillary permeability (K_f) was measured after an equilibration period of 20 minutes. The measurement was again made 40 minutes later. At the end of experiment, the volume of accumulated fluid in the lower weighing pan (total filtrate outflow) was measured. The lungs were then separated from the heart and the wet weight of the lungs was determined. Dried lung weight was measured after the lungs had been dehydrated in an oven at 50°C for 3 days.

2.7 Experimental protocol for histological studies

The protocol used for determination of the leucocyte content of the lungs is shown in Figure 7.

2.8 Drugs and Chemicals

Drugs and chemicals used in this study included: *E. coli* endotoxin (Lipopolysaccharide 0127:B8) from Difco Laboratories; bepafant (WEB 2170) from

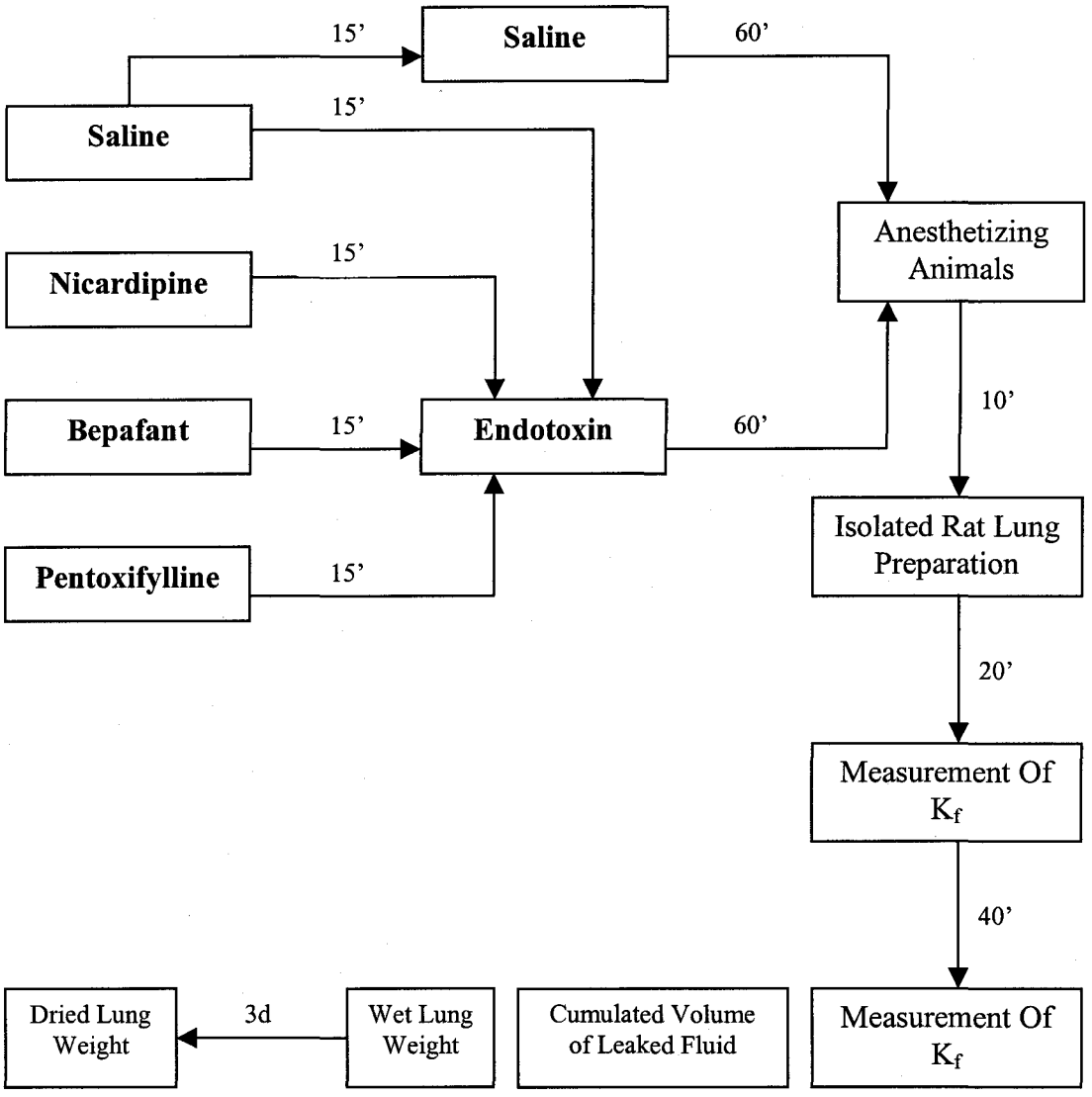


Figure 6. The procedure of perfused rat lung.

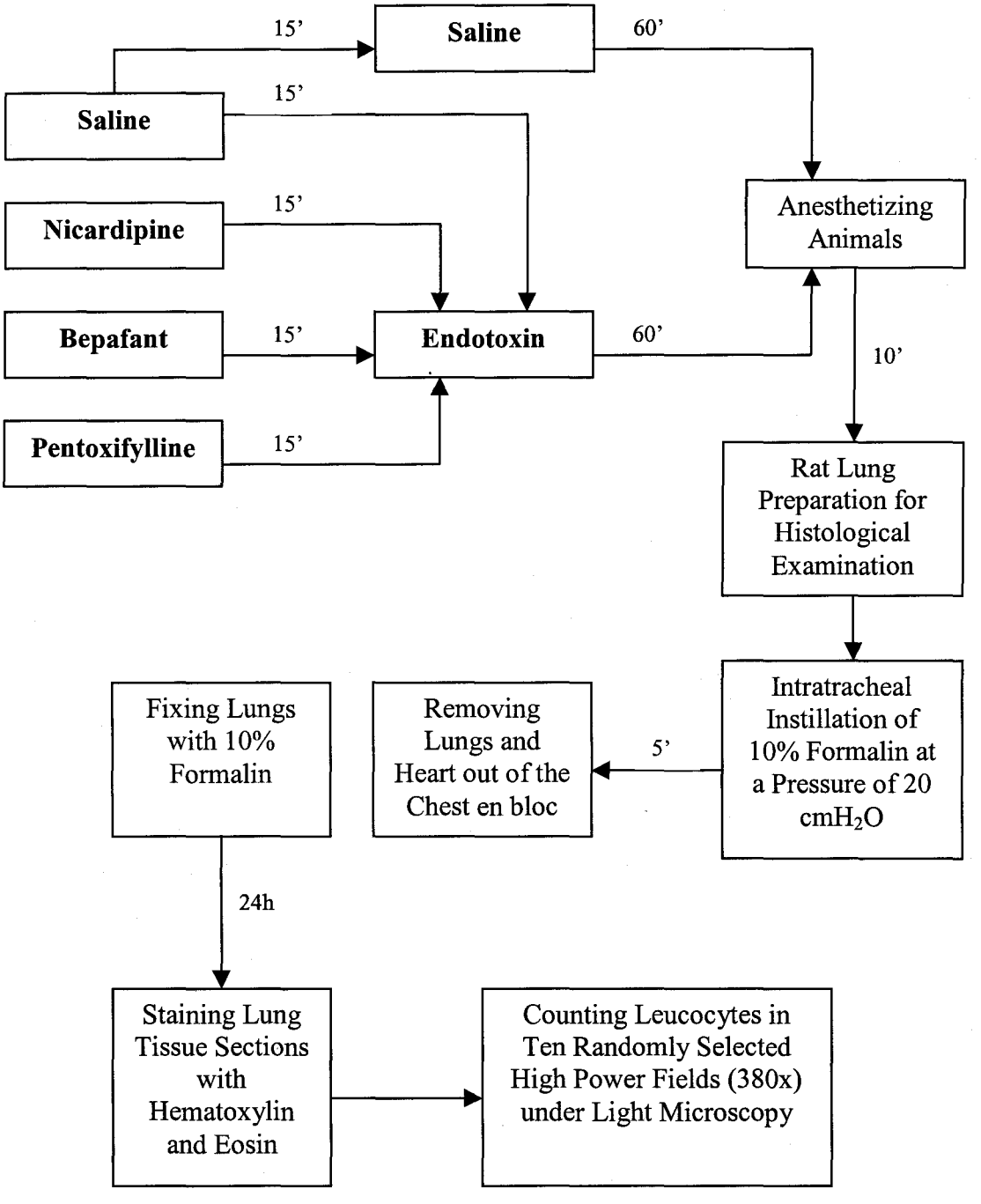


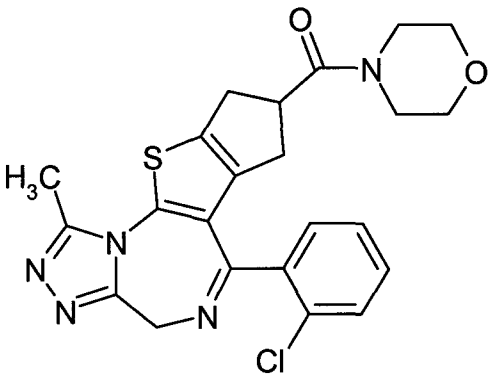
Figure 7. The procedure of histological examination.

Boerhinger Ingelheim KG; sodium heparin from ICN Biochemicals; nicardipine from Syntex Research; pentoxifylline from Hoechst-Russel Pharmaceuticals Inc.; and sodium pentobarbital from Abbott Laboratories. The chemical structures of bepafant, nicardipine and pentoxifylline are showed in Figure 8.

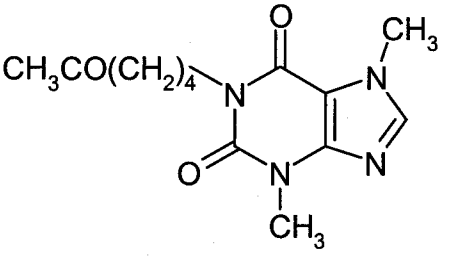
Lipopolysaccharide B (100 mg) was dissolved into normal saline to make a concentration of 10 mg/ml. Pentoxifylline and bepafant (WEB2170) were dissolved in normal saline at a concentration of 50 mg/ml and 1 mg/ml, respectively. Nicardipine was dissolved in a 50% aqueous-ethanol solution at a concentration of 1 mg/ml.

2.9 Data Analysis

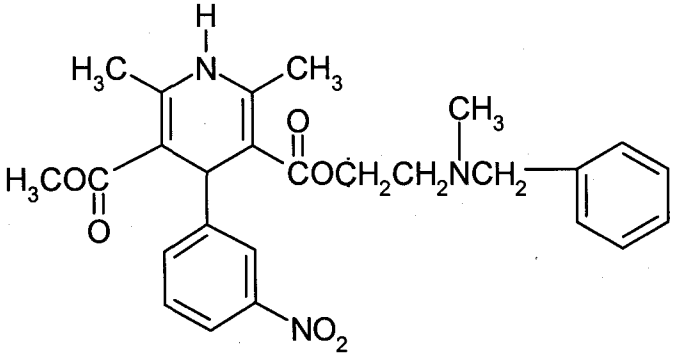
The results in the text, tables and figures were expressed as the mean ± standard error (S.E.). ANOVA with the Newman-Keuls Studentized range test for multi-group comparisons was used as appropriate. P values of less than 0.05 were considered significant.



Bepafant



Pentoxifylline



Nicardipine

Figure 8. Chemical Structures of Bepafant, Pentoxifylline and Nicardipine.

CHAPTER 3

RESULTS

3.1 Effects of endotoxin on pulmonary hemodynamics in rats anesthetized with pentobarbital

In these experiments, anesthetized rats were given ETX (10 mg/kg) i.v. One hour later, the lungs were removed and perfused at a constant rate with a buffered salt solution. Pulmonary arterial pressure, capillary pressure, total vascular resistance, arterial and venous segmental resistances and capillary permeability were all found to be significantly higher in rats treated with endotoxin than in control rats treated with saline. Lung water content, lung filtrate and lung weight were also higher in the endotoxin treated group than in the saline control group. These results are summarized in Table 4, 5 and Figures 9 to 16.

Table 4. Effects of saline and endotoxin on hemodynamic responses of the isolated rat lung.

Measurements	Time	Saline (10)	Endotoxin (11)
PAP (mmHg)	Baseline	8.81±1.27	10.76±0.99*
	40 Min	8.8±1.35	11.16±1.36*
Pc (mmHg)	Baseline	3.89±0.57	4.73±0.43*
	40 Min	3.87±0.59	4.91±0.60*
Rt (mmHg/ml/min)	Baseline	0.78±0.13	0.98±0.12*
	40 Min	0.77±0.13	1.02±0.12*
Ra (mmHg/ml/min)	Baseline	0.43±0.07	0.55±0.07*
	40 Min	0.43±0.07	0.57±0.069*
Rv (mmHg/ml/min)	Baseline	0.34±0.06	0.43±0.05*
	40 Min	0.34±0.06	0.45±0.05*
K_f ($g \cdot min^{-1} \cdot cmH_2O^{-1} \cdot 100^{-1}$)	Baseline	0.3387±0.08	0.8799±0.28*
	40 Min	0.4497±0.14	1.381±0.35*

PAP: pulmonary arterial pressure; Pc: capillary pressure; Rt: total vascular resistance; Ra: arterial segmental resistance; Rv: venous segmental resistance; K_f: filtration coefficient. * $p < 0.05$ compared with endotoxin group.

Table 5. Effect of endotoxin on lung weight, lung water content and filtrate after giving endotoxin in the isolated rat lung.

Measurements	Saline (10)	Endotoxin (11)
Lung Weight (g)	1.731±0.21	2.687±0.57*
Lung Water Content (%)	83.057±1.74	86.79±1.23*
Filtrate (ml)	0.87±0.52	3.573±1.88*

*, $p < 0.05$ as compared to Saline group

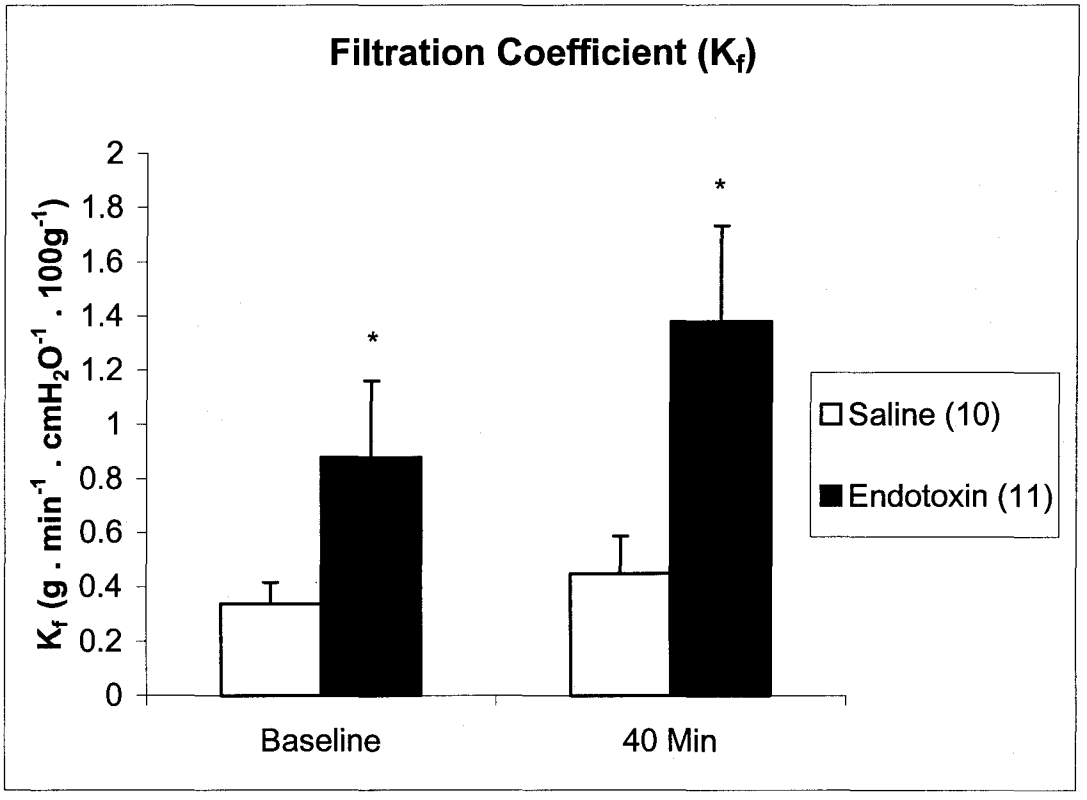


Figure 9. Effect of endotoxin on lung capillary permeability (K_f).

Saline and endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group.

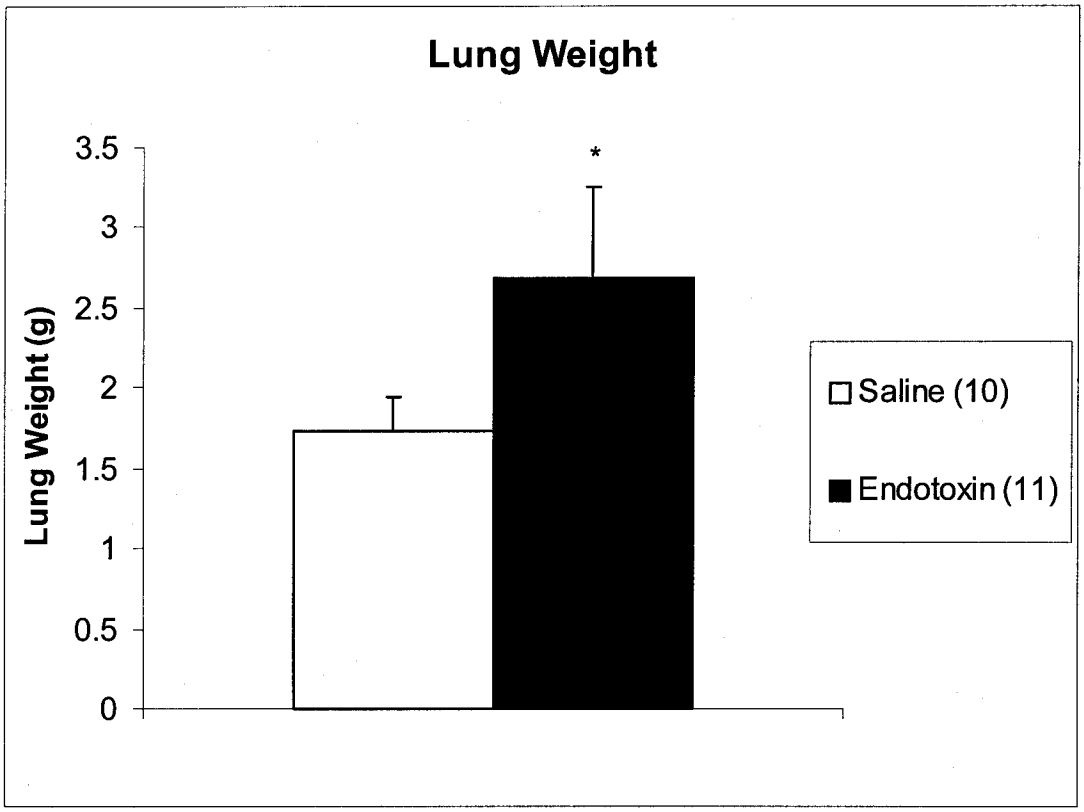


Figure 10. Effect of endotoxin on lung weight.

Values represent wet lung weight at the end of the experiment. Saline and endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin was administered. Number of animals is in parenthesis.
* Significantly different from value of the saline group.

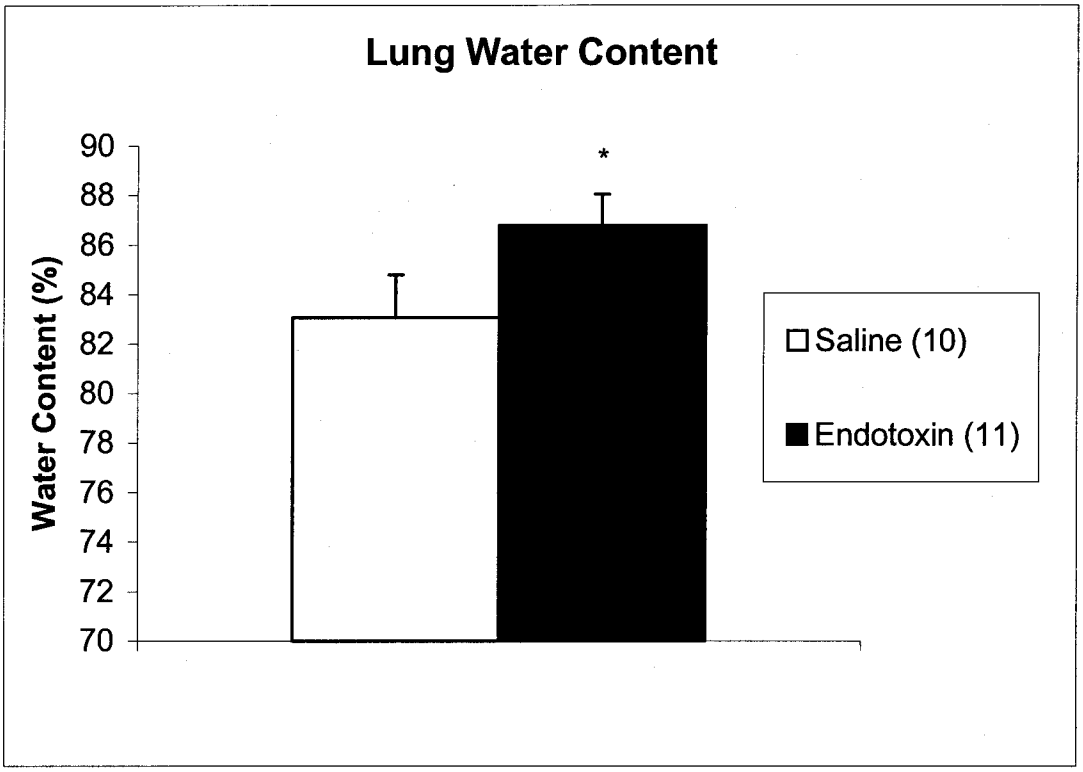


Figure 11. Effect of endotoxin on lung water content.

Values represent lung water content as percent of lung wet weight at the end of the experiment. Saline and Endotoxin were given i.v.. Lungs were isolated and perfused one hour after Endotoxin was administered. Number of animals is in parenthesis.
* Significantly different from value of the saline group.

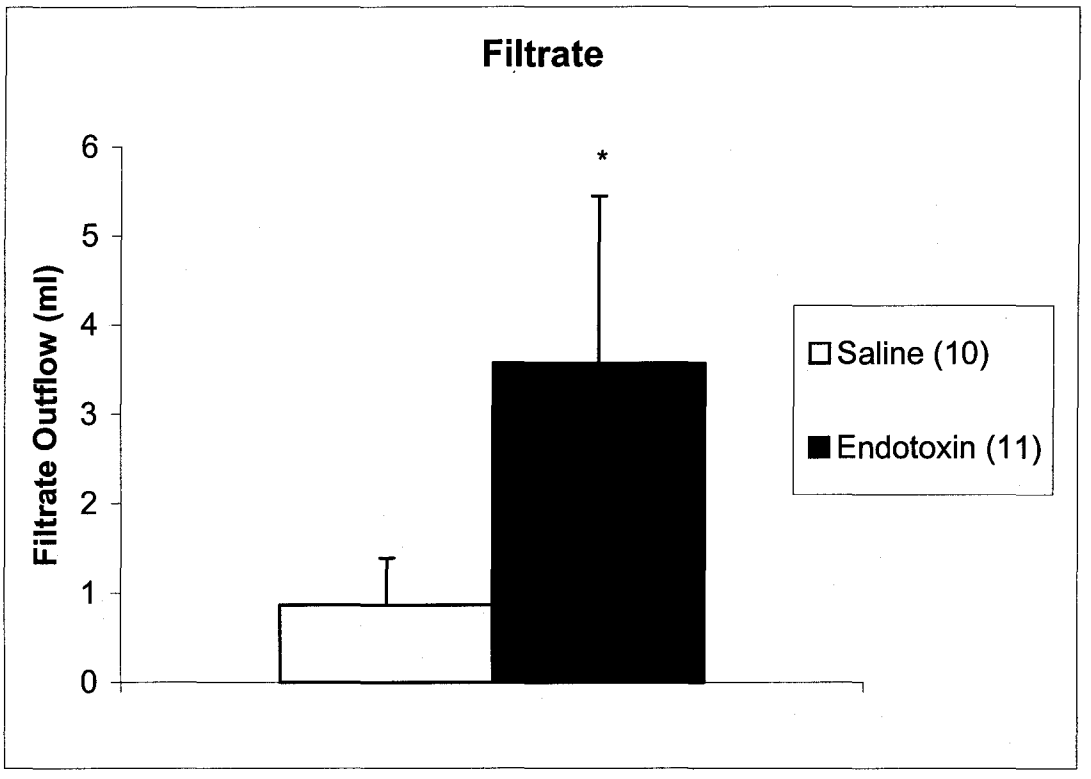


Figure 12. Effect of endotoxin on outflow of lung filtrate.

Saline and endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin was administered. Filtrate outflow volume is the total outflow recorded at the end of the experiment. Number of animals is in parenthesis. * Significantly different from value of the saline group.

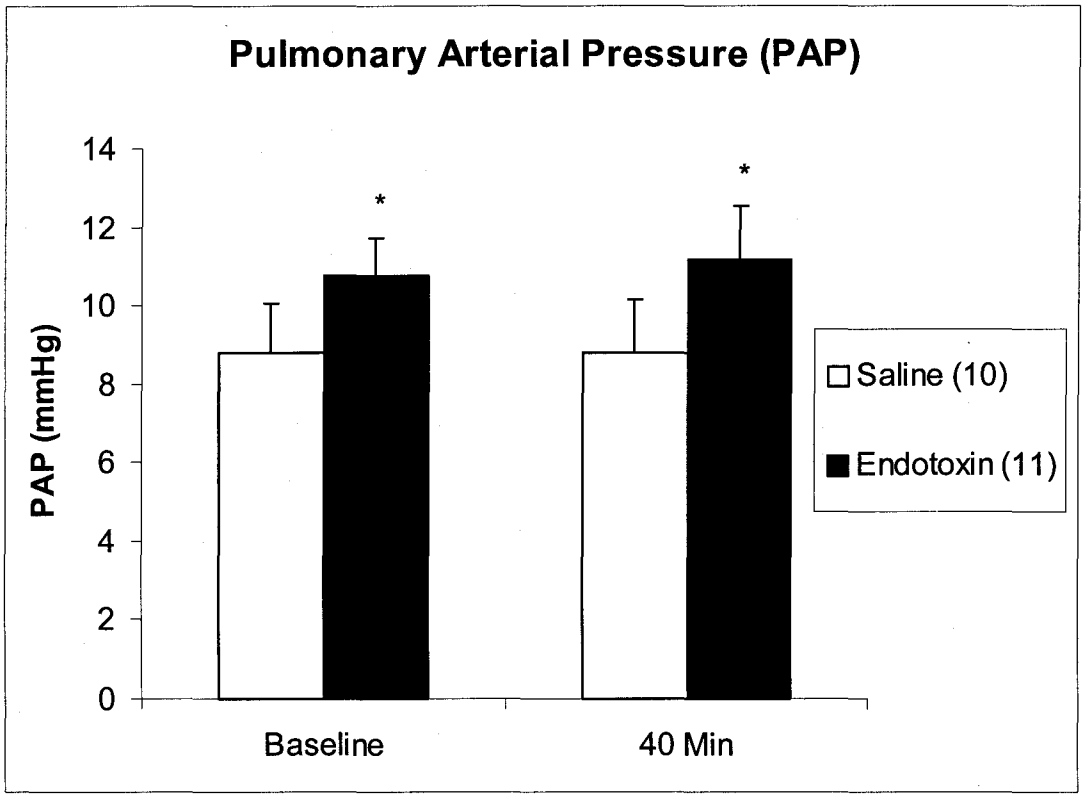


Figure 13. Effect of endotoxin on pulmonary arterial pressure.

Saline and endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin was administered. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals in parenthesis. * Significantly different from value of the saline group.

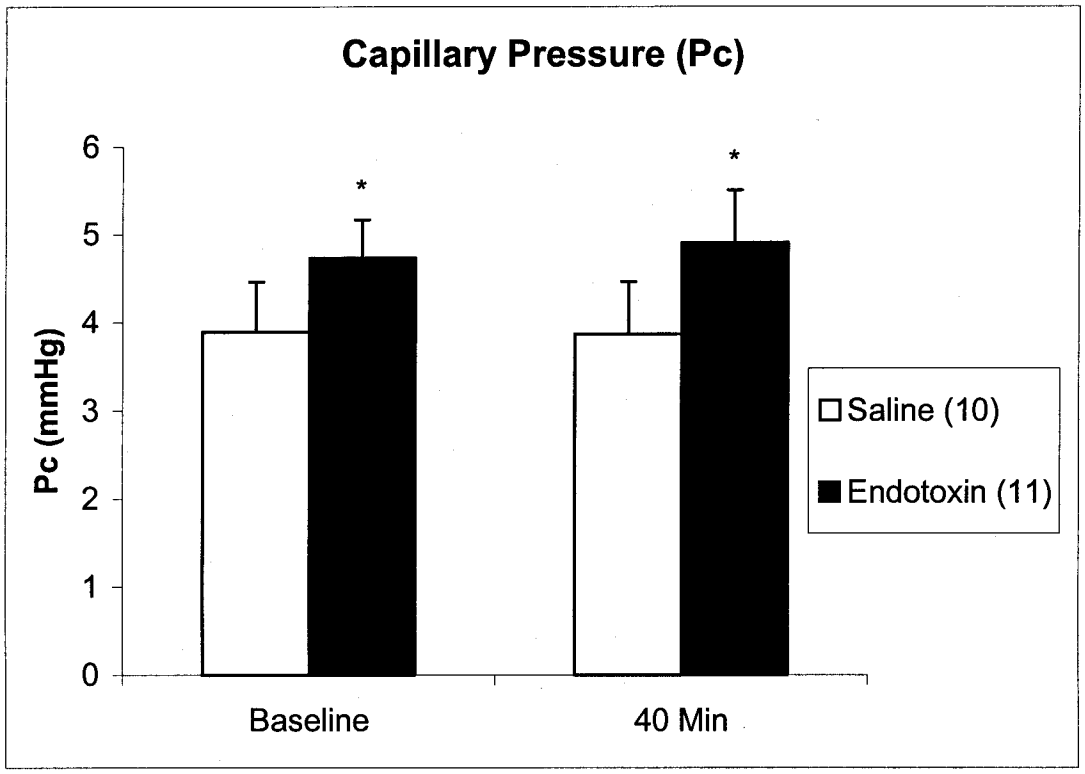


Figure 14. Effect of endotoxin on pulmonary capillary pressure.

Saline and Endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin was administered. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group.

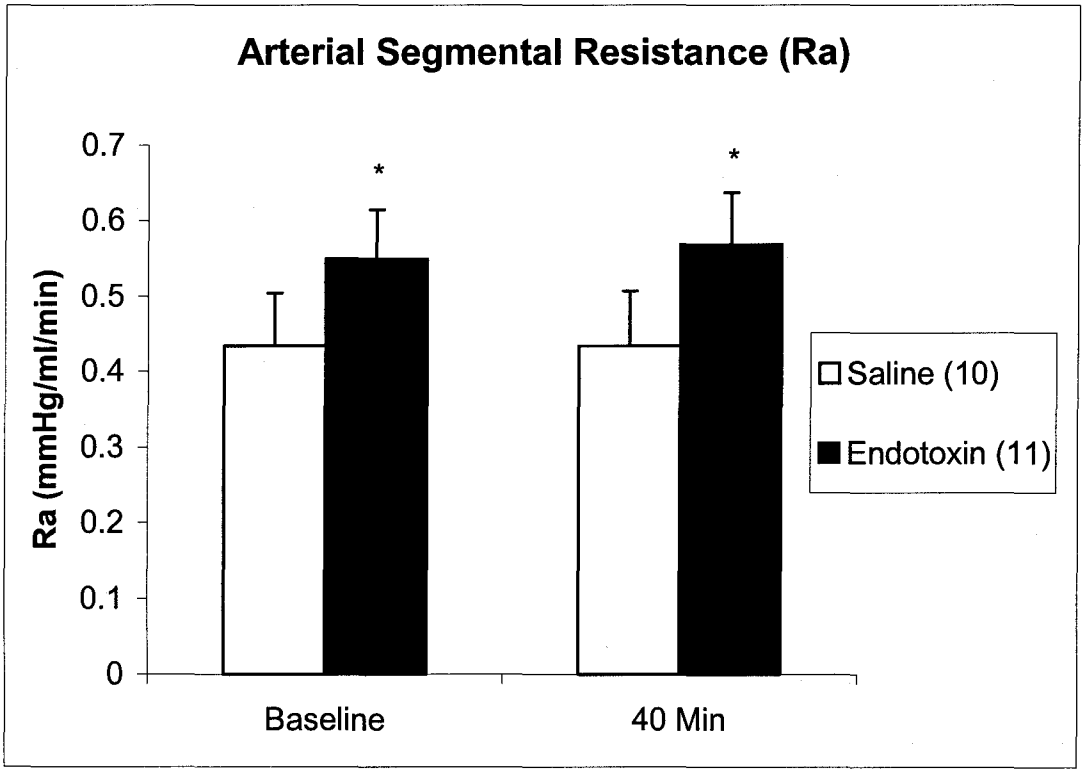


Figure 15. Effect of endotoxin on pulmonary arterial segmental resistance.

Saline and endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin was administered. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group.

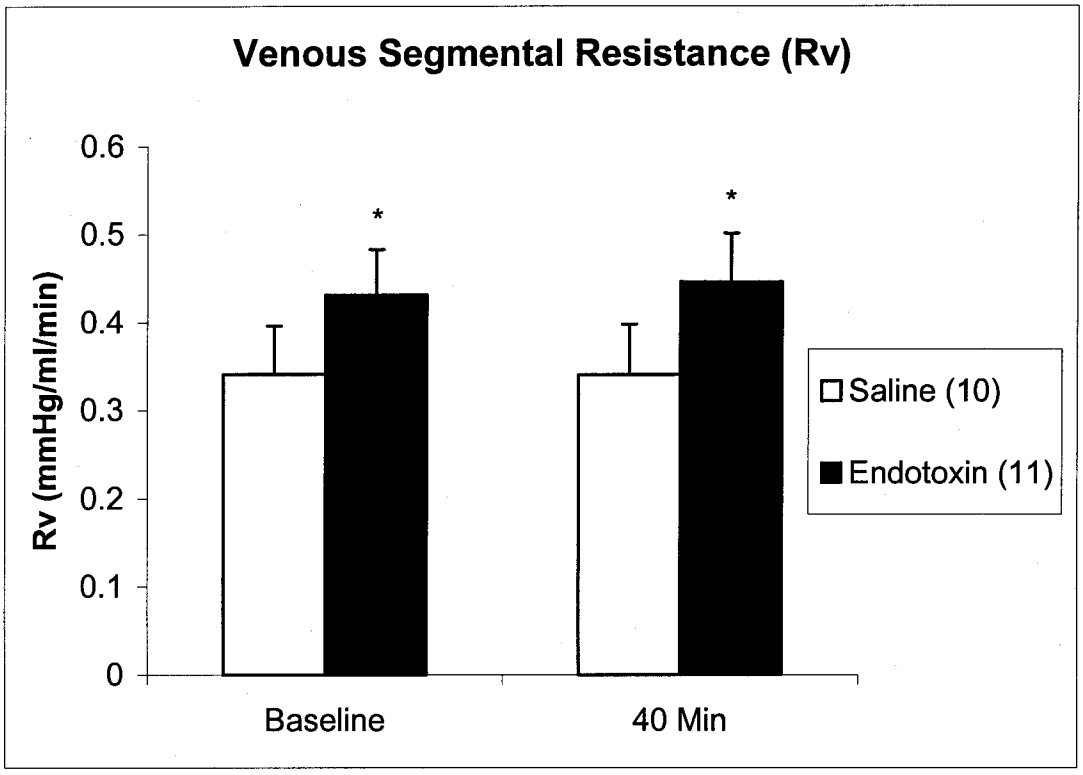


Figure 16. Effect of endotoxin on pulmonary venous segmental resistance.

Saline and endotoxin were given i.v.. Lungs were isolated and perfused one hour after endotoxin was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group.

3.2 Effects of nicardipine on rat lung injury induced by endotoxin

In the nicardipine group, the drug (1 mg/kg) was given 15 minutes before injecting endotoxin (10 mg/kg, iv). Nicardipine significantly reduced the endotoxin-induced increases in capillary permeability (K_f) and the outflow of lung filtrate but did not significantly affect the endotoxin-induced increases in pulmonary arterial pressure, capillary pressure, total vascular resistance and arterial and venous segmental resistances. Nicarpidine also did not affect the endotoxin-induced increases in lung weight and lung water content. These results are summarized in Table 6, Table 7 and Figures 17 to 24.

Table 6. Effects of pretreatment with nicardipine on the hemodynamic changes induced by endotoxin in the isolated rat lung.

Measurements	Time	Endotoxin (11)	Nicardipine (7)
PAP (mmHg)	Baseline	10.76±0.99	9.56±0.63
	40 Min	11.16±1.36	9.90±0.45
Pc (mmHg)	Baseline	4.73±0.43	4.21±0.28
	40 Min	4.91±0.60	4.36±0.20
Rt (mmHg/ml/min)	Baseline	0.98±0.12	0.94±0.06
	40 Min	1.02±0.12	0.98±0.05
Ra (mmHg/ml/min)	Baseline	0.55±0.07	0.53±0.03
	40 Min	0.57±0.069	0.55±0.03
Rv (mmHg/ml/min)	Baseline	0.43±0.05	0.41±0.02
	40 Min	0.45±0.05	0.43±0.02
K_f ($g \cdot min^{-1} \cdot cmH_2O^{-1} \cdot 100g^{-1}$)	Baseline	0.8799±0.28	0.4926±0.10*
	40 Min	1.381±0.35	0.6573±0.19*

PAP: pulmonary arterial pressure; Pc: capillary pressure; Rt: total vascular resistance; Ra: arterial segmental resistance; Rv: venous segmental resistance; K_f: filtration coefficient. * $p < 0.05$ compared with endotoxin group.

Table 7. Effects of pretreatment with nicardipine on the changes in lung weight, lung water content and filtrate outflow induced by endotoxin in the isolated rat lungs.

Measurements	Endotoxin (11)	Nicardipine (7)
Lung Weight (g)	2.687±0.57	2.188±0.43
Lung Water Content (%)	86.79±1.23	85.627±2.13
Filtrate (ml)	3.573±1.88	0.9857±0.23*

*, $p < 0.05$ as compared to Endotoxin group

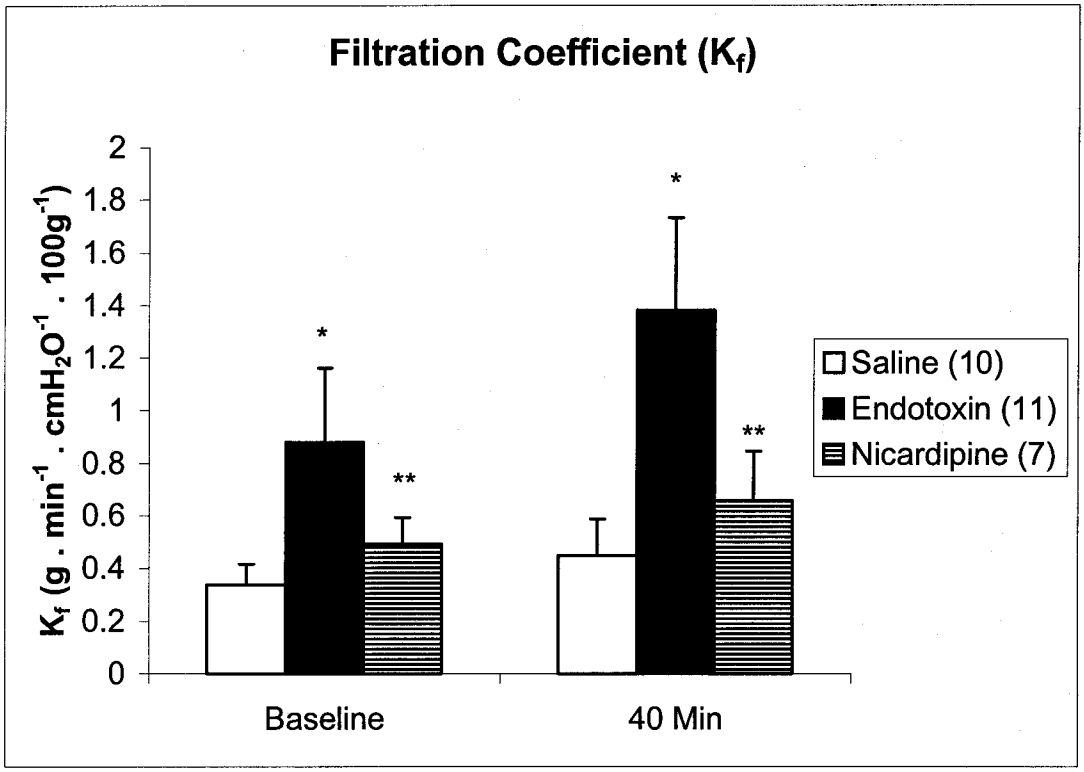


Figure 17. Effect of nicardipine on endotoxin-induced increase in lung capillary permeability (K_f).

Nicardipine (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

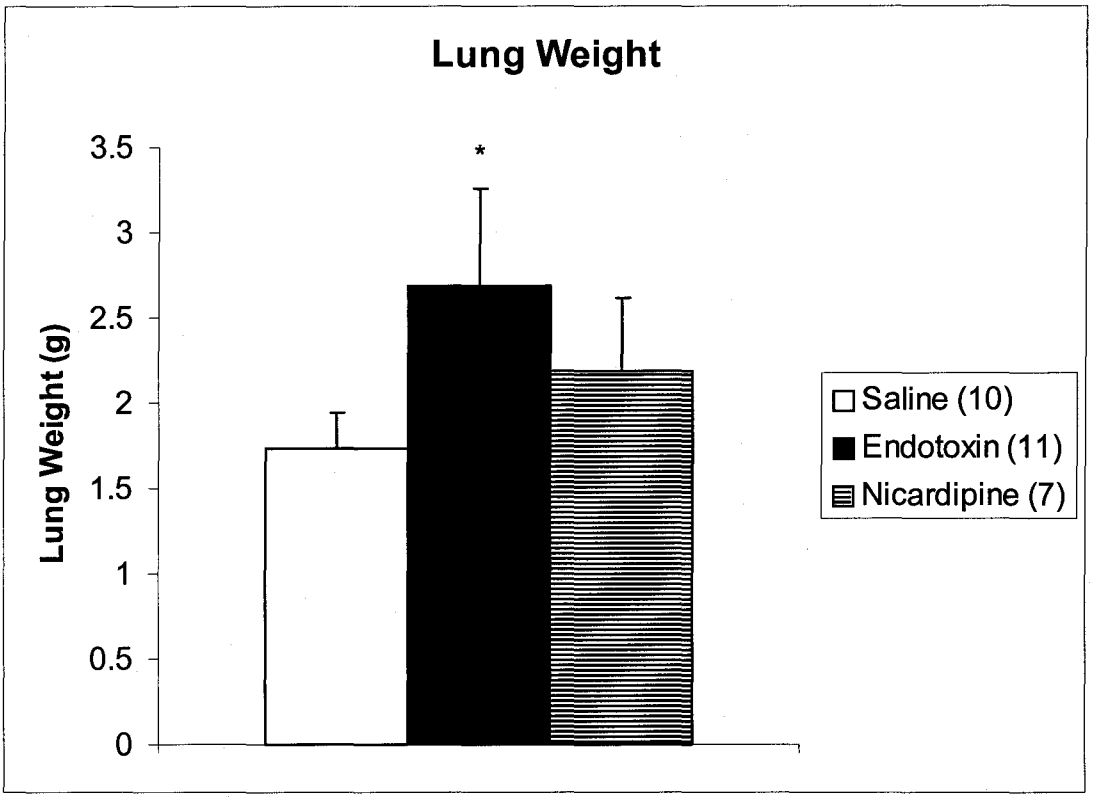


Figure 18. Effect of nicardipine on endotoxin-induced increase in lung weight.

Values represent wet lung weight at the end of the experiment. Nicardipine (1 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

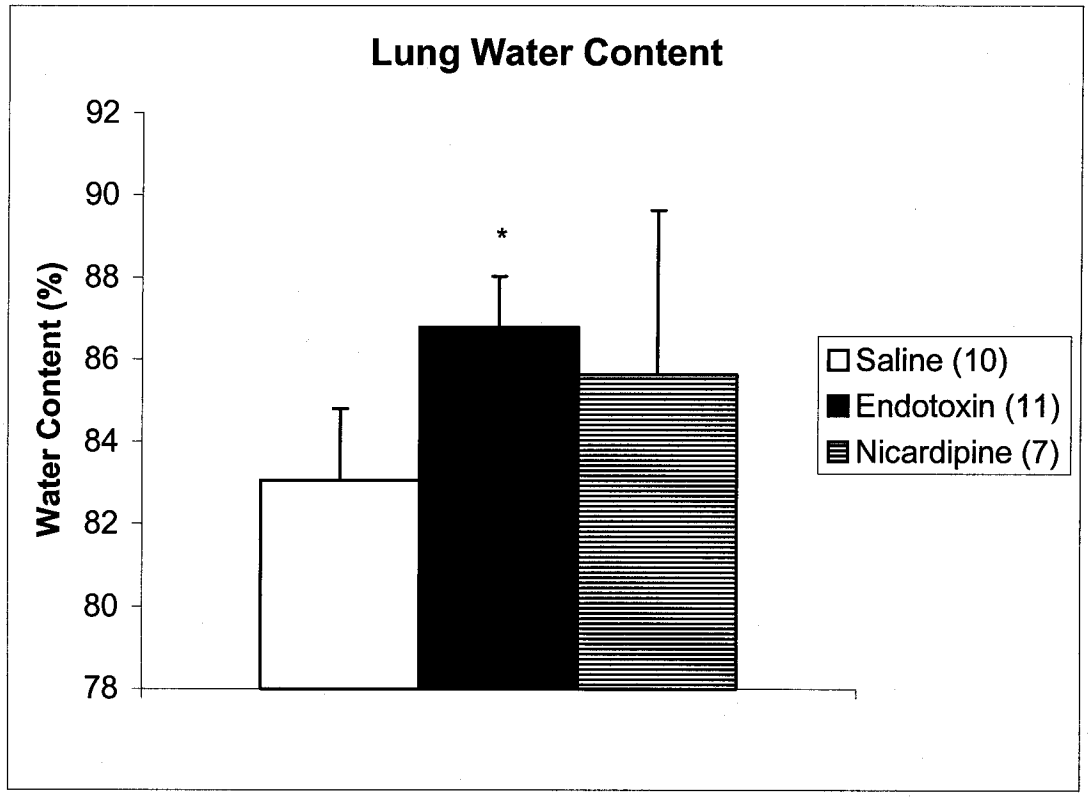


Figure 19. Effect of nicardipine on endotoxin-induced increase in lung water content.

Values represent lung water content as percent of lung wet weight at the end of the experiment. Nicardipine (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Number of animals is in parenthesis. Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

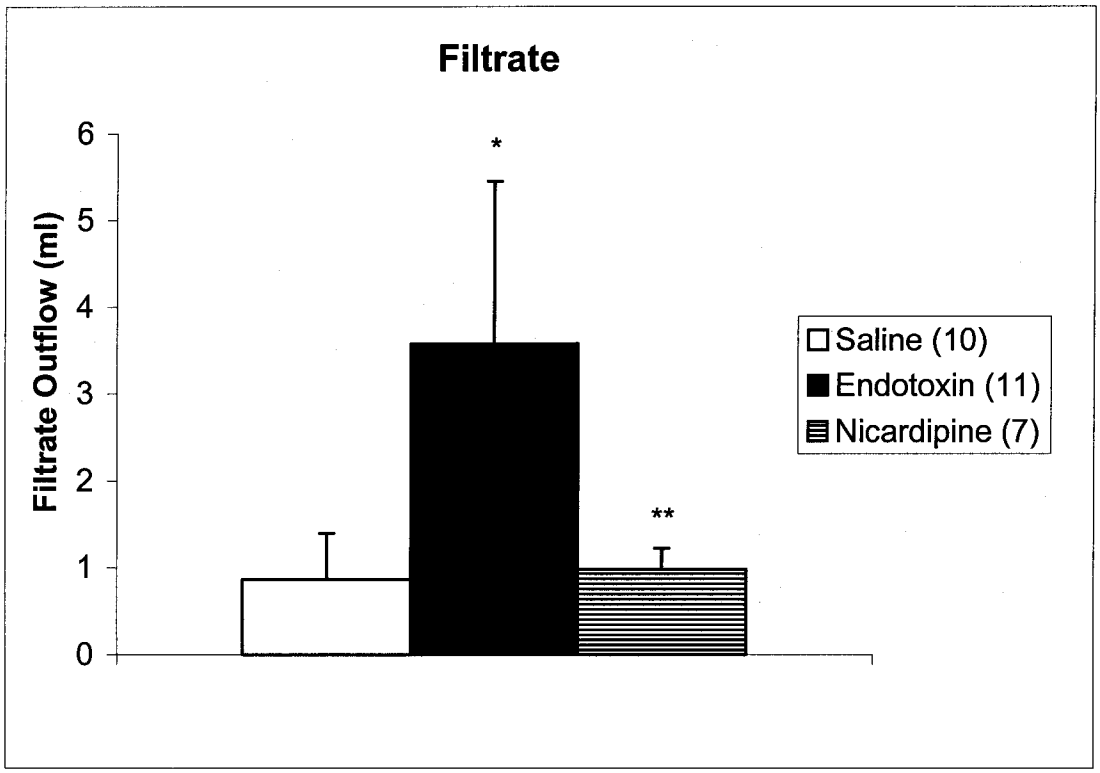


Figure 20. Effect of nicardipine on endotoxin-induced increase in outflow of lung filtrate.

Nicardipine (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Filtrate outflow volume is the total outflow recorded at the end of the experiment. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

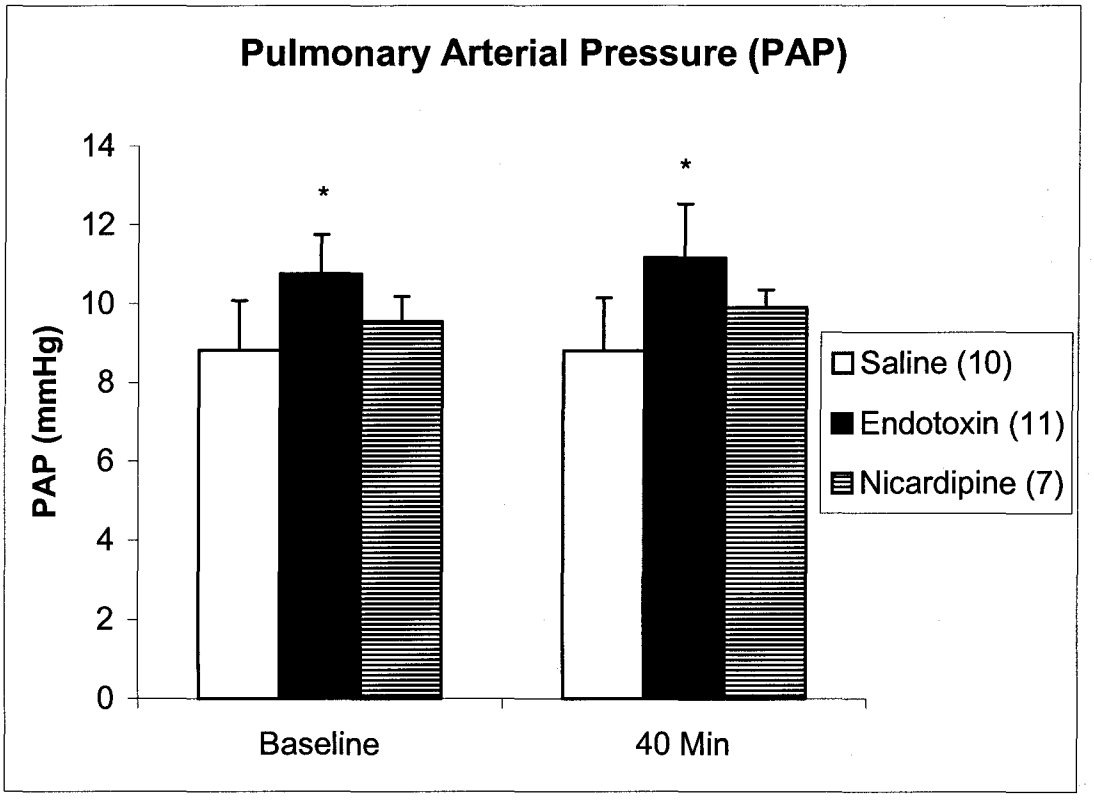


Figure 21. Effect of nicardipine on endotoxin-induced increase in pulmonary arterial pressure.

Nicardipine (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

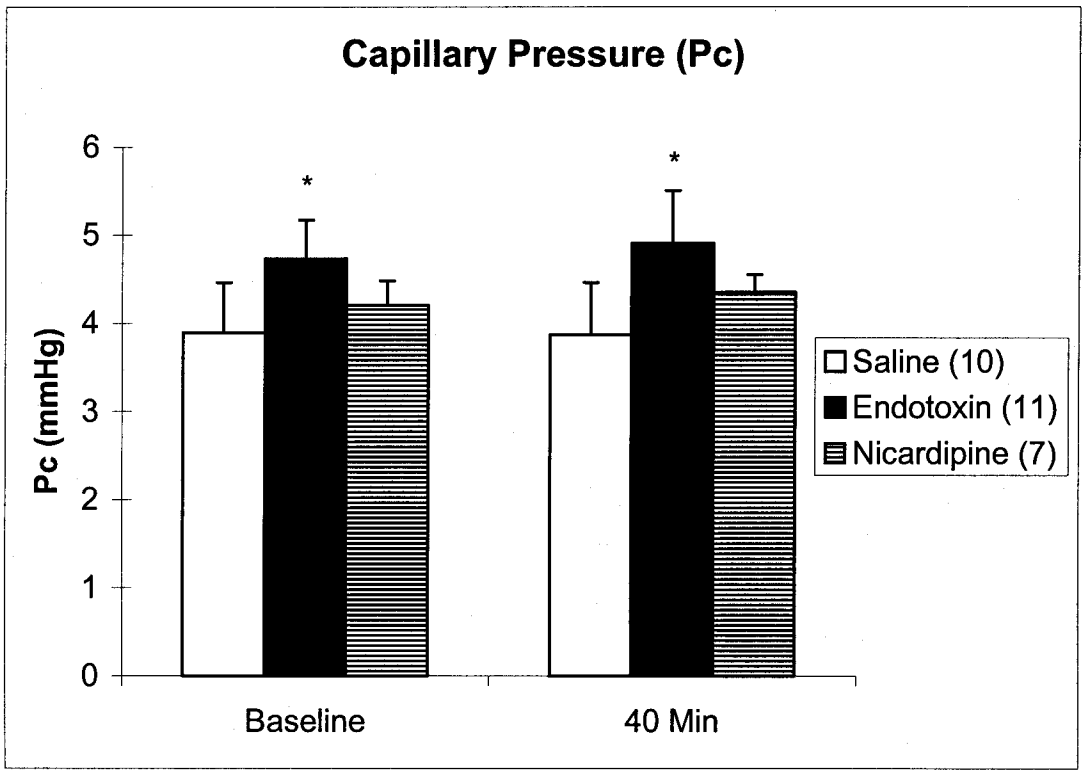


Figure 22. Effect of nicardipine on endotoxin-induced increase in pulmonary capillary pressure.

Nicardipine (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

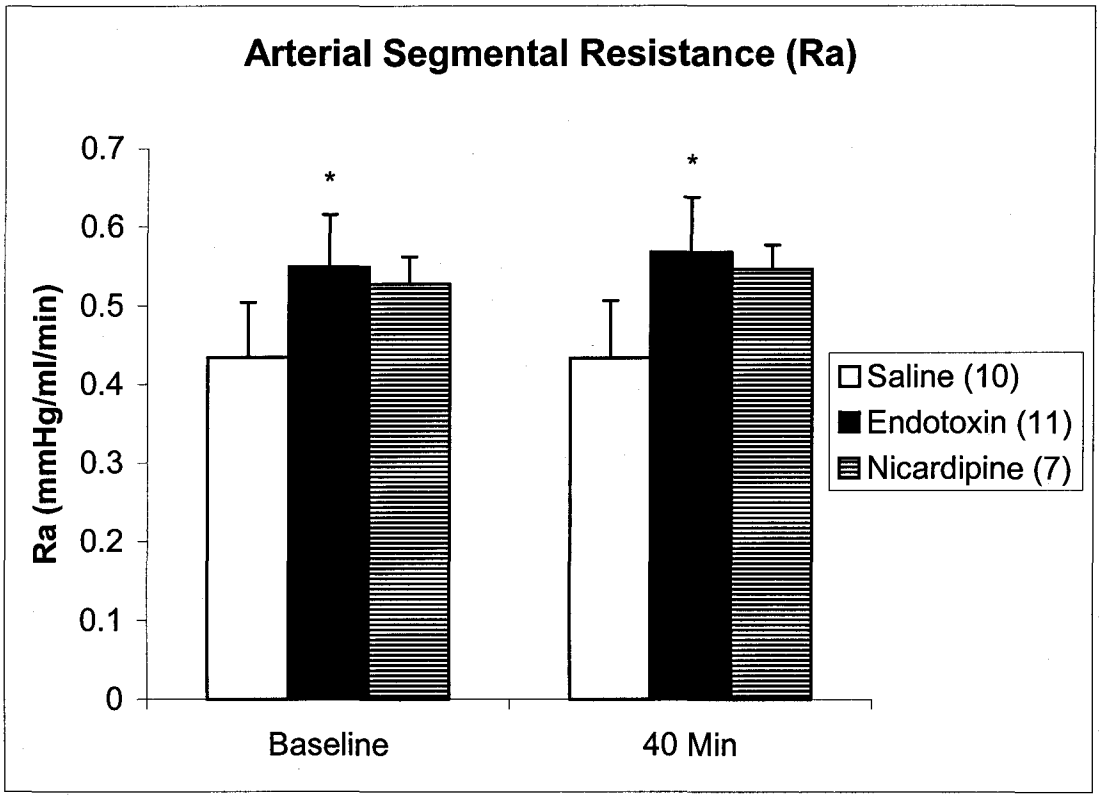


Figure 23. Effect of nicardipine on endotoxin-induced increase in pulmonary arterial segmental resistance.

Nicardipine (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

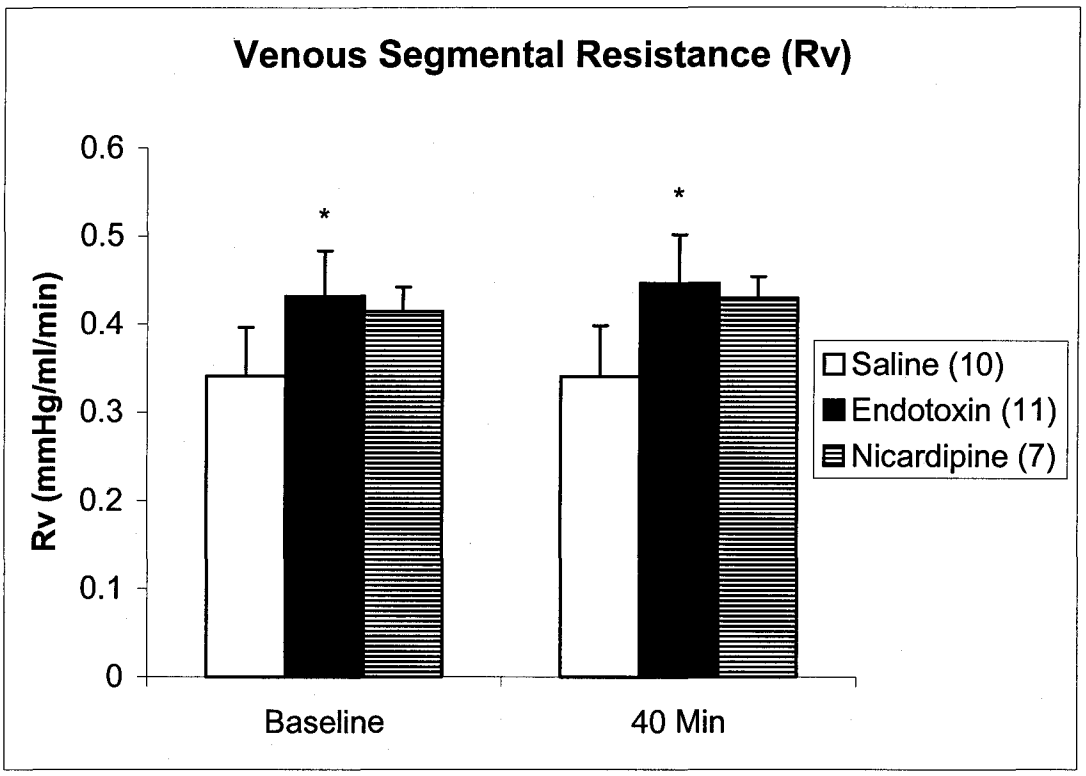


Figure 24. Effect of nicardipine on endotoxin-induced increase in pulmonary venous segmental resistance.

Nicardipine (1 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

3.3 Effects of bepafant on rat lung injury induced by endotoxin

In these experiments, bepafant (1 mg/kg, iv) was given 15 minutes before injecting endotoxin (10 mg/kg, iv). Compared to the group given endotoxin alone, bepafant pretreatment significantly reduced the endotoxin-induced increases in capillary permeability (K_f) and the outflow of lung filtrate. However, the drug failed to significantly affect the other endotoxin-induced changes, including the increases in pulmonary arterial pressure, capillary pressure, total vascular resistance and arterial and venous segmental resistances as well as the increases in lung weight and lung water content. Table 8, Table 9 and Figures 25 to 32 summarize the parameters obtained from the bepafant group as compared to the endotoxin control group.

Table 8. Effects of pretreatment with bepafant on the hemodynamic changes induced by endotoxin in the isolated rat lung.

Measurements	Time	Endotoxin (11)	Bepafant (7)
PAP (mmHg)	Baseline	10.76±0.99	9.79±0.99
	40 Min	11.16±1.36	10.29±0.70
Pc (mmHg)	Baseline	4.73±0.43	4.36±0.52
	40 Min	4.91±0.60	4.53±0.31
Rt (mmHg/ml/min)	Baseline	0.98±0.12	0.91±0.10
	40 Min	1.02±0.12	0.95±0.04
Ra (mmHg/ml/min)	Baseline	0.55±0.07	0.51±0.06
	40 Min	0.57±0.069	0.53±0.023
Rv (mmHg/ml/min)	Baseline	0.43±0.05	0.40±0.04
	40 Min	0.45±0.05	0.42±0.02
K_f ($g \cdot min^{-1} \cdot cmH_2O^{-1} \cdot 100g^{-1}$)	Baseline	0.8799±0.28	0.4889±0.19*
	40 Min	1.381±0.35	0.8581±0.33*

PAP: pulmonary arterial pressure; Pc: capillary pressure; Rt: total vascular resistance; Ra: arterial segmental resistance; Rv: venous segmental resistance: K_f: filtration coefficient. * $p < 0.05$ compared with endotoxin group.

Table 9. Effects of pretreatment with bepafant on the changes in lung weight, lung water content and filtrate outflow induced by endotoxin in the isolated rat lung.

Measurements	Endotoxin (11)	Bepafant (7)
Lung Weight (g)	2.687±0.57	2.558±0.60
Lung Water Content (%)	86.79±1.23	86.56±1.44
Filtrate (ml)	3.573±1.88	1.571±0.94*

*, $p < 0.05$ as compared to Endotoxin group

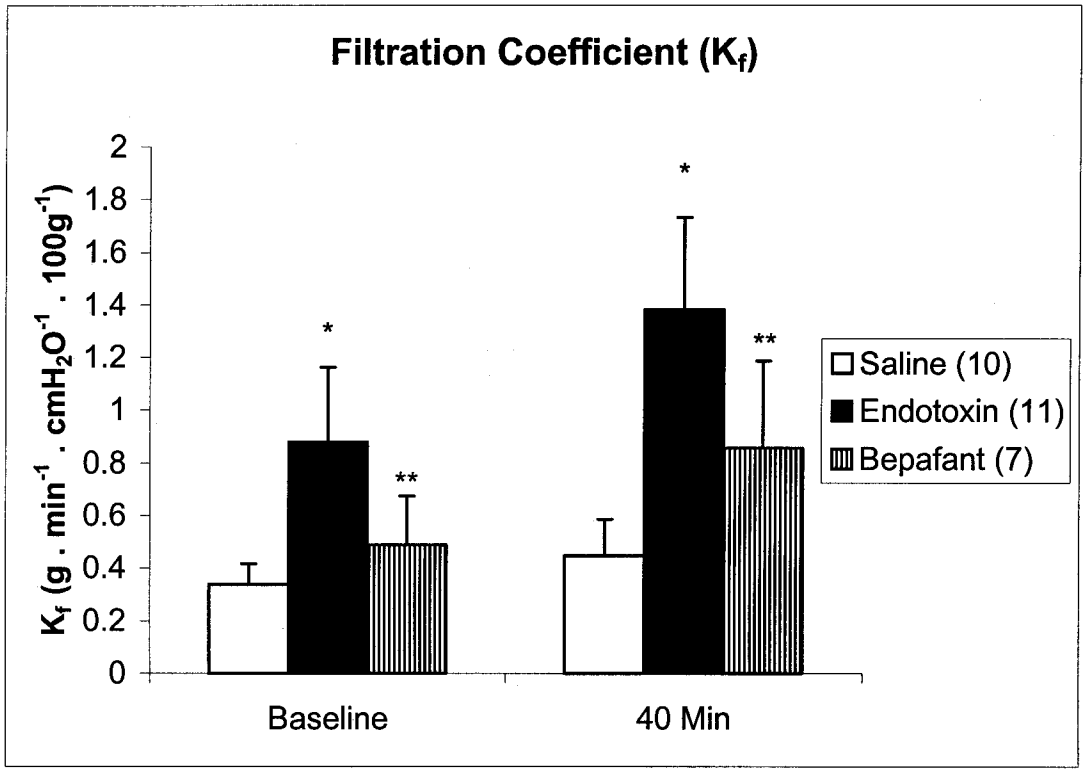


Figure 25. Effect of bepafant on endotoxin-induced increase in lung capillary permeability (K_f).

Bepafant (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

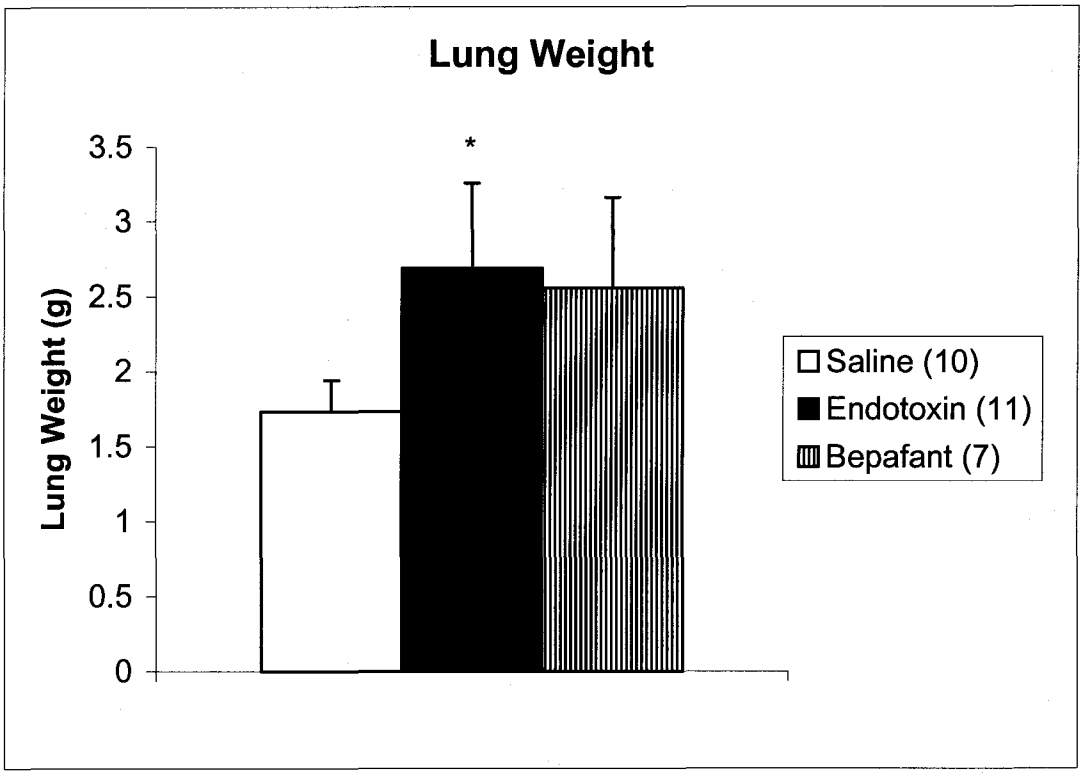


Figure 26. Effect of bepafant on endotoxin-induced increase in lung weight.

Values represent wet lung weight at the end of the experiment. Bepafant (1 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

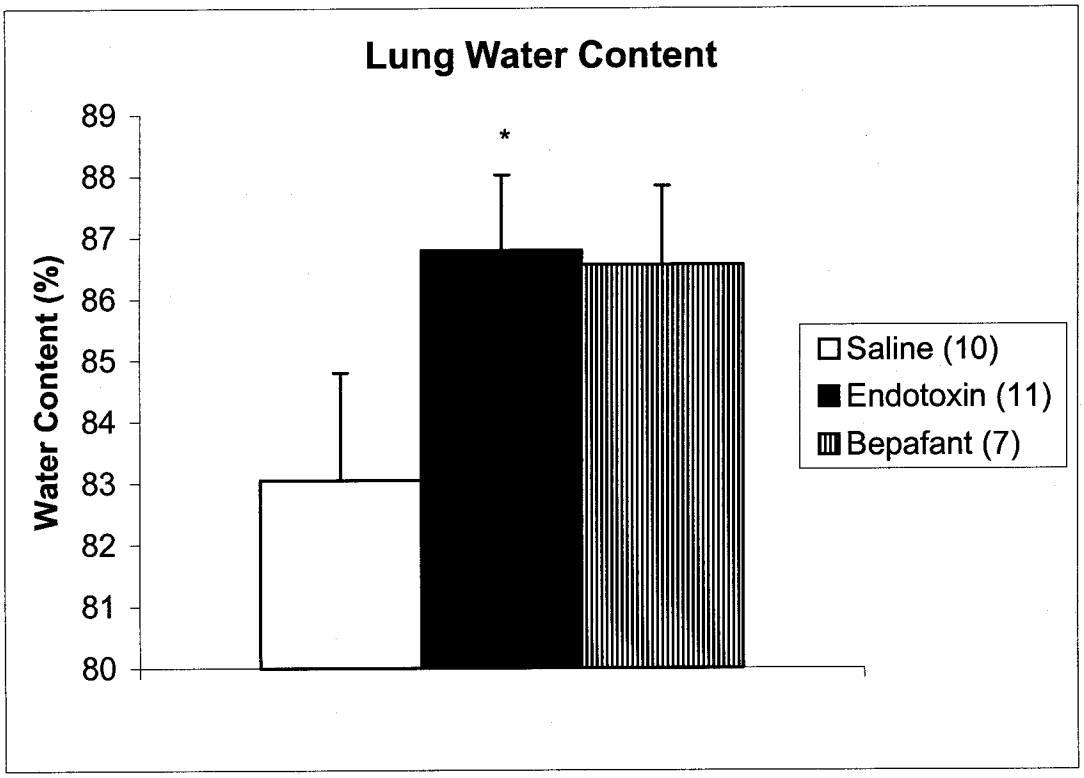


Figure 27. Effect of bepafant on endotoxin-induced increase in lung water content.

Values represent lung water content as percent of lung wet weight at the end of the experiment. Bepafant (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Number of animals is in parenthesis. Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

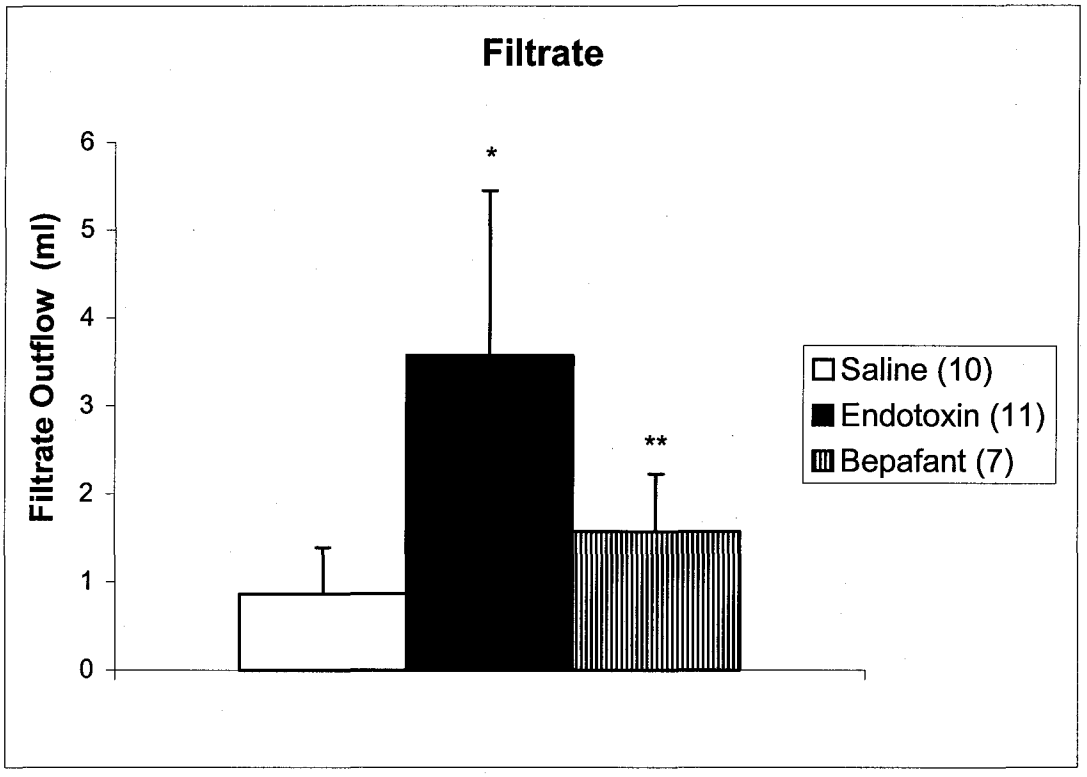


Figure 28. Effect of bepafant on endotoxin-induced increase in outflow of lung filtrate.

Bepafant (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Filtrate outflow volume is the total outflow recorded at the end of the experiment. Number of animals is in parenthesis. * Significant different from value of the saline group. ** Significantly different from value of the endotoxin group.

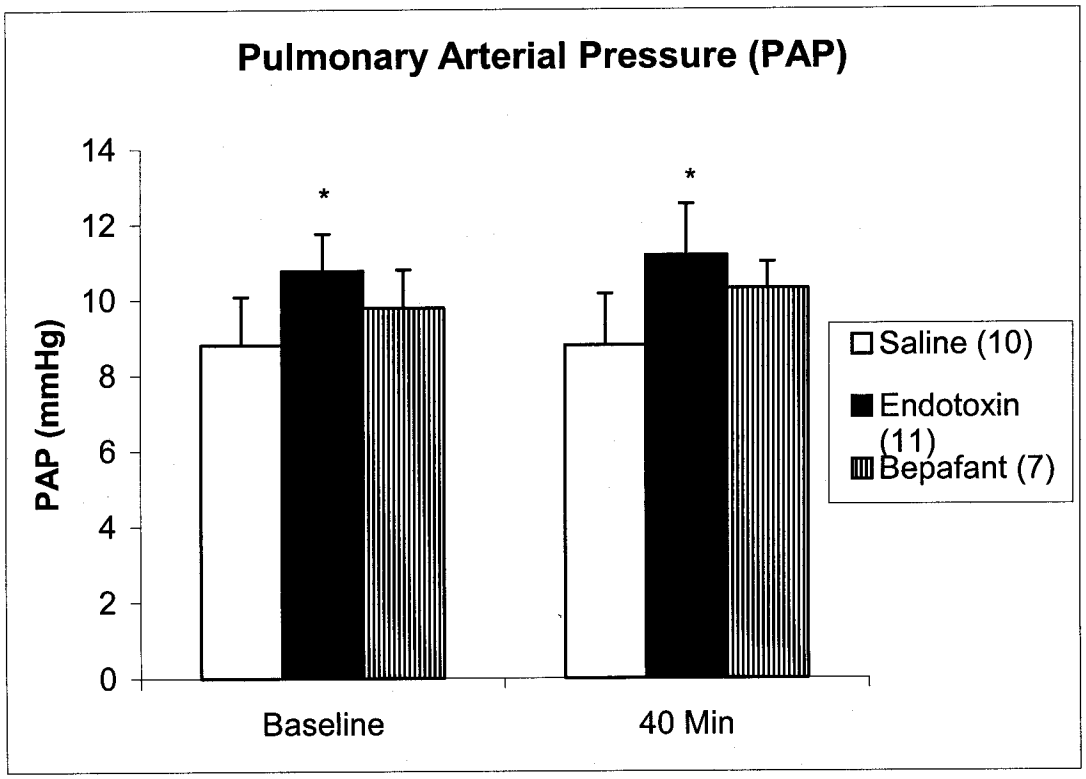


Figure 29. Effect of bepafant on endotoxin-induced increase in pulmonary arterial pressure.

Bepafant (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

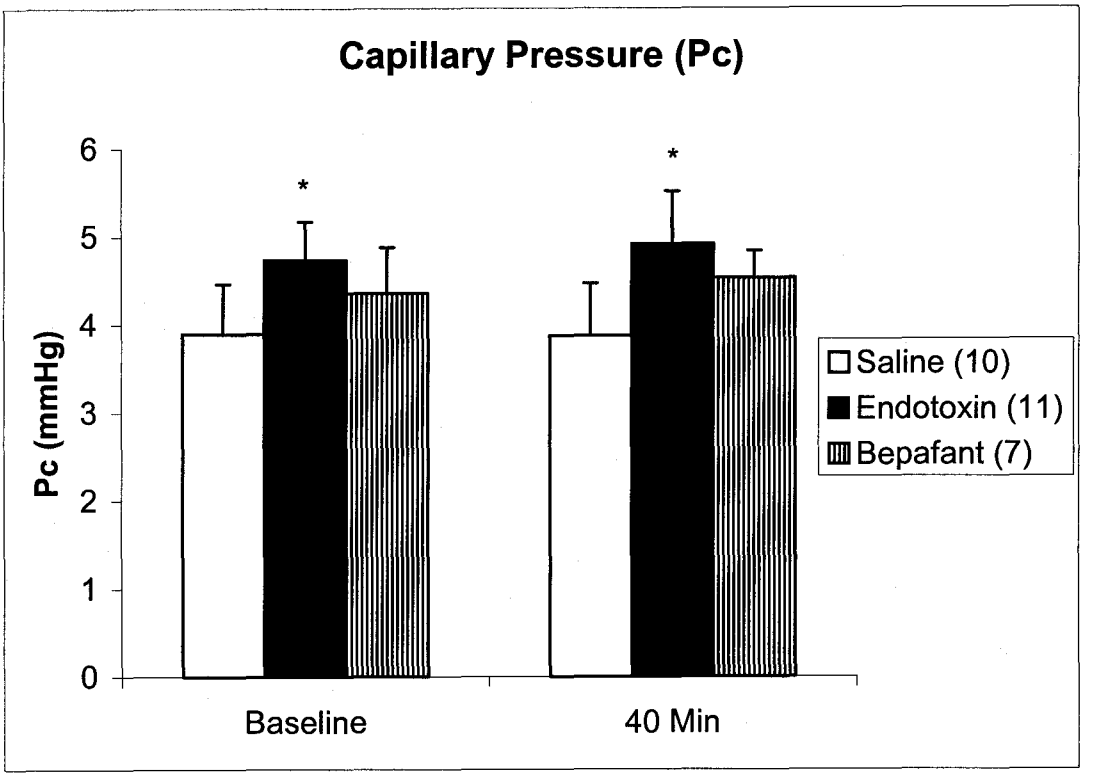


Figure 30. Effect of bepafant on endotoxin-induced increase in pulmonary capillary pressure.

Bepafant (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

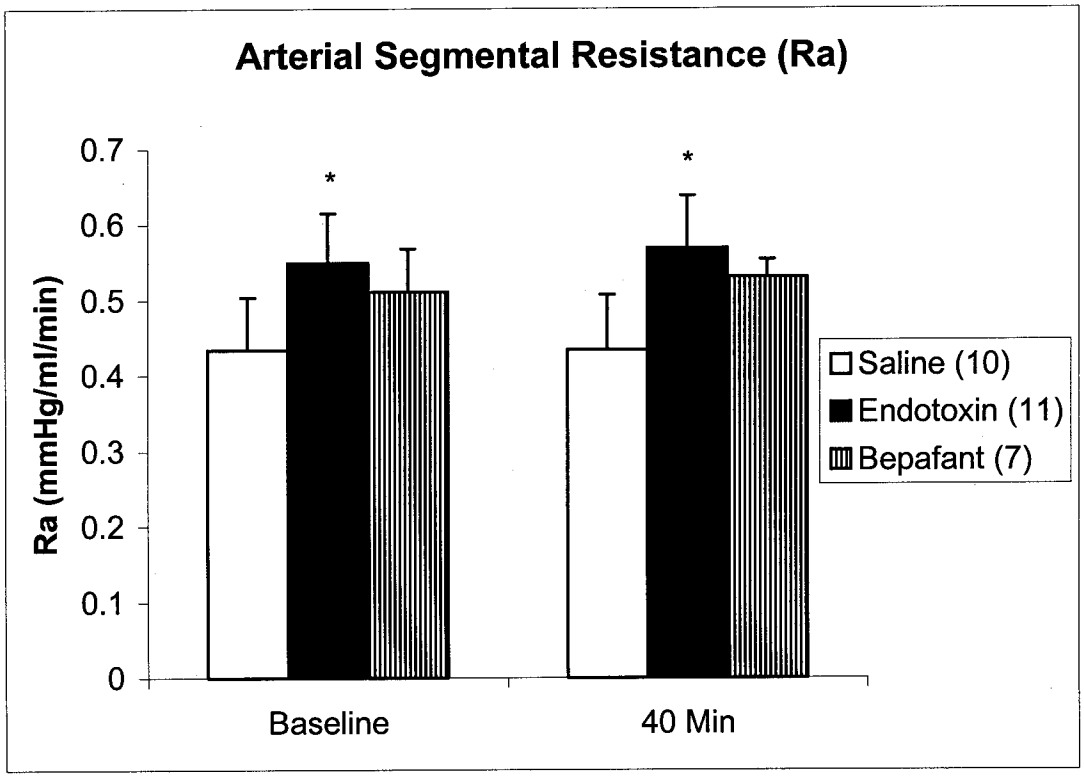


Figure 31. Effect of bepafant on endotoxin-induced increase in pulmonary arterial segmental resistance.

Bepafant (1mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

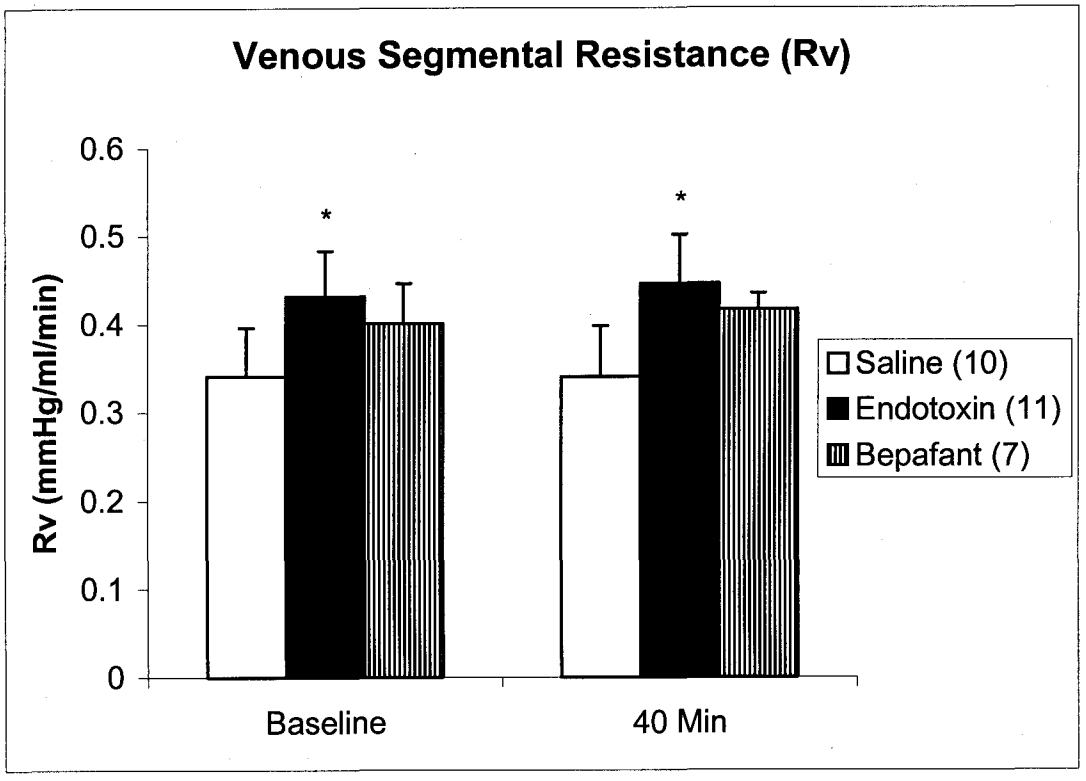


Figure 32. Effect of bepafant on endotoxin-induced increase in pulmonary venous segmental resistance.

Bepafant (1 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

3.4 Effects of pentoxifylline on rat lung injury induced by endotoxin

In these experiments, rats were pretreated with pentoxifylline (50 mg/kg, iv), 15 minutes before injecting endotoxin (10 mg/kg, iv). Pentoxifylline significantly reduced the endotoxin-induced increases in capillary permeability (K_f) and the outflow of lung filtrate. The drug also significantly reduced the endotoxin induced increases in pulmonary arterial pressure, capillary pressure, total vascular resistance, and arterial and venous segmental resistances. The drug, however, failed to affect the endotoxin-induced increases in lung weight and lung water content. The results are shown in Table 10, Table 11 and Figure 33 to 40.

Table 10. Effects of pretreatment with pentoxifylline on hemodynamic changes induced by endotoxin in the isolated rat lung

Measurements	Time	Endotoxin (11)	Pentoxifylline (7)
PAP (mmHg)	Baseline	10.76±0.99	8.86±1.55*
	40 Min	11.16±1.36	8.86±1.07*
Pc (mmHg)	Baseline	4.73±0.43	3.90±0.68*
	40 Min	4.91±0.60	3.90±0.47*
Rt (mmHg/ml/min)	Baseline	0.98±0.12	0.84±0.20
	40 Min	1.02±0.12	0.84±0.16*
Ra (mmHg/ml/min)	Baseline	0.55±0.07	0.47±0.11
	40 Min	0.57±0.069	0.47±0.09*
Rv (mmHg/ml/min)	Baseline	0.43±0.05	0.37±0.09
	40 Min	0.45±0.05	0.37±0.07*
K_f ($g \cdot min^{-1} \cdot cmH_2O^{-1} \cdot 100g^{-1}$)	Baseline	0.8799±0.28	0.5847±0.29*
	40 Min	1.381±0.35	0.8119±0.31*

PAP: pulmonary arterial pressure; Pc: capillary pressure; Rt: total vascular resistance; Ra: arterial segmental resistance; Rv: venous segmental resistance; K_f: filtration coefficient. * $p < 0.05$ compared with endotoxin group.

Table 11. Effects of pretreatment with pentoxifylline on the changes in lung weight, lung water content and filtrate outflow induced by endotoxin in the isolated rat lung.

Measurements	Endotoxin (11)	Pentoxifylline (7)
Lung Weight (g)	2.687±0.57	2.346±0.20
Lung Water Content (%)	86.79±1.23	85.986±1.22
Filtrate (ml)	3.573±1.88	1.543±0.94*

*, $p < 0.05$ as compared to Endotoxin group

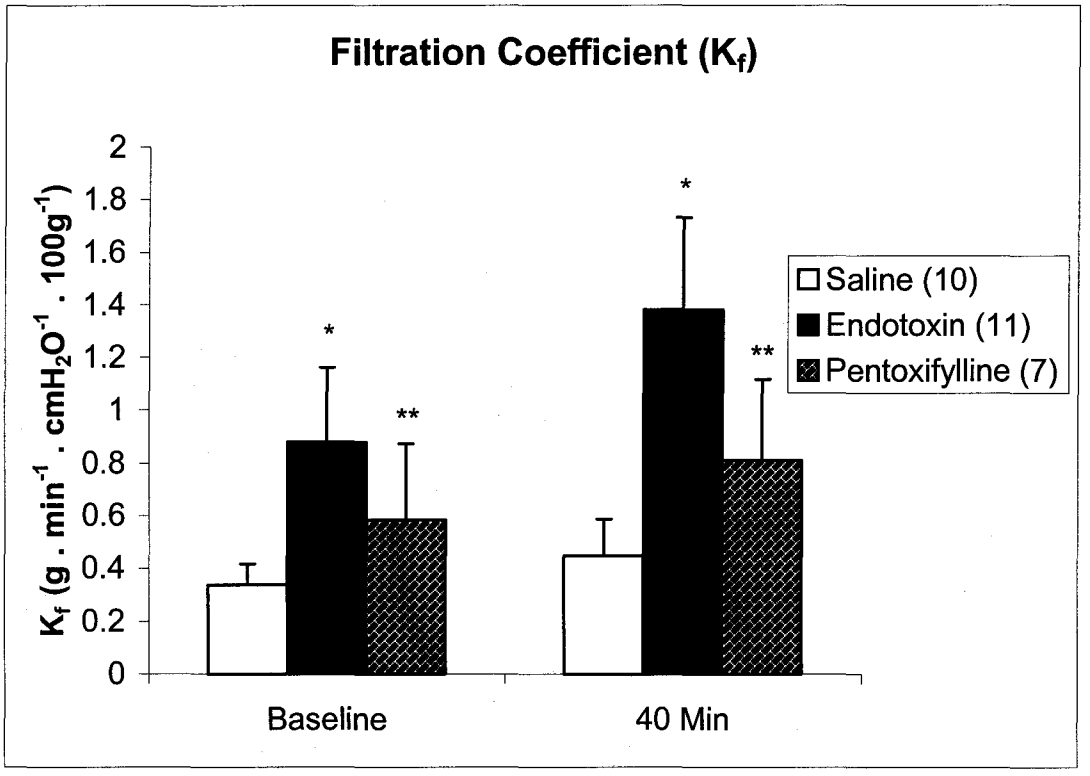


Figure 33. Effect of pentoxifylline on endotoxin-induced increase in lung capillary permeability (K_f).

Pentoxifylline (50mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

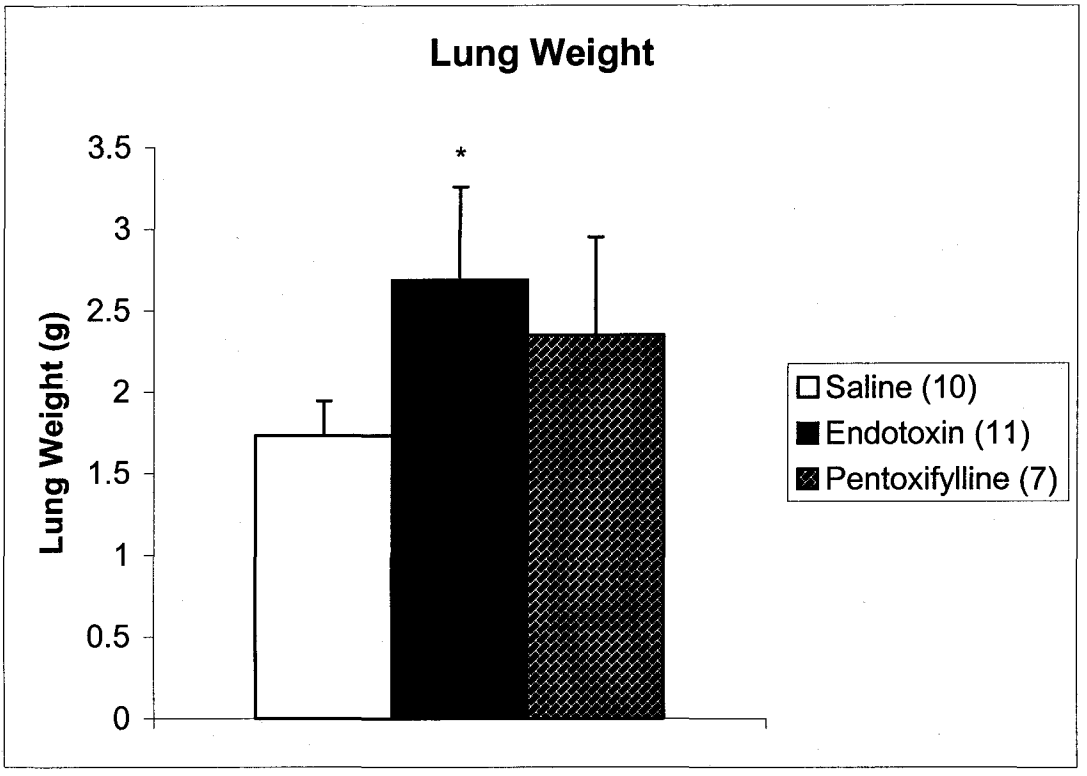


Figure 34. Effect of pentoxifylline on endotoxin-induced increase in lung weight.

Values represent wet lung weight at the end of the experiment. Pentoxifylline (50 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

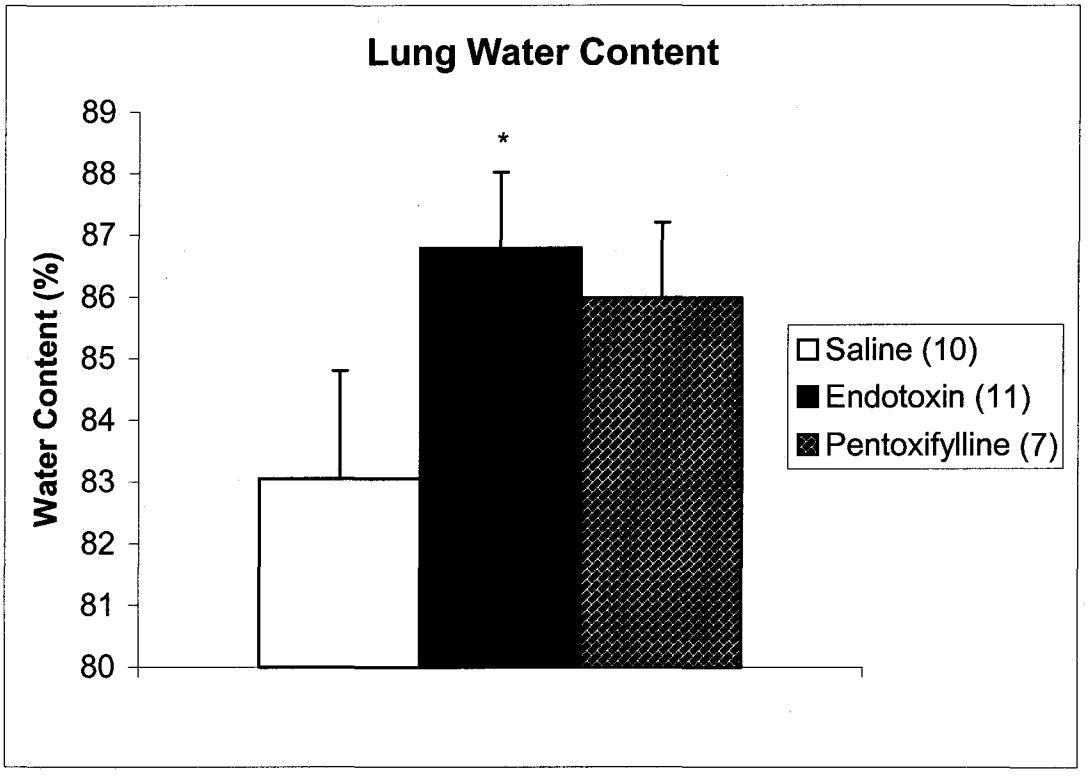


Figure 35. Effect of pentoxifylline on endotoxin-induced increase in lung water content.

Values represent lung water content as percent of lung wet weight at the end of the experiment. Pentoxifylline (50mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Number of animals is in parenthesis. Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

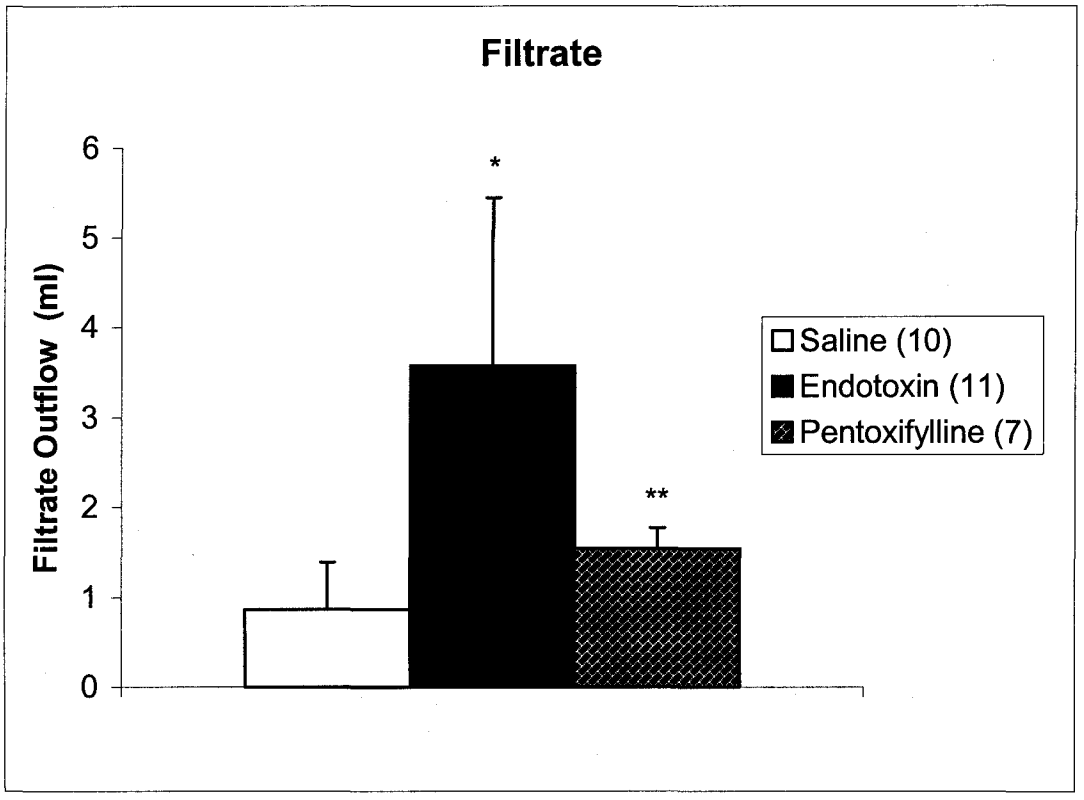


Figure 36. Effect of pentoxifylline on endotoxin-induced increase in outflow of lung filtrate.

Pentoxifylline (50 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. Filtrate outflow volume is the total outflow recorded at the end of the experiment. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

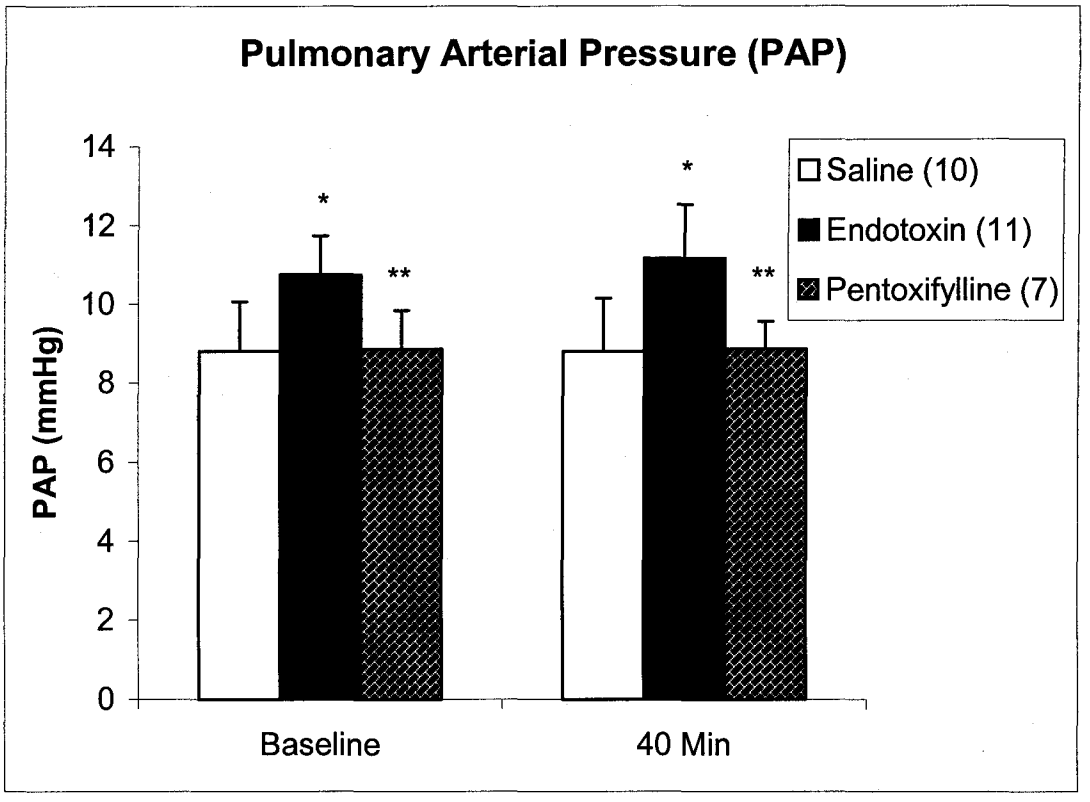


Figure 37. Effect of pentoxifylline on endotoxin-induced increase in pulmonary arterial pressure.

Pentoxifylline (50mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

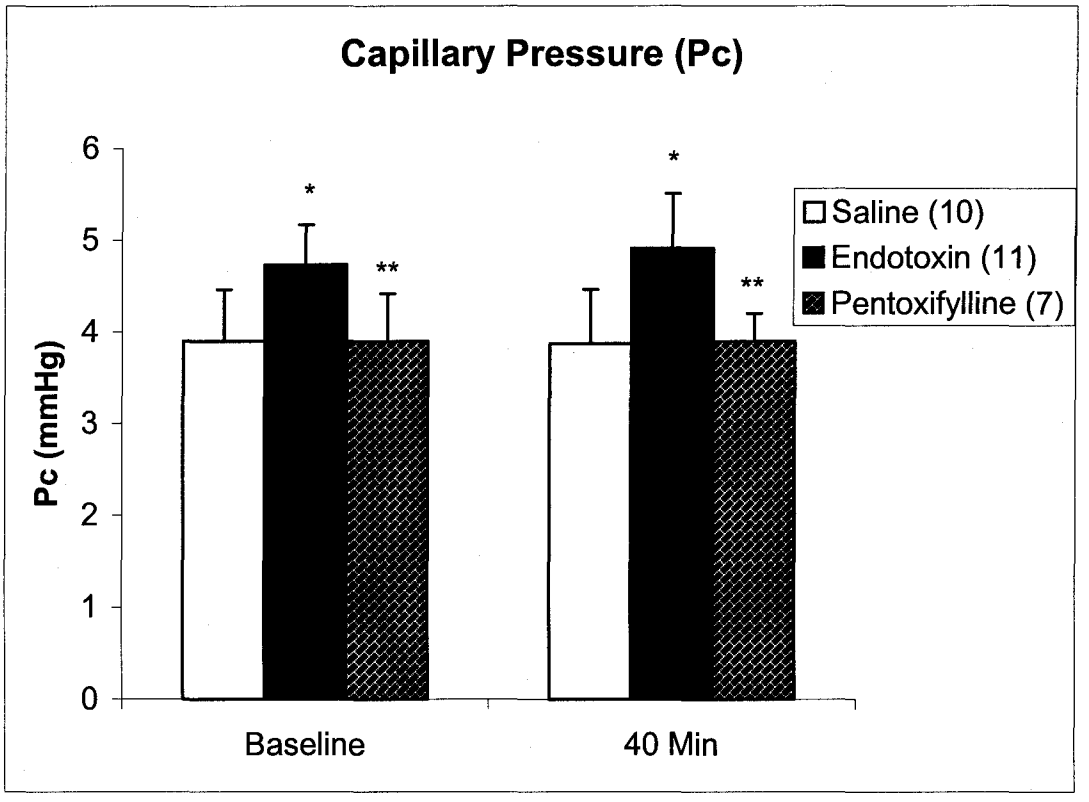


Figure 38. Effect of pentoxifylline on endotoxin-induced increase in pulmonary capillary pressure.

Pentoxifylline (50 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baselines*** were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

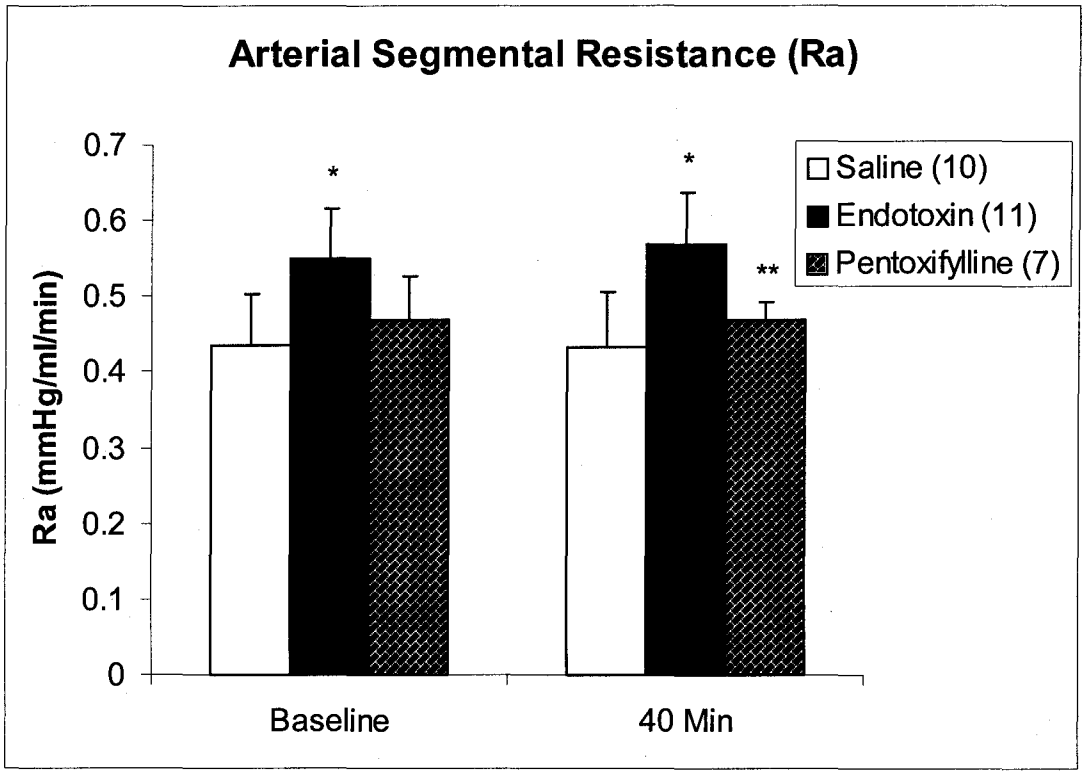


Figure 39. Effect of pentoxifylline on endotoxin-induced increase in pulmonary arterial segmental resistance.

Pentoxifylline (50 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

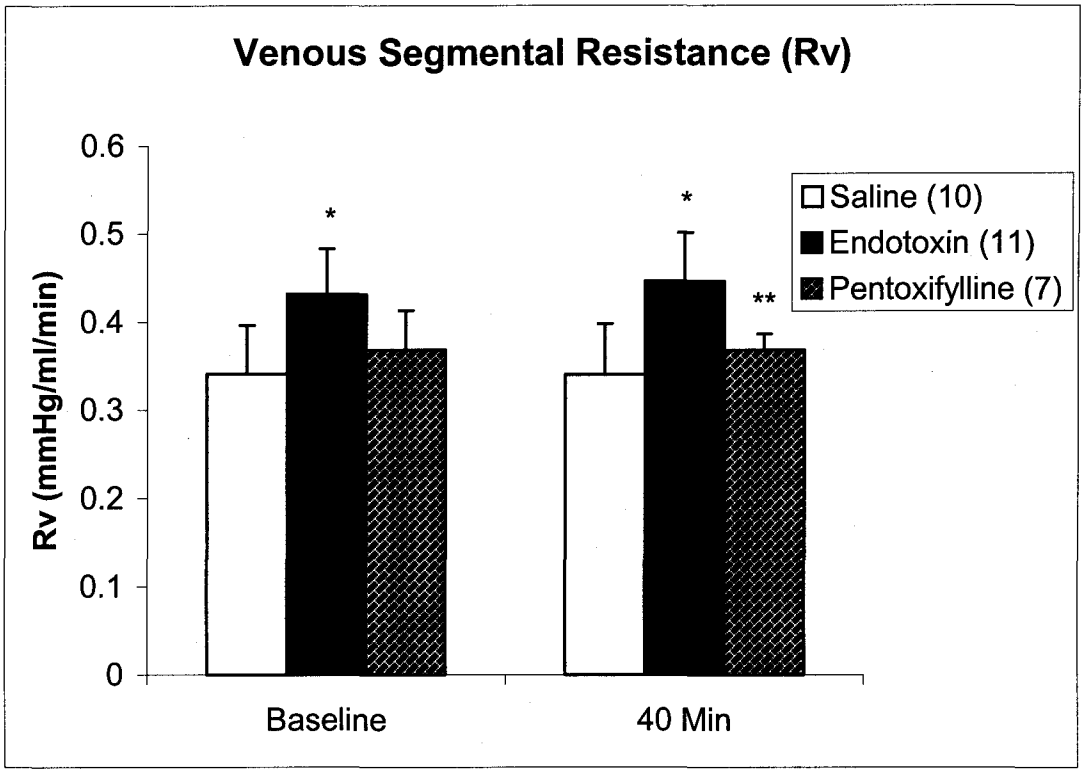


Figure 40. Effect of pentoxifylline on endotoxin-induced increase in pulmonary venous segmental resistance.

Pentoxifylline (50 mg/kg) was given as pretreatment, 15 minutes before endotoxin (ETX). Drug and ETX were given i.v.. Lungs were isolated and perfused one hour after ETX was administered. ***Baseline***s were values obtained 15 to 20 min after the start of perfusion, while ***40 min*** values were those obtained 40 minutes after baseline. Number of animals is in parenthesis. * Significantly different from value of the saline group. ** Significantly different from value of the endotoxin group.

3.5 Studies on the effects on endotoxin on pulmonary leucocyte count

In these experiments, the lungs were removed for histological examination, 60 minutes after the administration of endotoxin (10 mg/kg, iv). Endotoxin caused a significant increase in the leucocyte content of the lungs as compared to saline control animals (Table 12 and Fig. 41). Pretreatment with nicardipine (1 mg/kg) and bepafant (1 mg/kg) significantly reduced the endotoxin-induced increase in the pulmonary leucocyte count (Table 12 and Fig. 42 – 44). However, pretreatment with pentoxifylline (50 mg/kg) failed to affect the endotoxin-induced increase in the pulmonary leucocyte count.

Table 12. Pulmonary leucocyte count in the various groups of animals.

Chemical / Sample	Saline (8)	Endotoxin (8)	Nicardipine (8)	Bepafant (8)	Pentoxifylline (8)
1	83.8	180.3	132.6	131.8	127.5
2	77.8	177	155.7	116.9	166.3
3	86.2	183.6	115.4	161.3	142.8
4	67.8	163.7	111.1	133	142.3
5	70.5	137.4	113	123.8	151.7
6	88	132	136.1	133.4	139
7	75.2	175	165.5	158.7	163.9
8	73.8	168	144.7	179.4	145.3
Mean	77.9	164.6 †	134.3 *	142.3 *	147.4
Std Error	2.63	6.92	7.19	7.64	4.56

†, $P < 0.05$ as compared to Saline group; *, $P < 0.05$ as compared to Endotoxin group.

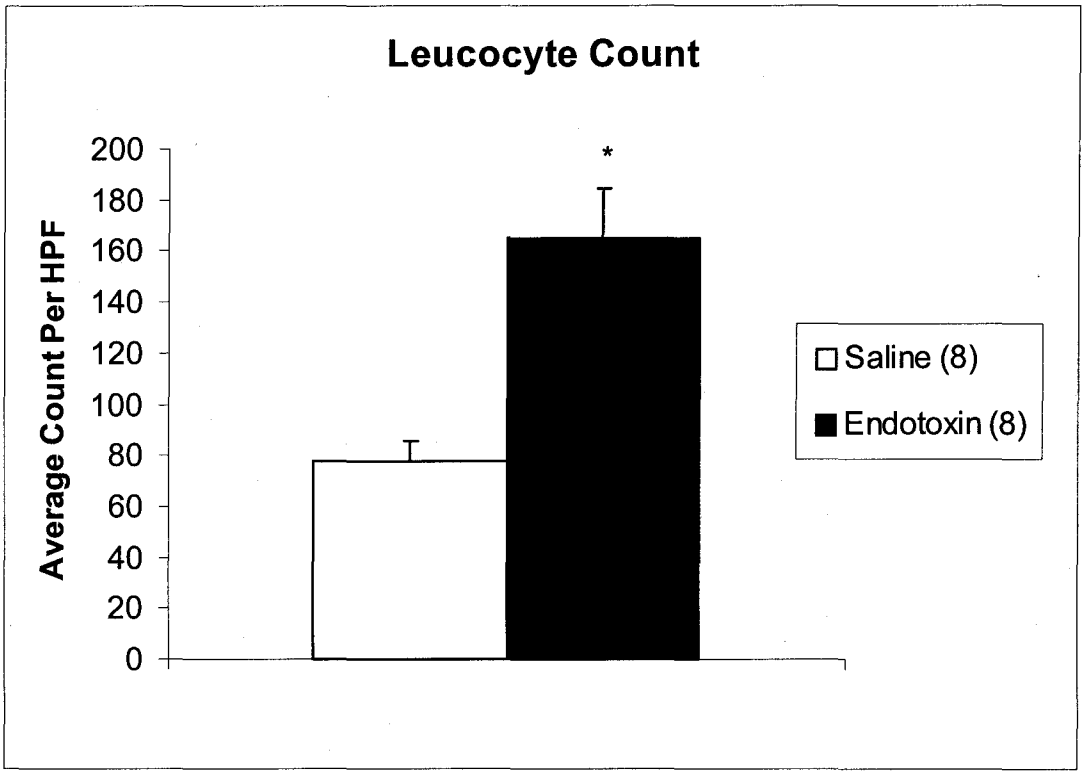


Figure 41. Endotoxin-induced increase in pulmonary leucocyte count.

Saline and endotoxin were given i.v.. Lungs were removed one hour after endotoxin, fixed in Formalin solution and stained with Hematoxylin and eosin. Leucocytes were counted in 10 randomly selected fields under high power light microscopy. Ordinate denotes the average count per high power field (HPF). Number of animals is in parenthesis. * Significantly different from value of saline group.

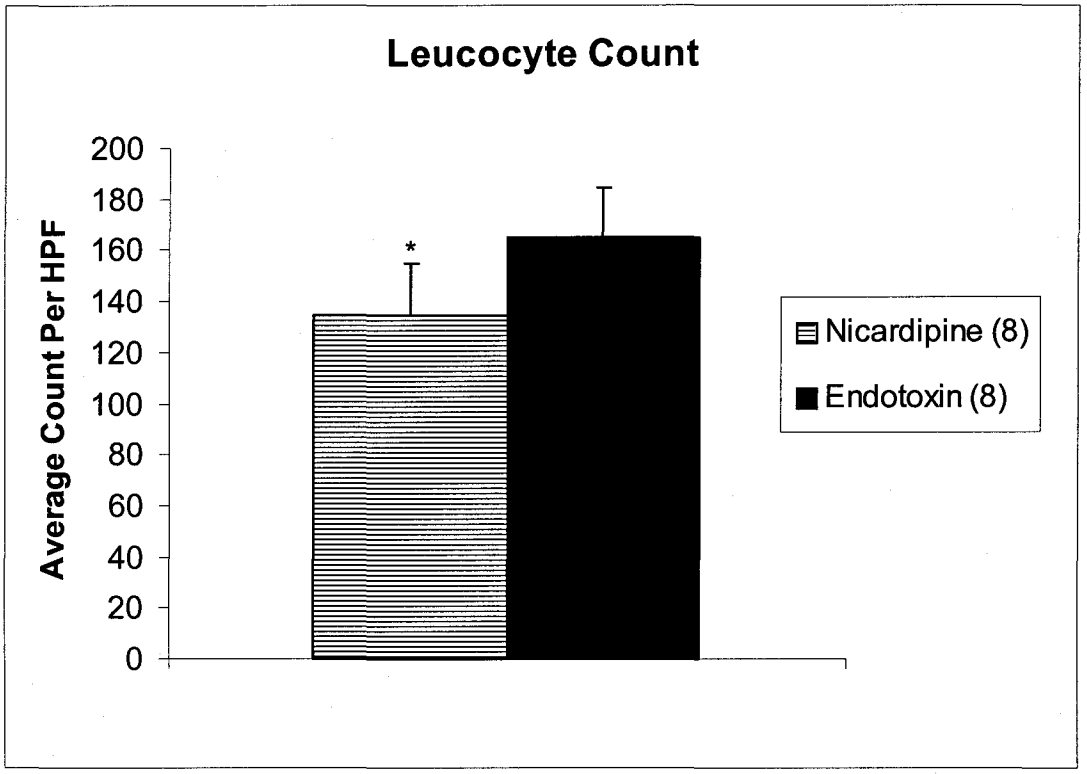


Figure 42. The effect of nicardipine on endotoxin-induced increase in pulmonary leucocyte count.

Nicardipine and endotoxin were given i.v.. Lungs were removed one hour after endotoxin, fixed in Formalin solution and stained with Hematoxylin and eosin. Leucocytes were counted in 10 randomly selected fields under high power light microscopy. Ordinate denotes the average count per high power field (HPF). Number of animals is in parenthesis. * Significantly different from value of endotoxin group.

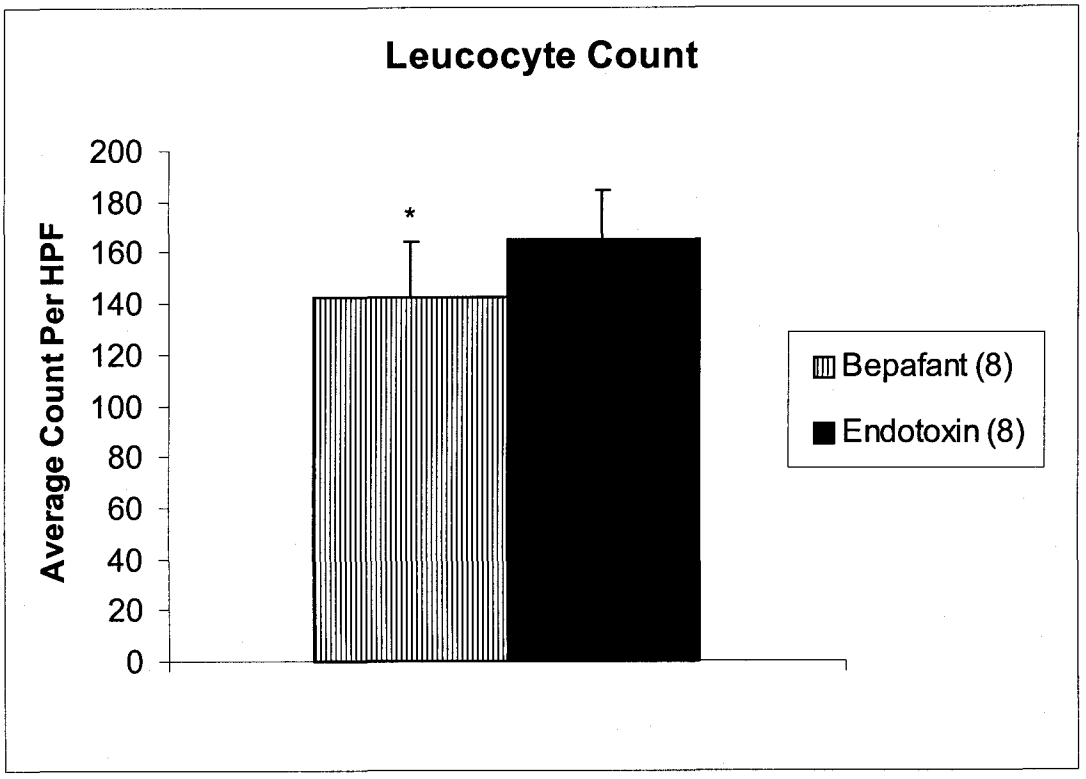


Figure 43. The effect of bepafant on endotoxin-induced increase in pulmonary leucocyte count.

Bepafant and endotoxin were given i.v.. Lungs were removed one hour after endotoxin, fixed in Formalin solution and stained with Hematoxylin and eosin. Leucocytes were counted in 10 randomly selected fields under high power light microscopy. Ordinate denotes the average count per high power field (HPF). Number of animals is in parenthesis. * Significantly different from value of endotoxin group.

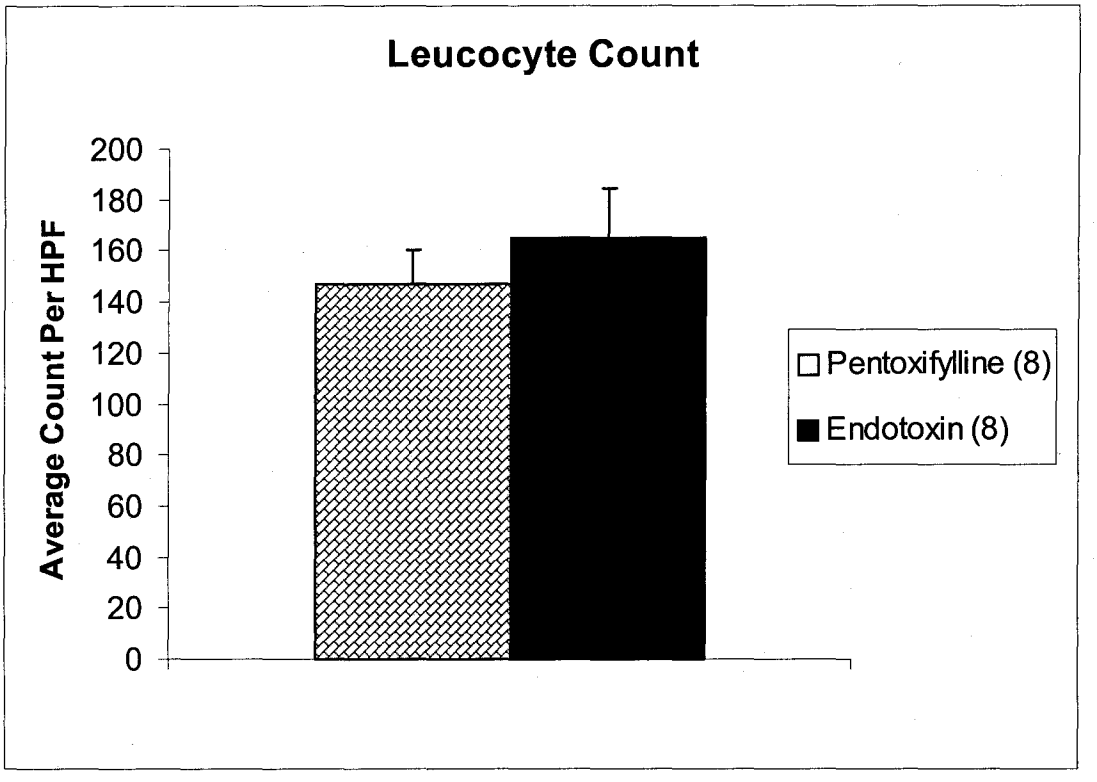


Figure 44. The effect of pentoxifylline on endotoxin-induced increase in pulmonary leucocyte count.

Pentoxifylline and endotoxin were given i.v.. Lungs were removed one hour after endotoxin, fixed in Formalin solution and stained with Hematoxylin and eosin. Leucocytes were counted in 10 randomly selected fields under high power light microscopy. Ordinate denotes the average count per high power field (HPF). Number of animals is in parenthesis. * Significantly different from value of endotoxin group.

Chapter 4

Discussion

4.1 Introduction

Endotoxin is known to cause pathological changes and functional derangements in a variety of organ systems. In previous experiments conducted in our laboratory, endotoxin was found to produce hematological manifestations of disseminated intravascular coagulation (DIC) including thrombocytopenia, hypofibrinogenemia, elevation of serum fibrin degradation products as well as evidence of gross visceral hemorrhages in rats. Pretreatment with nicardipine (a calcium channel blocker), and bepafant (a PAF antagonist) and pentoxifylline (a methylated xanthine that increases blood cell deformability) were found in those previous studies to reduce the endotoxin-induced mortality as well as prevent most of the manifestations of DIC [95 - 100].

The purpose of the present study was to determine whether these same agents (nicardipine, bepafant and pentoxifylline) would also have a protective effect against lung injury that endotoxin is known to produce. In the present experiments, we found that endotoxin (10 mg/kg) given i.v. in rats produced pulmonary changes indicative of lung injury including increases in capillary permeability (K_f), lung weight, lung water content and the outflow of lung filtrate. The lipopolysaccharide also produced changes in pulmonary pressure and vascular dynamics, including increases in pulmonary arterial pressure, capillary pressure and arterial and venous segmental resistances. Pretreatment with nicardipine (1.0 mg/kg), bepafant (1.0 mg/kg) and pentoxifylline, 15 minutes before

endotoxin, significantly reduced the endotoxin-induced increases in capillary permeability and the outflow of lung filtrate. Endotoxin also caused a significant increase in the leucocyte content of the lungs as compared to saline control animals. The increase was significantly reduced by pretreatment with nicardipine (1.0 mg/kg) and bepafant (1.0 mg/kg) but not with pentoxifylline (50 mg/kg).

Several facets of the present study will be discussed below, including: (1) the nature of the endotoxin-induced changes in pulmonary dynamics and its modification by pretreatment with the three drugs; (2) the possible role of leucocytes in the pulmonary injury; (3) the possible mechanisms of the protective action of the three drugs.

4.2 Endotoxin-induced lung injury

Many researchers have reported [195 - 197] that an increase in pulmonary artery pressure and an increase in pulmonary vascular resistance are features of the response to endotoxin in the rat pulmonary circulation. Pulmonary hypertension occurs mainly due to rise in cardiac output with little change in pulmonary vascular resistance after endotoxemia. Furthermore, this increase in pulmonary artery pressure is not due to back pressure from a dysfunctional left ventricle because left atrial pressure in general is not elevated in sepsis before volume resuscitation. Some clinical results show that the pulmonary artery diastolic-to-wedge pressure gradient is increased, suggesting upstream causes of vascular obstruction [101].

The causes of the elevations in pulmonary artery pressure and pulmonary vascular resistance after endotoxemia have not been clearly delineated. Possibilities for these

changes include reduction in cross-sectional area due to vascular obstruction by thrombosis, plugging by leukocytes and platelets, vasoconstriction, edema, and airway changes that may over distend alveoli and compress the capillary bed. In addition, microatelectasis related to diffuse lung injury and surfactant abnormalities may elevate pulmonary vascular resistance by reducing the cross-sectional area because of collapse and crinkling of the alveolar walls. Vasoconstriction is another major potential cause of the increased pulmonary artery pressure and pulmonary vascular resistance. Although pulmonary artery pressure in septic lung injury does not appear to correlate with clinical outcome, it is clear that a degree of active pulmonary vasoconstriction occurs in patients with septic lung injury [102].

In our experiments, endotoxin was given i.v. and one hour later, the lungs were isolated and perfused at a constant rate with a physiological saline solution containing Ficoll, a substance used to substitute for albumin. The pulmonary arterial pressure, and segmental arterial and venous resistances, as well as the capillary pressure were found to be significantly higher in animals given endotoxin than in the saline controls. Capillary permeability (K_f) was also found to be increased. Since the rate of perfusion was kept constant in our experiments, the increased pulmonary arterial pressure could only have been caused by the observed increase in segmental arterial resistance. The segmental venous resistance was also increased by endotoxin. The former (increased arterial resistance) would be expected to cause a *decrease* in capillary pressure, while the latter (increased venous resistance) would *increase* the capillary pressure. The observed

increase in capillary pressure is thus explicable on the basis that the increase in venous resistance was greater than the increase in arterial segmental resistance.

Lung weight, lung water content and the total outflow of lung filtrate at the end of the experiments (after a total of 55 to 60 minutes of perfusion) were significantly greater in lungs from endotoxin treated animals than from saline controls. The increased lung weight and lung water content are indicative of an increased amount of fluid in the pulmonary interstitium (pulmonary edema) while the increased outflow of lung filtrate would have been manifested by an increase in pulmonary lymph flow in the intact animal. These findings, which are indicative of loss of fluid from the capillaries, are attributable to both the increased capillary pressure and the increased capillary permeability (K_f) caused by the endotoxin.

Pretreatment with all three agents (nicardipine, bepafant and pentoxifylline) exerted a protective effect against the lung injury induced by endotoxin. Nicardipine and bepafant significantly reduced the endotoxin-induced increase in capillary permeability (K_f) without significantly affecting the endotoxin-induced changes in pulmonary arterial and capillary pressures or changes in vascular segmental resistances. Pentoxifylline differed from the other two drugs in that it inhibited the pulmonary hypertension and the increases in capillary pressure and vascular resistances as well as the increase in capillary permeability induced by the lipopolysaccharide.

Inhibition of the endotoxin-induced increase in capillary permeability by the three drugs was associated with a reduction in the total outflow of filtrate but not in the lung weight or lung water content. The latter were unexpected since a reduced permeability

would be expected to reduce lung weight and lung water content as well as filtrate outflow. However, this may be explicable on the basis of dose-response relationships. The dose of endotoxin used in our experiments produced a copious loss of fluid from the capillaries. The doses of the three agents that we employed were the same as the doses used in our previous studies on endotoxin-induced DIC. These doses were found to prevent most of the manifestations of DIC and to significantly reduce mortality caused by the lipopolysaccharide. In the present study, the three drugs apparently were able to reduce the loss of capillary fluid to an extent such that it was manifested by a reduction in filtrate outflow but not lung weight or lung water content. It is likely that the drugs would have reduced all three parameters had a smaller dose of endotoxin or larger doses of the three drugs been used. Although pentoxifylline reduced the endotoxin-induced increase in capillary pressure, nicardipine and bepafant failed to have this effect. This may have dampened the ability of the latter two drugs to antagonize the endotoxin-induced loss of fluid from the capillaries.

As discussed in Chapter 1 (Introduction), the pulmonary injury induced by endotoxin may be related to pulmonary sequestration and migration of neutrophils with subsequent degranulation and release of reactive oxygen species and enzymes that interact with endothelial cells, cytokines and complement components. In the present study, histological examination of lung tissue under high power light microscopy showed that endotoxin caused a significant increase in the number of leucocytes. While the method did not allow a differential count of the leucocytes, it is logical, on the basis of reports by other investigators, to speculate that the increase was at least partly if not

mainly related to increased numbers of neutrophils. Nicardipine and bepafant were found to significantly reduce the endotoxin-induced increase in pulmonary leucocyte count. Pentoxifylline failed to have such an effect. The protective effect of the former two agents, but not the latter, may thus in part be due to decreased pulmonary sequestion/migration of leucocytes.

4.3 Pentoxifylline

Pentoxifylline is a methylated xanthine derivative chemically related to theophylline and caffeine. It is a drug known to increase blood cell deformability and has been in clinical use for many years, especially in the treatment of intermittent claudication [103].

Leukocytes, with a mean diameter of about 12 to 14 μm, are much larger than the diameter of the capillary lumen (approximately 5 μm). Despite the size discrepancy, unimpeded flow of leukocytes through the capillaries occurs because of the remarkable deformability of blood cells. Endotoxin can decrease this deformability of leukocytes [104]. This may be one of the reasons why leukocytes are sequestrated in the lungs after the administration of endotoxin. Microvascular entrapment of leukocytes may be the initiating event for oxidative injury to endothelial and parenchymal cells. Previous results from our laboratory showed that pretreatment with pentoxifylline could improve blood cell deformability [99]. In the present study, pretreatment with pentoxifylline was found to reduce the endotoxin-induced increases in pulmonary hypertension, pulmonary capillary pressure, vascular resistances, capillary permeability and outflow of lung filtrate. We

considered the possibility of these salutary effects of pentoxifylline might be related to diminished endotoxin-induced pulmonary sequestration of leucocytes because of improved blood cell deformability. However, this did not prove to be the case since the drug did not affect the endotoxin-induced increase in leucocyte count.

Another important action of pentoxifylline is that it can inhibit phosphodiesterase (PDE), an enzyme that catalyzes the transformation of cAMP to adenosine 5' monophosphate, thereby increasing intracellular cAMP levels [105]. Elevation of cAMP in vascular smooth muscle causes vasodilation. In endothelial cells, increased cAMP levels can cause a decrease in capillary permeability by relaxing the endothelial cytoskeleton, thus decreasing the intercellular gaps [106]. This may be a possible explanation for our observation that pentoxifylline inhibited the increase in capillary permeability induced by the lipopolysaccharide.

Pentoxifylline has been reported to down regulate the production of tumor necrosis factor alpha (TNF-α) [107, 108]. The latter is a 17-kDa protein produced by macrophages and appears to have a central role in the pathogenesis of Gram-negative shock. It increases neutrophil margination and activates monocytes, macrophages, neutrophils, and eosinophils. Activated neutrophils can cause lung injury by releasing its contents, including enzymes and superoxide anions. TNF-α induces its own production and release, as well as those of IL-1β and IL-6 [12]. It can also up-regulate the expression of adhesion molecules including ICAM-1, P-selectin, and E-selectin, thereby increasing neutrophil--endothelium interactions [34, 109]. Additionally, it can decrease the chemotactic ability and deformability of polymorphonuclear neutrophils. Given as a purified

preparation, TNF-α evokes most of the effects of LPS in animals, including fever, shock and death [110]. Passive immunization against TNF-α [111] or administration of chimeric TNF-α inhibitors [112-114] have been reported to protect against the lethal effect of TNF-α.

Pentoxifylline is inactive on endotoxin-induced IL-6 production and on TNF-induced thromboplastin activity in endothelial cells. Furthermore, it does not inhibit activation of endothelial cells by TNF-α. This latter action of TNF stimulates transendothelial migration of polymorphoneclear neutrophils [115]. These observations thus suggest that endothelial cells are not a primary target of pentoxifylline [116]. However, pentoxifylline inhibits TNF activation of leucocytes by down-regulating its production and thus increases the chemotactic movement of leukocytes. This may therefore result in the trapping of leukocytes in the lungs and may be the reason why there is no significant decrease in leukocyte numbers in pentoxifylline pretreated animals as compared with the endotoxin controls, despite the fact that the drug decreases blood cell deformability.

Pentoxifylline also appears to have an anti-inflammatory action. Cermak, et al. [117] found pentoxifylline to inhibit the synthesis of C-reactive protein. The latter is an inflammatory acute-phase reactant which stimulates the production of tissue factor by monocytes. A decrease in tissue factor levels may be beneficial when inflammation is complicated by thrombosis.

4.4 Bepafant

Bepafant (WEB 2170) is a PAF (platelet-activating factor) receptor antagonist. Many antagonists of the PAF receptor have been described including compounds that are structural analogs as well as others without any structural relationship to PAF. A number of natural PAF antagonists have been identified, including BN 52021 and kadsurenone. More recently, several synthetic PAF receptor antagonists were also developed. They include 1) phospholipid analogs; 2) tetrahydrofuran derivatives; and 3) triazolobenzodiazepine derivatives such as WEB 2086, WEB 2170, BN 50726, and BN 50739.

PAF is one of most potent and versatile mediators found in mammals. The compound is thought to be a mediator of cell-to-cell communication and to function either as an intercellular or an intracellular messenger [118]. Numerous cell types and tissues have been shown to produce PAF upon appropriate stimulation [119]. In particular, PAF is produced by a variety of cells that may participate in inflammatory reactions, including monocytes/macrophages, polymorphonuclear neutrophils (PMN), eosinophils, basophils, and platelets [120 - 123]. In addition, human endothelial cells were found to produce PAF after stimulation by a myriad of inflammatory mediators including thrombin [124 - 127], angiotensin II [124], vasopressin [124], leukotrienes C_4 and D_4 [128], histamine [128], bradykinin [128], elastase [129], cathepsin G [129], hydrogen peroxide [130, 131], plasmin [132, 133], interleukin (IL)-8 [134] and IL-1β, and tumor necrosis factor (TNF)-α [135 - 141]. Most of the cells that produce PAF also possess PAF receptors [142 - 145] and are targets for PAF action. In addition to activating platelets, PAF promotes the aggregation,

chemotaxis, granule secretion, and oxygen radical generation from leukocytes as well as the adherence of leukocytes to the endothelium [146 - 150]. PAF increases the permeability of single layer endothelial cells in vitro [151] and stimulates smooth muscle contraction [152 - 154].

PAF has also been found to stimulate the release of arachidonic acid in various cell types by different mechanisms [155 - 159]. Furthermore, PAF induces an elevation of cytosolic free calcium in several cell types including vascular smooth muscle cells [160, 161]. The two main mechanisms involved in PAF-induced increase in cytosolic free calcium are 1) the mobilization of calcium from the intracellular stores as a result of inositol trisphosphate generation and 2) the influx of extracellular calcium through a membrane-associated channel, regulated either directly by PAF or indirectly by intracellular second messenger such as lipoxygenase-derived metabolites of arachidonic acid [118].

Our present data showed that pretreatment with bepafant inhibited the endotoxin-induced increase in pulmonary capillary permeability. This finding is in agreement with the observations by Leonardo et al. [162] that PAF-induced pulmonary edema in rats can be inhibited by PAF antagonists. Additionally, PAF has been reported to enhance the permeability of cultured single layer of human endothelial cell and induce changes of the cell cytoskeleton leading to cell retraction and formation of intercellular gaps [151]. PAF receptor antagonists inhibited these effects.

Bochenski et al. [163] showed both PAF and thromboxane A_2 mediated the rise in pulmonary arterial pressure caused by LPS; On the other hand, the LPS-induced fall in

systemic arterial pressure is mediated by PAF. The PAF antagonist, bepafant, attenuated the systemic hypotension but was a weaker protector against LPS-induced pulmonary hypertension. These results are consonant with our observations that bepafant had no effect on the endotoxin induced increases in pulmonary arterial pressure or vascular resistances.

4.5 Nicardipine

At least three distinct mechanisms may be responsible for contraction of vascular smooth muscle cells. First, voltage-sensitive Ca^{2+} channels open in response to depolarization of the membrane, and extracellular Ca^{2+} moves down its electrochemical gradient into the cell. Second, agonist-induced contractions stem from the hydrolysis of membrane phosphatidylinositol with the resultant formation of inositol trisphosphate; the latter acts as a second messenger causing a release of intracellular Ca^{2+} from the endoplasmic reticulum. Third, receptor-operated Ca^{2+} channels allow the entry of extracellular Ca^{2+} in response to receptor occupancy. An increase in cytosolic Ca^{2+} results in enhanced binding of Ca^{2+} to the protein, calmodulin. The Ca^{2+}-calmodulin complex in turn activates myosin light-chain kinase. The latter phosphorylates the light chain of myosin, allowing actin to interact with myosin and thus produce contraction of smooth muscle. Ca^{2+} channel blockers inhibit the voltage-dependent Ca^{2+} channels in vascular smooth muscle at significantly lower concentrations than are required to interfere with agonist-induced release of intracellular Ca^{2+} or to block receptor-operated Ca^{2+} channels. Our present data showed that nicardipine did not inhibit the increased pulmonary vascular

resistance caused by endotoxin. The drug is a dihydropyridine derivative, a class of calcium channel blockers that is vasoselective. Neely, et al. [164] found that the more vasoselective the calcium channel blocker, the greater is its effect on pulmonary vasoconstriction. However, calcium channel blockers are not selective for the pulmonary vascular bed. They are less effective in producing pulmonary vasodilatation than many other vasodilator drugs, including prostaglandin E_1, isoproterenol, prostacyclin, and nitroglycerin. Moreover, the effect of vasoselective calcium channel blockers on pulmonary vascular resistance is secondary to the effects of these agents on systemic vascular resistance.

Many researchers [165 - 180] report that sepsis/endotoxemia produces an increase in $[Ca^{2+}]_i$ and that this may be due to multiple factors and may be different, depending on the cell type. Evidence exists that transient increases in $[Ca^{2+}]_i$ are essential for migration of neutrophils (PMNs) [181] and calcium antagonists have been reported to inhibit the ability of PMNs to migrate to and destroy pathogens [182 - 184]. Our finding that nicardipine reduced the endotoxin-induced pulmonary leucocyte count is consonant with these reports.

In addition to reducing PMN mobility, calcium antagonists can prevent the oxidative burst that is necessary for killing of some types of bacteria [182, 183]. In experiments on LPS-stimulated PMNs, both calcium channel blockers and drugs that inhibit Ca^{2+} release from intracellular stores decreased superoxide anion production by more than 50 percent [184].

Calcium antagonists can also decrease production or release of cytokines. The study of Fukuzawa et al [185] showed nicardipine has an inhibitory effect on the in vivo production of TNF-α, The mechanism of the latter effect is not clear but may relate to inhibition of phosphodiesterase by nicardipine. Drugs that increase cAMP level in mononuclear cells are known to suppress TNF-α production. Thus, nicardipine may be similar to pentoxifylline in this regard.

Calcium regulation of IL-1 formation has been extensively investigated. While it has been reported that a calcium-mediated process was central in the production IL-1 [186 - 188], other reports concluded that calcium had no role in the induction or release of IL-1 [189]. There is evidence that Ca^{2+} is involved in the production of a number of other cytokines including IL-2, IL-6, IL-8, granulocyte macrophage colony-stimulating factor, etc [190 - 193]. Some calcium channel blockers, such as amlodipine, diltiazem, and verapamil, were found to inhibit IL-6 promoter activity in vascular smooth muscle cells [185]. IL-6 is produced by activated macrophages and stimulates acute-phase responses in the liver. IL-6 production is induced in part by TNF-α and IL-1β and it has been proposed that IL-6 "integrates" signals produced early in the inflammatory response [194]. Consequently, it is possible that drugs which decrease $[Ca^{2+}]_i$ may exert a more global dampening effect on cytokines than a single cytokine antagonist.

4.6 Summary

A review of the literature reveals that the pathophysiology of endotoxin-induced lung injury is highly complex and incompletely understood and appears to be

multifactorial, with neutrophils playing a central role. Pentoxifylline (an agent that increases blood cell deformability), bepafant (a PAF antagonist) and nicardipine (a calcium channel blocker) were observed to exert a beneficial action against the lung injury induced by the lipopolysaccharide. In previous studies, pretreatment with the three drugs was found to reduce endotoxin-induced mortality as well as to prevent most of the manifestations of disseminated intravascular coagulation. The present results suggest that a protective effect on the lungs may also contribute to the salutary effect of these agents on endotoxin-induced lethality. The mechanism(s) by which these agents exert this protective effect in the lungs is not clear. The results with nicardipine and bepafant suggest that these agents may in part act by inhibiting the pulmonary migration/sequestration of leucocytes. Based on what is known in the literature, it is also possible to postulate a number of other ways by which these drugs might exert their beneficial action. These are illustrated in figure 45.

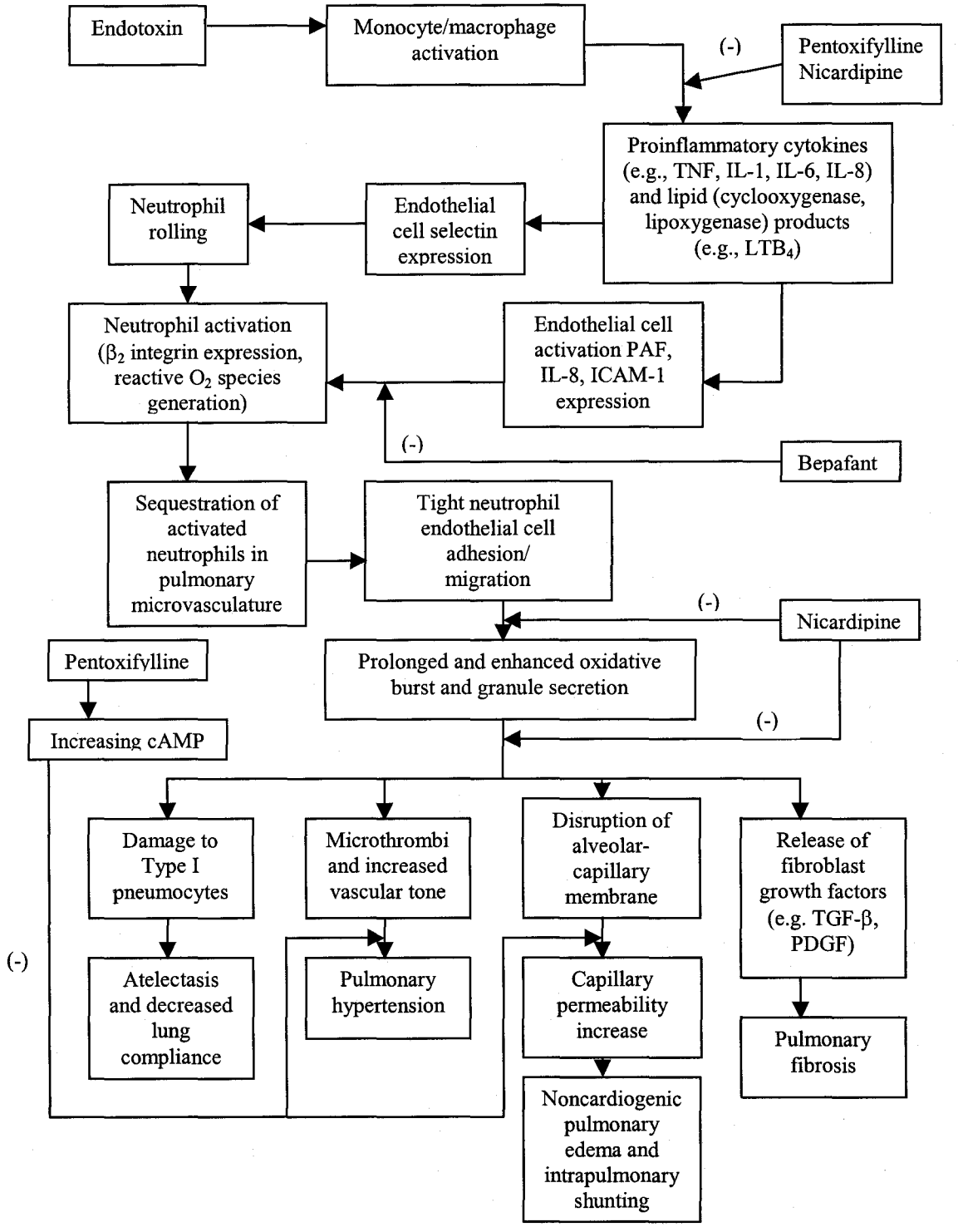


Figure 45. The steps detailed describe a likely mechanism that leads to acute lung injury and effect of three drugs. TNF = tumor necrosis factor; IL = interleukin; LTB_4 = leukotriene B_4; PAF = platelet-activation factor; ICAM-1 = intercellular adhesion molecule-1; O_2 = oxygen; TGF-β = transforming growth factor-β; PDGF = platelet derived growth factor.

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