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Influence of Atrial Natriuretic Peptide on inflammatory pathways in the lung

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dedicated to my family

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2 Introduction

2.1 Background and aim of the work

Atrial natriuretic peptide (ANP), which belongs to the family of natriuretic peptides is a peptide hormone mainly secreted by the heart in response to atrial stretch. It plays a fundamental role in electrolyte and volume homeostasis through potent biological effects including natriuresis, diuresis and vasorelaxation. Biological effects of ANP are mainly promoted through two major biochemically and functionally distinct classes of ANP receptors: natriuretic peptide receptor-A (NPR-A), which activates a particulate guanlyate cyclase and leads to rise of cyclic guanosin-3',5'-monophosphate and NPRC, which acts as clearance receptor and modulates additionally adenylate cyclase activity. The functions of ANP, however, are not only restricted to homeostasis of the reno-cardiovascular system, but also seem to play an important role in the immune system. ANP and its receptors were shown to be expressed in various organs of the immune system such as thymus, spleen, lymph nodes and macrophages. Expression is regulated by a variety of immunmodulating factors and further investigations revealed various effects of ANP on immune cells like macrophages or thymocytes. Additionally, elevated levels of plasma ANP could be detected in inflammatory states like acute asthma exacerbations, acute respiratory distress syndrome (ARDS) and septic shock. In the last years many investigations have been made regarding possible bronchoprotective effects of ANP in those pathophysiological conditions. The lung has the highest tissue concentration of specific ANP binding sites and is also a site of synthesis and release of ANP. Interestingly, up-to-date most efforts were maid to elucidate pulmonary effects of ANP regarding regulation of vascular tone and improvement of pulmonary endothelial cell function, but hardly any data exist concerning potential anti-inflammatory effects of ANP in airway inflammation. The lung can be divided into two distinct compartments, the vascular compartment, in which endothelial cells are mainly involved in inflammatory processes and the airway compartment, where epithelial cells have great importance in orchestrating the immune response. In previous studies, we demonstrated that

ANP inhibits TNF-α induced NF-κB activation and subsequent expression of adhesion molecules in human endothelial cells. Moreover, we were able to show, that ANP prevents NF-κB activation and TNF-α release in murine macrophages. The airway epithelium serves as first line of defence with respect to various external stimuli and mediates the extravasation of leukocytes in the alveolar space. So far, no investigations have been made concerning anti-inflammatory effects of ANP on alveolar epithelial cells.

Aim of the work was to elucidate whether ANP possess anti-inflammatory properties in airway inflammation. Therefore, we aimed to clarify the following question:

- Does ANP have effects on TNF-α induced signal transduction in alveolar epithelium?

- Does ANP show anti-inflammatory actions in the lung *in vivo* in a model of LPS-induced sepsis?

2.2 Atrial natriuretic peptide (ANP)

2.2.1 Discovery of natriuretic peptide family

The atrial natriuretic peptide was first described by de Bold et al in 1981 (de Bold *et al.*, 1981), who discovered the natriuretic and diuretic capability of an atrial extract injected in rats. The biological agent found responsible for this effect was a small cyclic peptide of 28 amino acids named atrial natriuretic peptide. In the following years other members of the so called natriuretic peptide family were discovered. BNP was isolated from porcine brain first and therefore named brain natriuretic peptide (Sudoh *et al.*, 1988). Along the lines of the first two peptides, the third discovered family member was named C-type natriuretic peptide (CNP) (Sudoh *et al.*, 1990). In 1992, another natriuretic peptide, dendroaspis natriuretic peptide (DNP) was first isolated from the venom of the green mamba (Dendroaspis angusticeps) (Piao *et al.*, 2004) and recently discovered also in humans (Richards *et al.*, 2002). In addition, Urodilatin, which has four additional amino acids in comparison to ANP, is a product of alternative processing of pro-ANP by renal cells (Forssmann *et al.*, 1998). ANP and BNP are mainly expressed in cardiac tissue, ANP in the atrium and BNP in the ventricle, while CNP is mainly expressed in the central nervous system and in the endothelium (Pandey, 2005).

2.2.2 Structure and synthesis of ANP

ANP is a cyclic 28 amino acid peptide with a disulfide bridge between two cysteine residues at position 7 and 23 (illustrated in ***figure 1***). This 17 amino acid loop is highly conserved and essential for biologic activity. All natriuretic peptides are synthesized as preprohormones.

Cleavage of 151 amino acid (aa) preproANP results in the 126 aa pro ANP, which is the predominant storage form (Suttner and Boldt, 2004). Corin, a transmembrane cardiac serine protease, cleaves ANP upon secretion to build the C-terminal active peptide (aa 99-126) (Yan *et al*., 2000). ANP is primarily expressed and stored in atrial granules, from which it is released in response to atrial wall stretch resulting from increased intravascular volume. This release into the circulation is mediated by exocytosis (Newman *et al*., 1991).

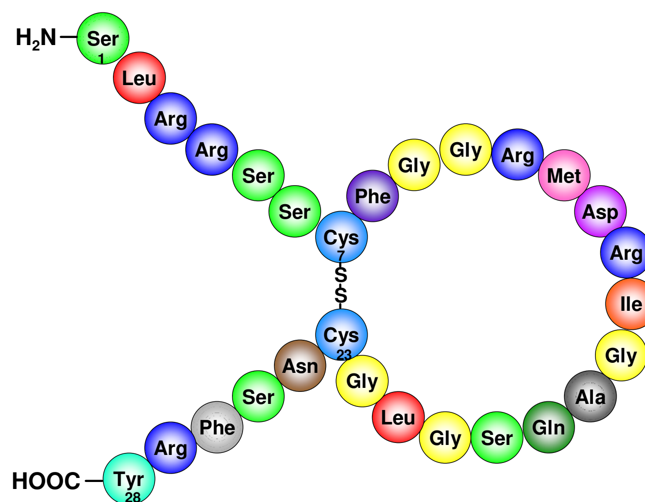

figure 1 Structure of ANP (human)

2.2.3 Receptors and signal transduction

Natriuretic peptides mediate their effects through three transmembran receptors named natriuretic peptide receptor-A (NPR-A), NPR-B and NPR-C (Kuhn, 2003; Misono, 2002; Tremblay et al., 2002). NPR-A and NPR-B have guanylate cyclase activity and lead to increasing cyclic-guanosin-5'-monophosphat (cGMP) levels while NPR-C may function as clearance receptor and causes declining cyclic adenosin-5'-monophosphat (cAMP) concentrations. ANP binds to and exert its effects preferentially through NPR-A and NPR-C

(see ***figure 2***). Binding to NPR-A results in the production of cGMP, a classic intracellular second messenger for which three classes of binding proteins are known: cGMP dependent proteinkinases (PKGs), cGMP binding phosphodiesterases and cyclic nucleotide-gated ion channels (Potter et al., 2005).

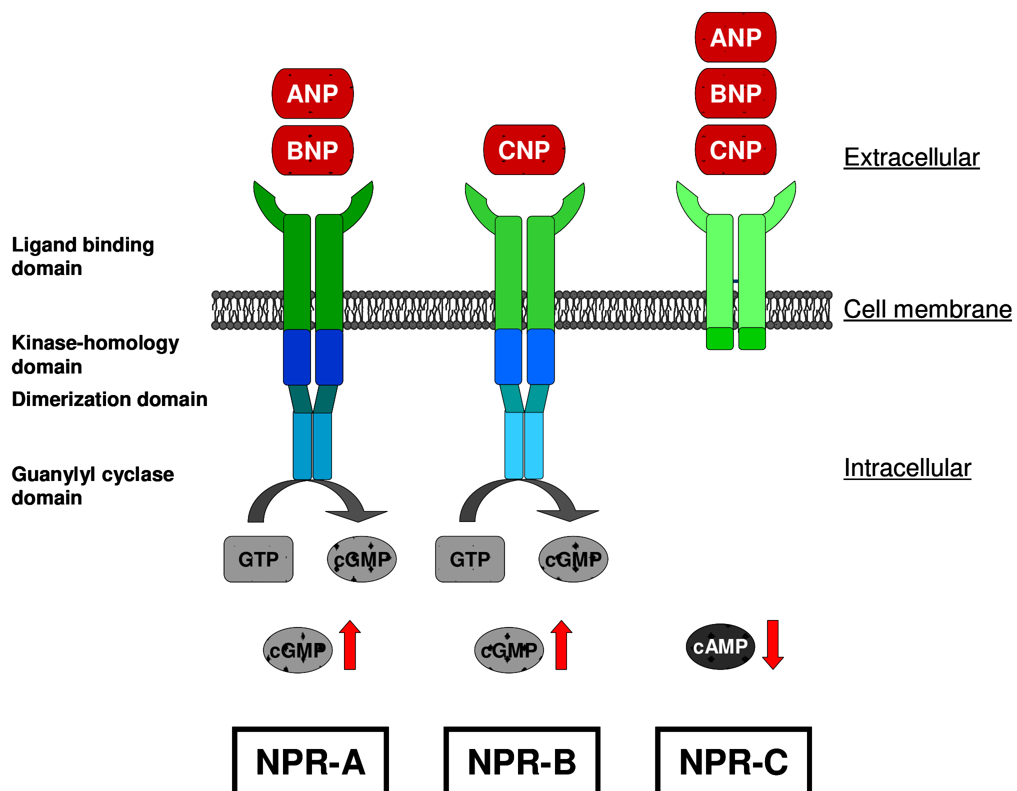

figure 2 Natriuretic peptide receptors

2.2.4 Effects of ANP on blood pressure

ANP release provokes a remarkable decrease in blood pressure. A combined effect on microvascular permeability, vasorelaxation, natriuresis and diuresis mediates the hypotensive property of ANP (Potter et al., 2005).

ANP contributes to the blood volume homeostasis by changes in fluid balance and endothelial permeability. In the kidney, ANP increases glomerular filtration rate, inhibits sodium and water reabsorption, and reduces renin secretion (Nishikimi *et al.*, 2005b). ANP also has direct effects on the heart. Mice lacking ANP or NPR-A suffer from cardiac hypertrophy, which is the result of a prolonged systemic hypertension and the loss of local inhibitory effect of heart growth (Nishikimi et al., 2005a; Kuhn, 2005).

2.2.5 Effect of ANP on the immune system

Besides its cardiovascular effects the natriuretic peptide system possesses various ascendancies on both the innate and adaptive immune system (Vollmar, 2005). First evidence for an involvement of ANP in immune regulation was gained when natriuretic peptide receptors where found in immune organs such as thymus, tonsil, spleen and macrophages (Vollmar and Schulz, 1990). In addition, macrophages were found to synthesize and release ANP in response to several inflammatory stimuli (Vollmar and Schulz, 1994). In the innate immune system, ANP is able to facilitate the defence of macrophages in response to pathogens by improving their release of reactive oxygen species and by enhancing their phagocytosis activity (Mattana and Singhal, 1993). For an appropriate immune response it is not only important to have an inflammatory response in the beginning, but also to resolve the inflammation. An overwhelming inflammatory response is as harmful as the infection itself, because of its ability to destroy tissues and to lead to a

generalized inflammatory response, called sepsis. ANP has shown anti-inflammatory properties in macrophages and endothelial cells. In macrophages it could be demonstrated, that ANP inhibits LPS induced iNOS activation and resultant NO production on transcriptional, posttranscriptional and substrate availability level. This is regulated via an autocrine mechanism (Kiemer and Vollmar, 1998). Via NPR-C and abased cAMP levels, ANP reduces both COX-2 mRNA and protein expression in macrophages exposed to LPS (Kiemer et al., 2002c). Cytokines play an essential role in the inflammatory process. ANP was found not only to reduce LPS-induced TNF-α secretion from macrophages via inhibition of NF-κB and AP-1 (Kiemer and Vollmar, 2001), it also demonstrated major impact on TNF-α effector functions in endothelial cells such as expression of adhesion molecules, expression of MCP-1 and increase of endothelial cell permeability (Kiemer *et al.*, 2005). Additionally, ANP also seems to exert cytoprotective effects as seen in several models of ischemia/reperfusion injury (Gerwig et al., 2003).

2.2.6 Effects of ANP on the lung

ANP and it's receptors are also strongly expressed in the pulmonary system (Gutkowska and Nemer, 1989). NP receptors have been found in endothelial and smooth muscle cells as well as in alveolar cells throughout the lung (Perreault and Gutkowska, 1995). Various biological effects are provoked by ANP in pulmonary functions as illustrated in ***figure 3***. Alveolar type II cells as well as respiratory epithelial cells are capable of synthesizing ANP. In tracheal and bronchial smooth muscle cells a bronchorelaxation is induced, in pulmonary arteries and also in pulmonary veins an increase in cGMP levels and subsequent vasodilatation can be observed. On account of this, ANP is a prominent regulator of the pulmonary vascular tone (Perreault and Gutkowska, 1995). In addition, antiproliferative effects of ANP in human airway smooth muscle cells, mediated through both cGMP-dependent and cGMP-independent mechanisms, has been reported (Hamad et al., 2003). ANP has also shown to

ameliorate the capillary function of pulmonary endothelial cells in hypoxia induced inflammation and therefore demonstrates direct cytoprotective effects on lung epithelium (Irwin et al., 2005). Bronchomotor responses in asthmatic patients are accompanied by an elevation of plasma ANP.

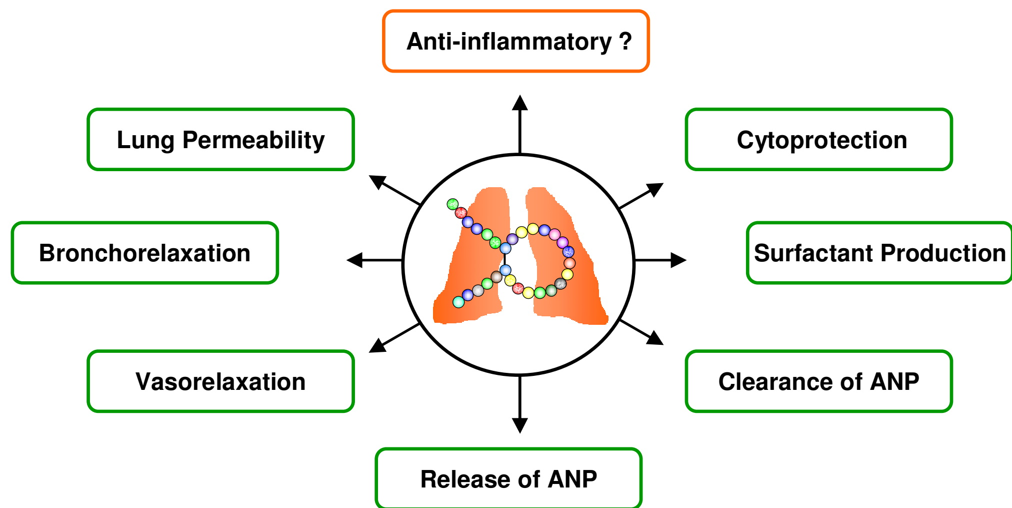

figure 3 Effects of ANP in the lung

Exogenous ANP reverses bronchoconstriction when given intravenously or by inhalation (Hamad et al., 2003). First evidence of an anti-inflammatory property of ANP in the lung was given with the report, that ANP gene transfer attenuates airway reactivity in a mouse model of allergic sensitization (Kumar *et al.*, 2002). In addition, ANP is elevated in patients suffering from septic shock and proANP can be utilised as a prognostic marker in sepsis (Morgenthaler *et al.*, 2005).

2.3 Lung and inflammation

2.3.1 Overview

The lung possesses the largest combined epithelial and endothelial surface area of any organ in the body. Additionally, it has a large capillary bed and an extensive pool of neutrophils and is therefore exceedingly vulnerable during inflammatory processes like pneumonia and sepsis (Crimi and Slutsky, 2004).

The lung can be divided into two major compartments, the vascular compartment, in which endothelial cells are mainly involved in the inflammatory response and the airway compartment where epithelial cells have great importance in orchestrating the immune response. Being the first tissue to encounter the external environment, the airway epithelium serves as first line of defence regarding to a variety of external stimuli (Martin *et al.*, 1997). In several studies, airway epithelial cells have shown their ability to express and secrete various immune molecules and mediators, such as cytokines, chemokines and adhesion molecules (Neff *et al.*, 2006). Additionally, there is increasing evidence that the alveolar epithelium has an important function in initiation and exacerbation of the immune response in the lung through interaction with alveolar macrophages and recruiting leukocytes into the alveolar space (Beck-Schimmer et al., 2004). The recruitment of neutrophils to sites of acute inflammation plays a crucial role during inflammatory response. In this process, the expression of adhesion molecules on epithelial cells can be regarded as major step in host response to inflammatory processes like bacterial infection by initiating extravasation of leukocytes (Beck-Schimmer et al., 2002).

2.3.2 Acute respiratory distress syndrome (ARDS)

ARDS is defined as clinical complication and severe form of acute lung injury with the following hallmarks according to the definitions of the American-European Consensus Conference Committee (AECCC): alveolar epithelial inflammation, non-cardiogenic pulmonary oedema, surfactant depletion, and inactivation and loss of normal endothelial reactivity (Artigas *et al.*, 1998). More than a million people worldwide are affected by ARDS each year with a mortality of 30-50 % of all patients. Inflammatory mediators play a key role in the pathogenesis of ARDS, in which two distinct categories of provoking events can be discerned. Directly lung associated events like aspiration or pneumonia can be the cause of ARDS as well as events causing lung injury in an indirect way, e.g. sepsis or shock (Bhatia and Moochhala, 2004).

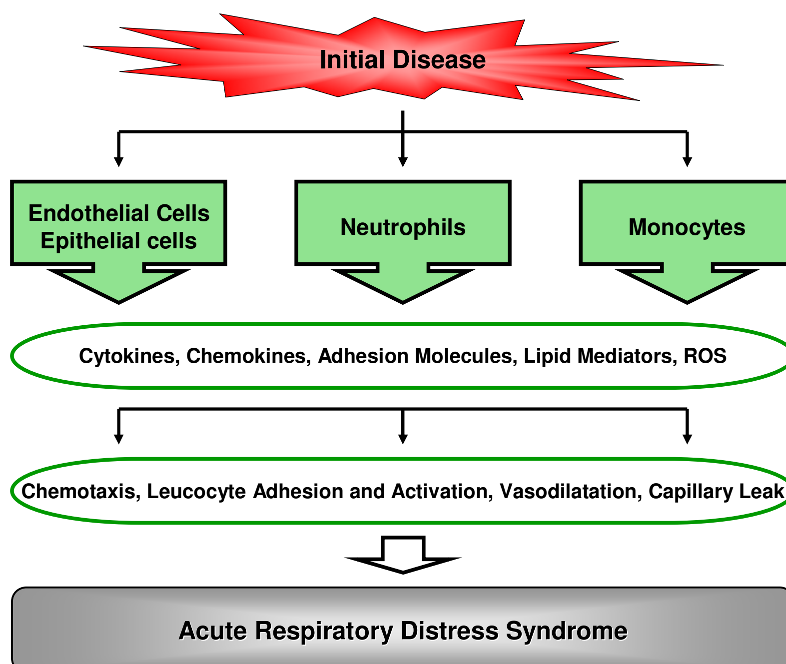

figure 4 ***The pathogenesis of ARDS***
(adapted from Bathia et al., 2004)

The inflammatory response involves the activation of alveolar macrophages, the additional recruitment of blood leukocytes and the production of a variety of mediators like cytokines, ROS, arachidonic metabolites, complement factors and the initiation of the coagulation cascade (Crimi and Slutsky, 2004). As a consequence of this inflammatory burst epithelial and endothelial disruption occurs, which leads to alveolar oedema, decreased lung compliance, and hypoxemia in the end. The complex process of pathogenesis of ARDS is illustrated in ***figure 4***. Up to now mechanical ventilation is still the most important therapeutic approach (Groeneveld, 2002).

2.3.3 Sepsis

Louis Pasteur showed for the first time in 1879/1880, that bacteria were present in blood from patients suffering of puerperal septicaemia. Later he came to the conclusion, that sepsis is a systemic response to fight off pathogens (Annane et al., 2005).

Nowadays sepsis is defined as a systemic inflammatory response syndrome (SIRS), which is caused by an infection, consisting of two ore more of the following syndromes: increased or decreased temperature or leukocyte count, tachycardia and rapid breathing (Levy et al., 2003). Normally the local inflammatory process is tightly regulated by the immune and neuroendocrine system. Sepsis develops when host response to an infection becomes amplified and subsequently dysregulated. In the onset of the disease systemic inflammation occurs converting the local infection to sepsis, severe sepsis or septic shock (Karima et al., 1999). The yearly incident rate of sepsis in the United States is 50-95 cases per 100 000 and has been increasing each year. This severe disease is responsible for 2 % of hospital admissions and 10 % of admissions to intensive care units (Annane et al., 2005). The mortality lies at approximately 30 %, and can rise up to 50 % in the group of elderly persons or in cases of septic shock. Sepsis mortality is numerically equivalent to mortality from acute myocardial infarction. In half of all cases of SIRS a microbiological diagnosis is made and the

definition of sepsis is fulfilled (Cohen, 2002). The commonest sites of infection which can lead to a sepsis are the lungs, abdomen, urinary tract and primary infection of the blood stream. 60 % of these cases were caused by gram-negative bacteria, the remainder by gram-positive bacteria. The most important pathogens provoking a gram-positive sepsis are *Staphylococcus aureus* strains and *Streptococcus pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* are the commonest gram-negative bacilli isolated from patients with sepsis, severe sepsis or septic shock (Annane et al., 2005). Invading microorganisms are detected by pattern recognition receptors expressed on the surface and in the cytosol of immune cells. They are able to recognize common structures of many microbial pathogens called pathogen associated molecular patterns (PAMPs), including endotoxins (LPS, see 2.5), peptidoglycan, lipoteichoic acid, lipopeptides, flagelline and viral RNA (Van Amersfoort et al., 2003). One major group of pattern recognition receptors is the Toll-like-receptor (TLR) family. TLR4 for instance is important for recognition of LPS and subsequent LPS-induced signal transduction, which is characterised by an excessive production of pro-inflammatory mediators such as TNF-α. These cytokines are now able to initiate secondary inflammatory cascades like production of reactive oxygen species (ROS), prostaglandins, other cytokines and the up regulation of adhesion molecules which leads to extravasation of leucocytes into tissue, further release of ROS and proteases and in the end causes tissue destruction (Karima et al., 1999). Additionally, these cytokines in combination with the occurring vascular injury are also able to interfere with coagulation pathways leading to microthrombosis and tissue hypoperfusion (Jagneaux et al., 2004; Esmon et al., 1999). Moreover, release of large amounts of nitric oxide (NO) causes vascular relaxation and impaired myocardial function causing an endotoxin derived state of shock (Kirkeboen and Strand, 1999). This combination of tissue hypoxia and tissue destruction can lead to multiple organ failure and succeeding death (see ***figure 5***).

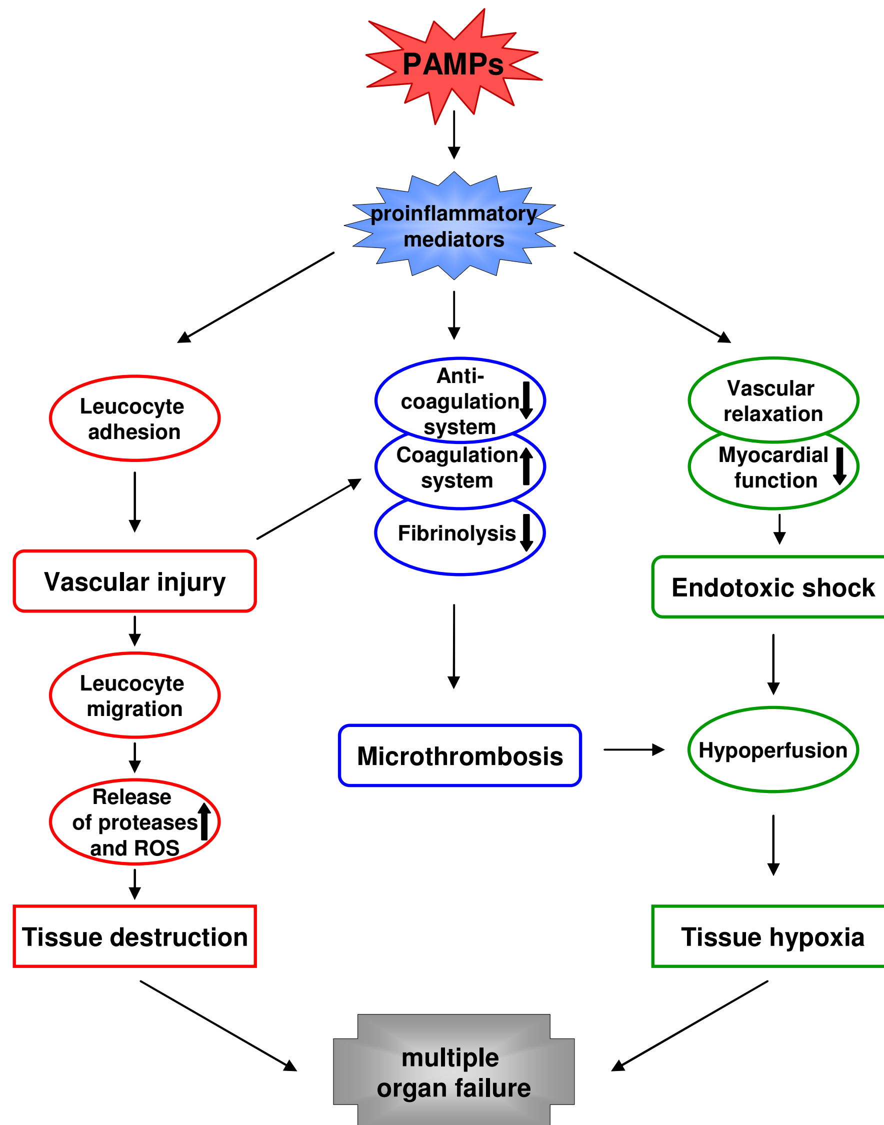

figure 5 The pathogenesis of multiple organ failure
PAMPs: pathogen associated molecular patterns

(adapted from Karima et al. 1999)

2.4 Tumour necrosis factor-α (TNF-α)

2.4.1 Overview

Tumour necrosis factor (TNF-α) is a potent cytokine produced by many cell types in response to inflammation, injury, infection and other environmental challenges. The 70kDa glycoprotein can trigger manifold organism and cellular responses, including leukocyte activation and migration, fever, acute phase response, cell proliferation, differentiation and apoptosis (Tracey and Cerami, 1993; Aggarwal et al., 1985). Most commonly, binding of TNF-α to its receptors lead to the activation of two prominent transcription factors, AP-1 and NF-κB, being responsible for gene induction important for inflammatory responses.

2.4.2 Receptors and signalling

TNF-α, being active as self-assembling, non-covalent bound trimer, exerts its effects through two different receptors called TNFR1 and TNFR2. Those receptors trimerize when TNF-α, which exists both as membrane integrated and as soluble form, is bound and several adaptor molecules are recruited (Chan *et al.*, 2000). Soluble TNF-α predominantly activates TNFR1, which has cytoplasmatic death domains (DD). In contrast, membrane bound TNF-α prefers TNFR2 mediating its effects through TRAF-interacting motifs (TIMs) in the cytoplasmatic domain (Grell *et al.*, 1995). After binding to TNFR1, the complex translocates to cholesterol and sphingolipid enriched membrane microdomains called lipid rafts, in which it associates with receptor-interacting protein (RIP), TNF-receptor associated factor 2 (TRAF2) and TNFR1-associated death domain protein (TRADD) forming a signalling complex (see ***figure 6***).

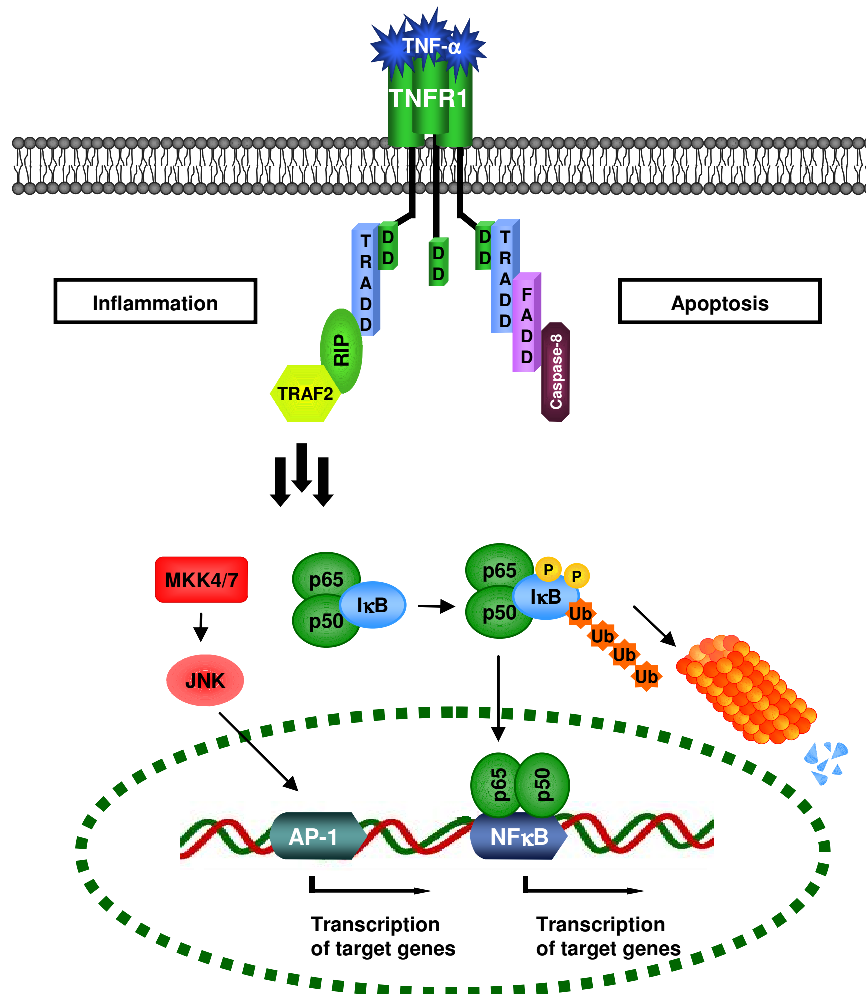

figure 6 TNFR1 signalling

This complex induces the activation and transcription of inflammatory genes like cytokines and adhesion molecules *via* the transcription factors NF-κB and AP-1 (Legler et al., 2003; Aggarwal, 2003). TNFR1 also activates both, pro- and anti-apoptotic pathways. This balance is tightly regulated at numerous levels including regulation of receptor/ligand expression, soluble decoy receptor expression and antiapoptotic ligand induction (Krippner-Heidenreich *et al.*, 2002).

2.5 Lipopolysaccharide (LPS)

2.5.1 Overview

In the end of the 19th century, in Robert Koch's laboratory a heat-resistant toxin was identified in the lysates of *Vibrio cholerae*, which was able to cause toxic shock in animals. Because it was not secreted by bacteria, it was called endotoxin. Nowadays the toxic principle is identified as a lipopolysaccharide being a major component of the outer membrane of gram-negative bacteria. LPS consists of a bisphosphorylated glycolipid (lipid A) and a hydrophilic polysaccharide. The latter is composed of a core and an O-specific chain, which has great importance in LPS heterogeneity between different bacteria.

2.5.2 Receptor and signalling

Once released, LPS binds to LPS-binding protein (LBP) and is delivered to the cell surface receptor CD14, from where it is transferred to Toll like receptor 4 (TLR4). Toll like receptors are an ancient family of pattern recognition receptors, which play a crucial role in early host defence against invading pathogens. They activate multiple steps in the inflammatory process, which are important to fight off the invading pathogens and to coordinate systemic defences. Up to present 10-15 different TLRs are known in diverse mammals, which can recognize various pathogen-associated molecular patterns (PAMPs) (Akira and Takeda, 2004). LPS forms a receptor complex consisting of MD-2, an adaptor protein and dimerized TLR4. This stimulation of TLR4 triggers the association of myeloid differentiation primary-response protein 88 (MyD88) and other adaptor proteins like TRIF, TRAM and Mal. This in turn leads to recruiting of Interleukin-1R-associated kinase-4 (IRAK-4). This event allows the association and phosphorylation of IRAK-1 by IRAK-4. Tumour-necrosis-factor-recetor-associated-factor-6 (TRAF6) binds to IRAK-1 and once phosphorylated the IRAK-

4/TRAF6 complex translocates into the cytoplasm. There transforming-growth-factor-β-activated kinase (TAK1), TAK1-binding protein 1 (TAB1) and TAB2 are bound. TAK1 gets activated and in turn phosphorylates and activates both mitogen-activated kinase kinases (MAPKK) like MKK3/6 or MKK4/7 and the inhibitor of nuclear factor-κB (IκB)-kinase complex (IKK complex), consisting of IKK1, IKK2 and nuclear factor-κB (NF-κB) essential modulator (NEMO) (Karin and Delhase, 2000). The IKK complex phosphorylates IκBα, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and the expression of target genes is induced (Ravid and Hochstrasser, 2004) as illustrated in ***figure 7***.

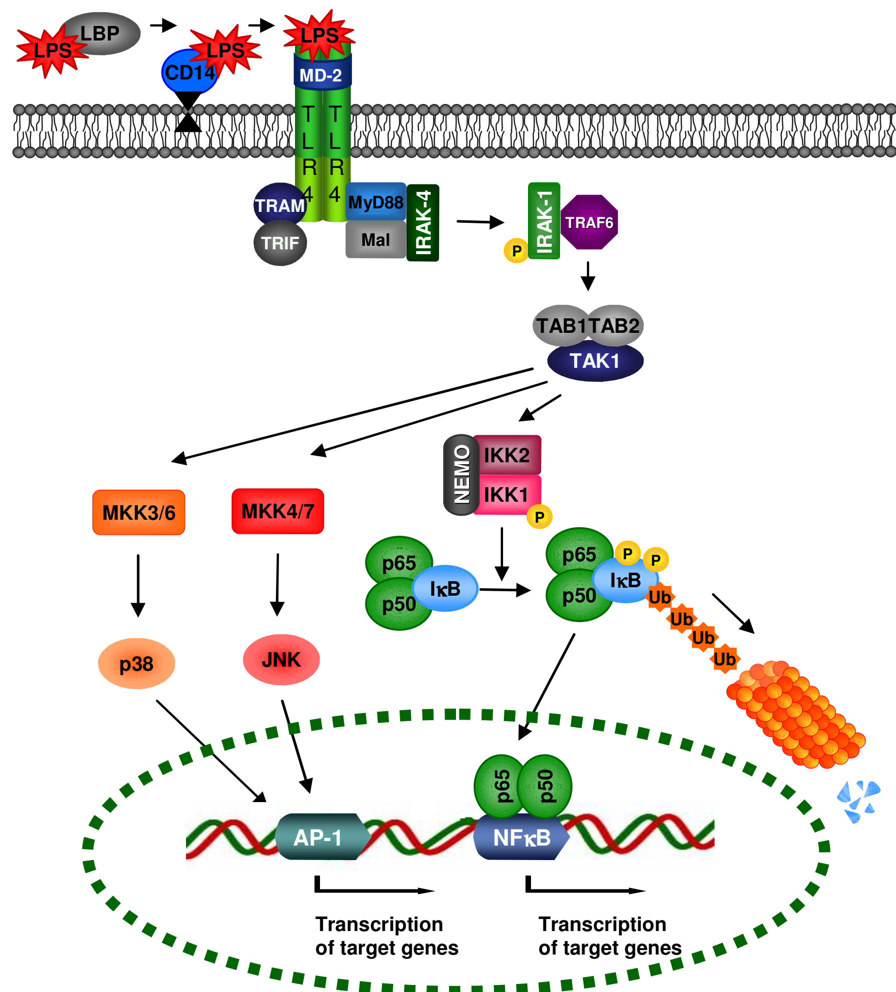

figure 7 ***LPS signalling***

2.6 p38 mitogen activated protein kinase (p38 MAPK)

The p38 MAPK is a member of the mitogen activated protein kinase family and its signalling transduction pathway has major impact in regulating various cellular responses including inflammation, cell differentiation, cell growth and death. MAPK are members of discrete signalling cascades which consist of three protein kinases, a MAPK and two upstream components, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). Up to now, four isoforms of p38 have been described in mammalian cells (p38α, p38β, p38γ and p38δ). Of these homologues p38α is the most physiologically relevant kinase involved in inflammatory processes. p38 was originally identified in LPS-stimulated murine macrophages (Han et al., 1994). For activation, p38 requires dual phosphorylation on Thr180 and Tyr182 by MKK3 and MKK6. These MAPKK are activated by several MAPKKK depending on the decisive stimulus (Kyriakis and Avruch, 2001; Obata et al., 2000). Recent studies revealed an additional mechanism of p38 activation, which is independent of upstream MAPKK and involves TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein 1) (Ge et al., 2002). In response to LPS, p38 phosphorylates and activates a variety of transcription factors, that include ATF-2 (Chen et al., 1998) and Elk-1 (Raingeaud et al., 1996). p38 kinase is essential for cytokine production following LPS treatment (Carter et al., 1999b) and inhibition of p38 has shown to attenuate the severity of pancreatitis-induced adult respiratory distress syndrome (Denham et al., 2000). Additionally, p38 regulates TNF-α mRNA stability and reduces TNF-α transcription by influencing transactivation of NF-κB (Campbell et al., 2004). Therefore, one aim of the present study was to elucidate potential effects of ANP on p38 MAPK in the mouse lung during LPS-induced sepsis.

2.7 Proteine kinase B / Akt

Protein kinase B (PKB), also known and in the following termed as Akt, is a serine/threonin kinase which plays a critical role in the modulation of cell development, growth and survival. Akt is an important downstream target of phosphatidylinositol 3-kinase (PI3K). Synthesis of 3'-phosphorylated inositides by PI3K after activation translocates Akt to the plasma membrane, where it is activated by a phosphoinositide-dependent kinase (PDK1) (Cantley, 2002). Akt is the homologue of the transforming oncogene of the AKT8 oncovirus (ν-Akt). Three mammalian members of this family have been isolated so far termed Akt 1/2/3. They share >80 % amino acid homology and contain a conserved domain structure: a pleckstrin homology (PH) domain which mediated binding of Akt to 3'-phosphoinositides, a catalytic kinase domain containing a phosphorylation site at Thr308 and a regulatory C-terminal domain with a second regulatory phosphorylation site at Ser473. Phosphorylation of Thr308 and Ser473 is essential for maximal Akt activation (Vivanco and Sawyers, 2002). Constitutive Akt signalling promotes proliferation and increased cell survival for example by phosphorylating and thereby inhibiting the pro-apoptotic protein BAD and by transcriptional regulation of pro and anti-apoptotic genes (Song et al., 2005). Recent reports revealed a growing evidence for participation of the PI3K/Akt pathway in LPS-induced inflammatory mechanisms (Guha and Mackman, 2002; Williams et al., 2004). Therefore, it seemed plausible to investigate whether Akt is involved in the effect of ANP on LPS-induced septic shock.

2.8 Adhesion Molecules

2.8.1 Overview

Two main pathways are responsible for regulating cell to cell communications: soluble factors like growth factors and cytokines, and a group of cell adhesion molecules (CAMs). These adhesion molecules can be subdivided into four groups: selectins, integrins, cadherins and immunoglobulin-like adhesion molecules. The latter is a family of more than 70 known members of cell surface glycoproteins being characterised by immunoglobulin homology units, which consist of two anti-parallel beta sheets connected through two cysteine residues. As an example, T-cell receptor, Immunoglobulins, MHC-antigens, CD4, CD8 and ICAM-1 are members of this family (Aplin et al., 1998).

2.8.2 ICAM-1

ICAM-1 is a 505 aa transmembrane glycoprotein that consists of five immunoglobulin-like domains, a transmembrane segment and a cytoplasmatic tail. It is constitutively expressed on cell surfaces of a variety of cell types, e.g. fibroblasts, leucocytes, endothelial and epithelial cells in a low manner. The adhesive interactions are mediated by binding to two integrins belonging to the β_2 subfamily, e.g. LFA-1 and Mac-1. ICAM-1 expression is predominantly transcriptional regulated. Up regulation occurs in response to a number of inflammatory mediators such as oxidative stress, virus infections or pro-inflammatory cytokines, and is associated with a variety of inflammatory diseases including asthma, atherosclerosis, ischemia reperfusion injury and ARDS (van de and van der Saag, 1996). The ICAM-1 promoter contains a large number of binding sites for inducible transcription factors, the most important of which is NFκ-B (Roebuck and Finnegan, 1999).

2.8.3 Role of ICAM-1 in lung inflammation

The recruitment of leucocytes is one of the fundamental mechanisms involved in inflammatory processes. The migration of monocytes into the alveolar compartment can be regarded as crucial step in the development of acute and chronic lung injury (Mulligan et al., 1993). This process requires the leucocytes to adhere and migrate through the vascular endothelium, through the extracellular matrix of endothelial and epithelial cells and in the end to cross the alveolar epithelial barrier. ICAM-1 has been shown to play a major role in recruiting leucocytes to sites of inflammation by mediating adherence of neutrophils to endothelial cells leading to subsequent extravasation (Bevilacqua et al., 1994). This adhesion is mediated through ICAM-1 in endothelial cells, and through CD11a/CD18 and CD11b/CD18 as receptors on neutrophils. Further, ICAM-1 is also expressed on alveolar epithelial cells on a low level and markedly upregulated in response to pro-inflammatory stimuli like TNF-α, LPS or IFN-γ (Beck-Schimmer et al., 2002; Paine, III et al., 1994). Recent studies revealed that ICAM-1 is also critically involved in target cell-effector cell interactions (Beck-Schimmer et al., 2004). These investigations indicate that ICAM-1 seems to be important for the adhesion of neutrophils and macrophages to stimulated alveolar epithelial cells and their subsequent cytotoxic actions. In addition, there is increasing evidence for the importance of soluble ICAM-1 in orchestrating the immune response in the airway compartment. Soluble ICAM-1 has been demonstrated to enhance alveolar macrophage production of macrophage inflammatory protein-1 and TNF-α.

3 Materials and methods

3.1 Cell culture

3.1.1 Materials

ANP (1-28, human) was purchased from Tocris (Westwood, Ellisville, USA), tumour necrosis factor-α (TNF-α) from tebu-bio (Offenbach, Germany). The protease inhibitor cocktail Complete® was from Roche (Mannheim, Germany). All other materials except antibodies were purchased from either Sigma (Deisenhofen, Germany), Carl-Roth GmbH (Karlsruhe, Germany) or VWR International (Munich, Germany). ANP and TNF-α were diluted in phosphate buffered saline (PBS) containing 0.1 % bovine serum albumine (BSA). If not stated otherwise, all solutions were prepared with double-distilled water.

3.1.2 Solutions

Phosphate buffered saline (PBS) pH7.4		Trypsin/EDTA (T/E)	
Na_2HPO_4	10.4 mM	Trypsin	0.05 g
KH_2PO_4	3.16 mM	(1:250 in PBS)	
NaCl	132.2 mM	Na_2EDTA	0.20 g
		PBS	ad 100.0 ml

3.1.3 Type II alveolar epithelial cell line A549

The human type II alveolar epithelial cell carcinoma A549 was graciously provided by Prof. Dr. E. Wagner (Department of Pharmacy, LMU Munich, Germany). This cell line provides most of the characteristics of type II alveolar epithelial cells (Nardone and Andrews, 1979; Smith, 1977)

3.1.4 Culture of A549

A549 were cultured in HAMs F12K medium (PAN Biotech, Aidenbach, Germany) supplemented with 10 % heat inactivated fetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin in an incubator (Heraeus, Hanau, Germany) in a humidified atmosphere at 5 CO_2 and 37℃. Cells were routinely tested for mycoplasma with the PCR detection kit VenorGeM (Minerva Biolabs, Berlin, Germany).

3.1.5 Passaging

For passaging of A549 cells, the medium was removed and the cells were washed three times with PBS. Afterwards A549 where incubated with 2.5 ml T/E solution per 75 cm^2 flask for 2 min at 37℃. The cells where gradually detached and the digestion of trypsin was stopped with HAMs F12K containing 10 % heat-inactivated FCS. After centrifugation at 150 x g, 4℃ for 8 min the supernatant was discarded and the pellet was resuspended in HAMs F12K supplemented with 10 % heat-inactivated FCS and penicillin (100 U/ml)/streptomycine (100 ng/ml). A549 were subcultured 1:10 in culture flasks or plates and grown until confluence.

3.1.6 Freezing and thawing

For long-time storage cells were grown to confluence in 150 cm^2 flasks, trypsinized, centrifuged and resuspended in ice-cold freezing medium, containing 10% DMSO as cryoprotectant.

<u>Freezing medium A549</u>

HAMs F12K	55 %
FCS	40 %
DMSO	10 %

The resuspended cells were directly transferred to cryo-vials and frozen at -20℃ for one day. Because successive freezing is required for survival of the cells, the vials were kept at -80℃ for another three days until long term storage in liquid nitrogen at -196℃.

For thawing, the content of a cryo-vial was defrosted rapidly by dissolving in 20 ml of prewarmed cell culture medium, centrifuged and resuspended in culture medium.

The culture was left to grow for at least 5 days before any experiments.

3.2 LPS model of murine sepsis

The following animal experiments were kindly performed by Dr. Martin Lehner (Biochemical Pharmacology, University of Konstanz) (see 3.2.3.1) and Ulla Gebert (Biochemical Pharmacology, University of Konstanz) as well as Melanie Keller (3.2.3.2).

3.2.1 Animals

Male BALB/c mice (pathogen-free, 22 ± 6 g) were provided by the in house Animal Breeding Facility of the University of Konstanz and housed in a temperature- and humidity-controlled room at 22℃ and 55 % humidity under a constant 12 h light/dark cycle. Animals had free access to water and chow (Ssniff, Soest, Germany), but were fasted with free access to water 12 h prior to the *in vivo* experiment. All studies were performed with the permission of the government authorities, in accordance with the German Legislation on Laboratory Animal Experiments and followed the directives of the University of Konstanz Ethical Committee.

3.2.2 Materials and solutions

ANP (1-28, rat) was purchased from Bachem (Heidelberg, Germany), Lipopolysaccharide (LPS) from *Salmonella abortus equi* S. from BIOCLOT (Aidenbach, Germany). Pentobarbital (Nembutal), which was used for anaesthesia of animals, was from Sanofi-Ceva (Hannover, Germany). All other materials were purchased from either Sigma (Deisenhofen, Germany) or VWR International™ (Munich, Germany). ANP and LPS were diluted in a total volume of 300 µl sterile 0.9 % saline solution containing 0.1 % human serum albumin (HSA).

3.2.3 Experimental setting and tissue sample generation

3.2.3.1 TNF-α measurement in plasma and tissue samples

In this experiment, mice received preconditioning with ANP prior to LPS challenge, in order to investigate potential effects of ANP pretreatment on the subsequent LPS-induced TNF-α expression. At the beginning of the *in vivo* experiment, either NaCl (0.9 %) or ANP (50 µg/kg b.w., 5 µg/kg b.w. or 0.5 mg/kg b.w.) was administered to mice intravenously. After a preconditoning period of 15 min, LPS (300 µg/kg b.w.) or NaCl (0.9%) were injected i.p.

figure 8 ***Experimental setting for TNF-α measurement in plasma and tissue samples (⇩organ withdrawal) in a model of ANP preconditioning in LPS-induced sepsis.*** *Animals were injected i.v. with either NaCl (Co) or ANP (50 µg/kg b.w., 5 µg/kg b.w. or 0.5 µg/kg b.w.) prior to i.p. LPS administration (300 µg/kg b.w.). After 120 min and lethal anaesthesia blood was obtained and lungs were excised and snap frozen in liquid nitrogen for TNF-α measurement.*

120 min afterwards blood and tissue samples were withdrawn after cardiac puncture and lethal i.v. anaesthesia and of mice with 150 mg/kg b.w. pentobarbital plus 0.8 mg/kg b.w. heparin and further handled as described in 3.10. Four treatment groups were generated, each group consisting of n = 4 animals.

3.2.3.2 Experimental setting for tissue sample generation

Mice were injected with either intravenous NaCl (0.9 %) or ANP (5 µg/kg b.w.). After a pretreatment period of 15 min animals received an i.p. injection of NaCl (0.9 %) or LPS (1 mg/kg b.w.). At the indicated times, blood and tissue samples were obtained after lethal intravenous anaesthesia of mice with 150 mg/kg b.w. pentobarbital plus 0.8 mg/kg b.w. heparin and snap-frozen in liquid nitrogen ***(figure 9)***. Four treatment groups were generated, each group consisting of n = 5 animals.

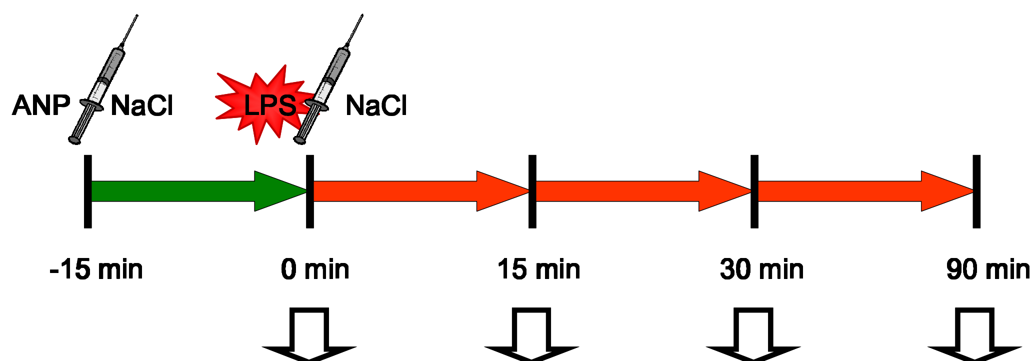

figure 9: ***Experimental setting for ANP preconditioning in vivo before LPS-induced septic shock (⇩ organ withdrawal).***
Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1mg/kg b.w.). or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p. After lethal anaesthesia lungs were excised 15 min, 30 min or 90 min after LPS injection and snap-frozen in liquid nitrogen.

3.3 Western Blot analysis of protein

3.3.1 Sample preparation

3.3.1.1 Solutions

Modified RIPA buffer (lysis buffer)

NaCl	150 mM
Tris-HCl	50 mM
Nonidet P-40	1.0 %
Sodium deoxycholat	0.25 %
SDS	0.1 %
Added freshly before use:	
For inhibition of proteases	
Complete®	4 %
PMSF	1 mM
For inhibition of phosphatases	
NaF	1 mM
Activated Na_3VO_4	1 mM

Lysis buffer for lung tissue

NaCl	137 mM
Tris	20 mM
Na_2EDTA	2 mM
Glycerol	10 %
$Na_4P_2O_7$	2 mM
$Na_2C_3H_7O_6P$	20 mM
Complete®	4 %
PMSF	1 mM
NaF	10 mM
Activated Na_3VO_4	2 mM

Laemmli sample buffer (3x)

Tris-HCl	187.5 mM
SDS	6.0 %
Glycerol	30 %
Bromphenolblue	0.015 %
Added freshly before use:	
β-Mercaptoethanol	5 %

3.3.1.2 Preparation of whole cell lysates

Cells were cultured in 6-well plates until 90 % confluence and were treated as indicated in the associated figure legend. Subsequently, cells were washed with ice-cold PBS, lysed in modified RIPA buffer, homogenized and centrifuged (14,000 rpm, 4℃, 10 min). The supernatants were handled further on as described in 3.3.1.3.

3.3.1.3 Preparation of whole organ lysates

Approximately 20 mg of lung tissue were hackled and homogenized with a dounce homogenizer in 300 µl of lysis buffer in order to get a homogenous suspension. Afterward the samples were centrifuged (14,000 rpm, 4℃, 10 min), 10 µl of the supernatants were further diluted and used for determination of protein content and the remaining supernatant was diluted with Laemmli sample buffer (3x) and boiled at 95℃ for 5 min. Samples were stored at -20℃ until Western Blot analysis.

3.3.1.4 Protein determination

Protein concentrations were determined in order to ensure equal amounts of protein in all samples analyzed by Western Blot. Quantification was performed using the bicinchoninacid assay (BC assay reagents, Interdim, Montulocon, France) as described by Smith and co-workers (Smith et al., 1985).

The blue complex was measured photometrically at 550 nm (TECAN Sunrise Absorbance reader, TECAN, Crailsheim, Germany). Protein standards were obtained by diluting a stock solution of Bovine Serum Albumin (BSA). Linear regression was used to determine the actual protein concentration of the samples.

3.3.2 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

3.3.2.1 Solutions

Separation gel (10 %)

PAA solution (30 %)	40 %
Tris-base pH 8.8	375 mM
SDS	0.1 %
TEMED	0.1 %
APS	0.5 %

Stacking Gel

PAA solution (30 %)	17 %
Tris-HCl pH 6.8	125 mM
SDS	0.1 %
TEMED	0.2 %
APS	1 %

Electrophoresis buffer (1x)

Tris	4.9 mM
Glycine	38 mM
SDS	0.1 %

3.3.2.2 Electrophoresis

The prepared lung and cell lysates described above were separated by denaturating sodium dodecylsulfat polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli method. SDS, a highly negative charged detergent binds to the hydrophobic parts of proteins and solubilizes them. After denaturating the proteins by reducing the disulfide binds with β-mercaptoethanol and boiling the samples at 95℃ for 5 min, the complexes of SDS with the denatured proteins have a large net negative charge that is roughly proportional to the mass of the protein. Their migration velocity during the electrophoretic separation is now roughly proportional to the mass of the protein. Equal amounts of protein were subjected to SDS-PAGE (Mini-Protean 3, Bio-Rad, Munich, Germany) on a discontinuous polyacrylamide gel, consisting of separation gel (10 %) and stacking gel. Electrophoresis was carried out at 100 V for 21 min for stacking and 200 V for 36 min for separation of the protein mixture. The

molecular weight of the investigated proteins was determined by comparison with prestained protein standards (Caleidoscope protein marker, Bio-Rad, Munich, Germany).

3.3.3 Western Blot

3.3.3.1 Solutions

Tris-buffered saline pH 8.0 containing 0.1 % Tween (TBS-T)

Tris	24.6 mM
NaCl	188 mM
Tween 20	0.2 %

Anode buffer

Tris	12 mM
CAPS	8 mM
Methanol	15 %

Cathode buffer

Tris	12 mM
CAPS	8mM
SDS	0.01 %

Coomassie staining solution

Coomassie brilliant blue G-250	3 %
Acetic acid (100 %)	10 %
Ethanol (96 %)	45 %

Coomassie destaining solution

Acetic acid (100 %)	10 %
Ethanol (96 %)	33 %

ECL solutions

Solution A

Luminol	25 mM
p-Coumaric acid	0.396 mM
Tris pH 8.5	100 mM

Solution B

H_2O_2 (30 %)	0.006 %
Tris pH 8.5	100 mM

3.3.3.2 Antibodies

Primary antibodies	*Diluted in*	*Dilution*	*manufacturer*
Rabbit anti IκBα	1 % Blotto in TBS-T	1: 1,000	Santa Cruz, Heidelberg, Germany
Rabbit anti phospho-IκBα(Ser 32)	5 % BSA in TBS-T	1:1,000	Cell signalling, Frankfurt/Main, Germany
Rabbit anti Akt	1 % Blotto in TBS-T	1:1,000	Cell signalling, Frankfurt/Main, Germany
Rabbit anti phospho Akt (Ser 473)	5 % BSA in TBS-T	1:2,000	Cell signalling, Frankfurt/Main, Germany
Rabbit anti p38 MAPK	5 % BSA in TBS-T	1:1,000	Cell signalling, Frankfurt/Main, Germany
Rabbit anti phospho p38 MAPK (Thr180/Tyr182)	5 % BSA in TBS-T	1:1,000	Cell signalling, Frankfurt/Main, Germany
Secondary antibodies	*Diluted in*	*Dilution*	*manufacturer*
Goat anti rabbit IgG (H+L)	1 % Blotto in TBS-T	1:20,000	Dianova, Hamburg, Germany

table 1: Primary and secondary antibodies used for Western Blot analysis

3.3.3.3 Semi-Dry blotting

Using a Transblot SD semidry transfer cell (Bio-Rad, Hercules, USA), the separated proteins were electrophoretically transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA), which was incubated in Methanol for 5 min and then stored for at least 30 min in anode buffer before usage. One sheet of thick blotting paper (Schleicher & Schüll) was soaked with anode buffer and rolled onto the anode. The prepared membrane and the gels

were added. After covering the stack with another sheet of thick blotting paper soaked with cathode buffer, the transfer was carried out for 1 h at 1,6 mA/cm^2. In order to saturate unspecific binding sites, the membrane was immersed for 1 h in 5 % non-fat dry milk in TBS-T at RT.

3.3.3.4 Protein detection

Membranes were incubated with the respective primary antibody solution (see ***table 1***) overnight at 4°C. After four washing steps (5 min TBS-T, pH 8,0), the suitable secondary horseradish peroxidase-labelled antibody was administered for 1 h at RT, followed by four additional washing steps (5 min in TBS-T, pH 8,0). All steps regarding the incubation of the membrane were performed under constant shaking. For visualizing of proteins, a freshly prepared mixture (1:1) of the two detection solutions was added to the membrane for 1 min. The appearing chemoluminescence (see ***figure 10***) was detected by exposure of the membrane to a X-ray film (Super RX, Fuji, Düsseldorf, Germany) and following development with a Curix 60 Developing system (AGFA, Cologne, Germany).

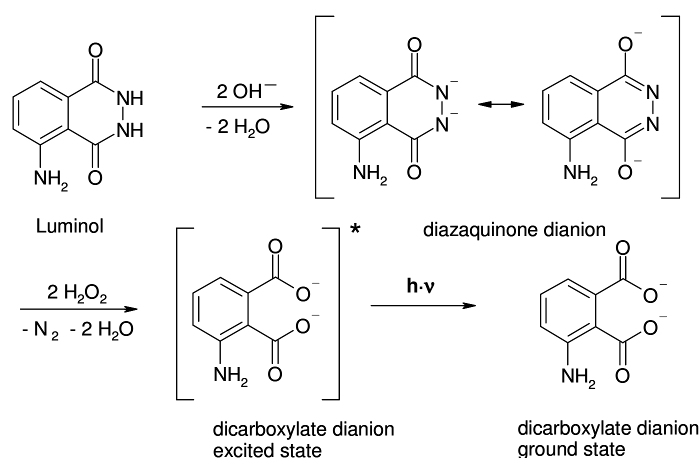

figure 10: Western Blot detection with Luminol

3.3.3.5 Coomassie blue staining

Gels were stained after protein transfer with Coomassie brilliant blue G solution for 20 min in order to ensure equal protein loading and blotting efficiency. This dye binds non-specific to nearly all kinds of proteins under complexation. Afterwards, gels were washed with destaining solution for 60 min until proteins appeared as blue bands.

3.3.3.6 Stripping and reprobing

In order to analyze different proteins on the same membrane, primary and secondary antibodies from former experiments have to be removed from the membrane. Therefore, blots were incubated in stripping buffer at 50℃, shaking for 30 min. After six washing steps in TBS-T (5 min, RT), stripping efficiency was confirmed by carrying out another development with ECL solution. When removal of antibodies was successful, the membrane was blocked again for 1 h with 5 % non-fat dry milk in TBS-T and then incubation with antibodies was performed as described in 3.3.3.4.

3.4 Electro Mobility Shift Assay (EMSA)

3.4.1 Solutions

Buffer A

HEPES pH7.9	10 mM
KCl	10 mM
EDTA	0.1 mM
EGTA	0.1 MM
DTT	1mM
PMSF	0.5 mM

Buffer B

HEPES pH7.9	20 mM
NaCl	400 mM
EDTA	1 mM
EGTA	0.5 mM
Glycerol	25 %
DTT	1 mM
PMSF	1 mM

DTT and PMSF were added to the Buffer A and B stock solutions directly before use.

<u>STE buffer pH 7.5</u>

Tris-HCl	10 mM
NaCl	100 mM
EDTA	1mM

<u>5x binding buffer</u>

Glycerol	20 %
$MgCl_2$	5 mM
EDTA	2.5 mM
NaCl	250 mM
Tris-HCl	50 mm

<u>Gel loading buffer</u>

Tris-HCl	250 mM
Bromphenolblue	0.2 %
Glycerol	40 %

<u>Reaction buffer</u>

DTT	2.6 mM
5x binding buffer	90 %
gel loading buffer	10 %

<u>10x TBE pH8.3</u>

Tris	0.89 M
Boric acid	0.89 M
Na_2EDTA	0.02 M

<u>non-denaturating polyacrylamide gel</u>

10x TBE	5.3 %
PAA solution (30 %)	15.8 %
Glycerol	2.6 %
TEMED	0.05 %
APS	0.08 %

3.4.2 Isolation of nuclear protein

3.4.2.1 Preparation from cells

A549 cells were grown in 6-well plates up to 90 % confluence and were treated as indicated in the respective figure legend. Subsequently, cells were washed twice with ice-cold PBS,

scraped off in PBS with a rubber cell scraper, centrifuged for 5 min at 1500 rpm and resuspended in 400 µl of ice-cold Buffer A for 15 min. Then 25 µl Nonidet P-40 was added and after intense vortexing the cell suspension was centrifuged (14,000 rpm, 4℃, 45 sec). The nuclear pellet was resuspended under continuous shaking for 15 min at 4℃ in Buffer B. The nuclear extract was centrifuged (14,000 rpm, 4℃, 5 min) and the supernatant was stored in aliquots at -85℃.

3.4.2.2 Preparation from lung tissue

Lung tissue (approximately 20 mg) was directly homogenized in 300 µl of Buffer A on ice with a dounce homogenizer in order to ensure a homogenous suspension. Samples were centrifuged at 1,000 rpm at 4℃ for 10 min and resuspendend in 300 ml Buffer A, followed by addition of 18 µl Nonidet P-40 (NP-40) and careful mixture of samples. After 10 min incubation on ice, samples were centrifuged at 14,000 rpm and 4℃ for 10 min. Subsequently the pellet was resolved and incubated in 50 µl Buffer B by shaking for 30 min at 4℃. After another centrifugation step (14,000 rpm, 10 min, 4℃) the supernatant containing nuclear proteins was frozen in aliquots at -85℃ until usage for EMSA.

3.4.3 Protein determination

Protein concentrations in isolated nuclear fractions were determined by the method of Bradford (Bradford, 1976) using coomassie brilliant blue G-250 as indicating dye (see ***figure 11***).

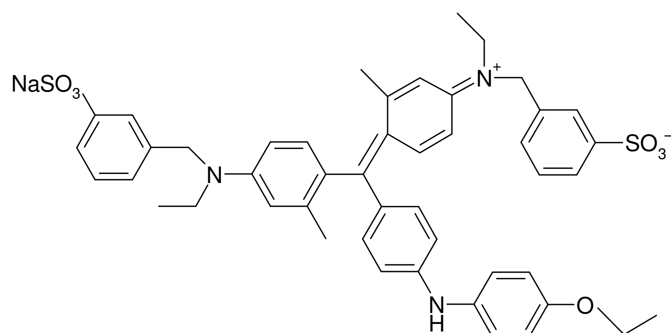

figure 11: Coomassie Brilliant Blue G-250

3.4.4 Radioactive labeling of consensus oligonucleotides

Double-stranded oligonucleotides, containing either the consensus sequence for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') (Promega, Heidelberg, Germany) were 5' end-labelled with [γ^{32}P]-ATP (3000 Ci/mmol, Amersham, Freiburg, Germany) using T4 polynucleotide kinase (USB, Cleveland, USA), which catalyzes the transfer of the terminal phosphate of ATP to the 5'-hydroxyl termini of the DNA. After incubation of oligonucleotides with T4 polynucleotide kinase for 10 min at 37°C, the reaction was terminated by addition of 0.5 M EDTA solution. The radioactive labelled DNA was separated from unlabelled DNA by using NucTrap probe purification columns (Stratagene, La Jolla, USA). Radioactive oligonucleotides were eluated from the column with 70 µl of STE buffer and frozen at -20°C.

3.4.5 Binding reaction and electrophoretic separation

Equal amounts of nuclear protein were incubated for 5 min in a total volume of 14 µl containing 2 µg poly(dIdC) and 3 µl reaction buffer at room temperature. Afterwards, 1ml of

the radio-labelled oligonucleotide was added. After incubation for 30 min at room temperature, the nucleoprotein-oligonucleotide complexes were resolved by gel electrophoresis (Mini-Protean 3, Bio-Rad, Munich, Germany) for approximately 70 min at 100 V on non-denaturating polyacrylamide gels (4.5 %) with 0.25 % TBE as electrophoresis buffer. The gel was autoradiographed with an intensifying screen at - 80℃. Signal detection and quantification was performed by phosphorimaging (Cyclone Storage Phosphor Screen; Canberra-Packard, Dreieich, Germany).

3.5 *In vitro* phosphorylation by p38 MAPK

Activity of p38 MAPK was examined with an *in vitro* phosphorylation assay. In this method, myelin basic protein (MBP) is used as substrate for p38 MAPK.

3.5.1 Solutions

Lysis buffer

Na_2EDTA	2 mM	added freshly before use:	
NaCl	137 mM	Activated Na_3VO_4	2 mM
Glycerol	10 %	PMSF	2 mM
$Na_2P_2O_7$	2 mM	Complete®	4 %
Tris-HCl	20 mM		
Triton®X-100	1 %		
$Na_2C_3H_7O_6P$	20 mM		
NaF	10 mM		

Kinase buffer

HEPES	20 mM	added freshly before use:	
$MgCl_2$	20 mM	Activated Na_3VO_4	2 mM
$Na_2C_3H_7O_6P$	25 mM	DTT	2 mM

ATP mix

Kinase buffer	X* µl
[γ^{32}P] ATP	10 mCi/ml (3000 Ci/mmol)
ATP	5 mM
$MgCl_2$	2 M

* buffer is added to adjust volume according to number of samples

Laemmli sample buffer

Tris-HCl	3.125 M
SDS	20 %
Glycerol	50 %
DTT	16 %
Pyronin Y	0.005 %

3.5.2 Immunoprecipitation

Approximately 30 mg of frozen lung tissue was homogenized in ice-cold lysis buffer with a dounce homogenizer and subsequently centrifuged (10,000 rpm, 4℃, 10 min). Protein concentrations were determined in the supernatant according the method of Pierce (Smith et al., 1985). Equal amounts of protein were incubated with 1.5 µl of anti-p38 polyclonal rabbit antibody (Cell signaling, Frankfurt/Main, Germany). After 2 h of incubation, immunoprecipitation was performed with protein A agarose (5 µl per probe) shaking overnight at 4℃. Then probes were centrifuged (10,000 rpm, 4 min, 4℃) and the precipitates were washed three times with lysis buffer and once with kinase buffer.

3.5.3 In vitro phosphorylation assay

After resuspension in 20 µl of kinase buffer, 3 µl of substrate solution containing 1 mg MBP dissolved in 300 µl kinase buffer and 10 µl ATP-mix were added. For phosphorylation this incubation mixture was incubated at 30℃ for 20 min under permanent shaking. Reaction

was stopped by adding 6 µl Laemmli buffer and subsequent heating at 90°C for 3 min. 30 µl of this reaction mixture were subjected to SDS-PAGE with a 12 % PAA gel at 200 V (for details see 3.3.2). Signal detection and quantification was performed by phosphorimaging (Cyclone Storage Phosphor Screen; Canberra-Packard, Dreieich, Germany).

3.6 Isolation and characterization of RNA

Total RNA was prepared using RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' description. Cells were washed with PBS 3 times and lysed in 350 µl RNA lysis buffer per 6 well plate. This buffer directly inactivates RNases.
Total RNA from lungs was isolated by homogenizing approximately 20 mg of lung tissue directly in RNA lysis buffer using a Polytron homogenizer (Kinematics, Luzern, Switzerland). Prior to RNA isolation with the guanidinium isothiocyanate RNA isolation method using RNeasy® mini columns, the lysat was loaded onto a Qiashredder® column (Quiagen, Germany) in order to ensure a homogenous suspension. Samples for quantification with real-time polymerase chain reaction (real-time PCR) were additionally subjected to DNase digestion (RNase-free DNase Set, Quiagen, Hilden, Germany) during RNA isolation, because real-time PCR is extremely sensitive to smallest amounts of DNA. The purified RNA was eluated from the column with 50 µl of RNase free water under low salt conditions. Samples were taken for quantification of total RNA and verification of RNA integrity and RNA was stored at -85°C. RNA concentration was determined by measuring the absorption at 260 nm (A_{260}) and 280 nm (A_{280}) (Lambda Bio 20 Photometer, Perkin Elmer, Überlingen, Germany). The amount of RNA was calculated from the A_{260} value, the ratio A_{260}/A_{280} was used to specify the purity of RNA with ideal values between 1.8 and 2.0. Protein contaminations would generate high values at 280 nm and therefore the ratio A_{260}/A_{280} would be too low. Integrity of isolated RNA was checked subjecting 1 µg of total RNA to agarose gel electrophoresis (see 3.7.4), ethidium bromide staining and densitometric analysis (Kodak

Image Station, Kodak, Rochester, USA). The intensity ratio of ribosomal 28S and 18S RNA was used for evaluation of RNA integrity.

3.7 Reverse transcription - polymerase chain reaction

3.7.1 Solutions

10x TBE buffer

Tris	50 mM
Boric acid	50 mM
Na_2EDTA	0.5 mM

3.7.2 Primers

All primers were designed with Primer Express 2.0 software (PE Applied Biosystems) and obtained from MWG Biotech AG (Ebersberg, Germany).

NPR-A forward (human):	5'-CCT CAA GTC ATC CAA CTG CGT-3'
NPR-A reverse (human):	5'-GCA TAA ACG GTG TGT CCT TGC-3'
NPR-C forward (human):	5'-TGC GGC CGA ATG TCA AAT A-3'
NPR-C reverse (human):	5'-AGG CCA CAT GAT TTG GAC G-3'

3.7.3 Reverse transcription and polymerase chain reaction

RT-PCR was performed using the Access RT-PCR System Kit (Promega, Mannheim, Germany), which incorporates AMV Reverse Transcriptase (AMV RT) for first strand cDNA synthesis and *Thermus flavus* (*Tfl*) DNA Polymerase for second strand cDNA synthesis and DNA amplification in a single-tube reaction. 1 µg of total RNA was used for RT-PCR in a volume of 50 µl containing 1 mM $MgSO_4$, 1x AMV/*Tfl* reaction buffer, 200 µM of each dNTP, 1 µM of each primer, upstream and downstream, and AMV RT and *Tfl* DNA polymerase 0.1u/ µl each. First strand cDNA synthesis was carried out at 48℃ for 45 min and AMV RT inactivation for 2 min at 94℃. Subsequently second strand synthesis and PCR amplification (94℃ for 30 sec, 60℃ for 1 min, 68℃ for 2 min, 40 cycles) was performed.

3.7.4 Agarose gel electrophoresis

The PCR products were separated by agarose gel (1.2 %) (Seakem LE Agarose, BioWhittaker, Rockland, USA) electrophoresis (Owl Seperation Systems, Portsmouth, USA). Ethidium bromide was directly added to the agarose gel solution (1.0 µg/ml) and TBE was used as electrophoresis buffer. 5 µl of PCR product was subjected to electrophoresis using 6x blue/orange loading dye and 100 bp DNA ladder (both Promega, Mannheim, Germany) and performed for 2 h at 100 V. Bands were visualized with an image station (Kodak Image Station, Kodak, Rochester, USA) at 254 nm.

3.8 Real time PCR

Real-time polymerase chain reaction (PCR) is a method which is able to monitor the progress of DNA amplification. For real-time detection the Taqman® assay system was used. In this assay, a fluorescent reporter dye is utilised for visualization of the increasing amount of PCR product. The probe is an oligonucleotide which is labelled with a reporter dye at the 5' end and with a quencher dye at the 3' end. There is no detectable fluorescence when the probe is intact, because the quencher dye is close enough to the reporter dye. Through an additional 5' → 3' – exonuclease activity the Taq polymerase cleaves the probe and leads to an increase in fluorescence emission. The fluorescent emission is measured at each cycle. The first significant increase in fluorescent intensity during the exponential phase of fluorescence augmentation correlates with the initial amount of target template. Quantification of the results was performed by using hypoxanthine phospho-ribosyltransferase (HPRT) as a housekeeping gene (Pfaffl, 2001).

3.8.1 Primer and probe

All primers and probes were designed with Primer Express 2.0 software (PE Applied Biosystems) and obtained from biomers.net GmbH (Ulm, Germany).

TNF-α forward (mouse): 5'-TGG CCT CCC TCT CAT CAG TTC - 3'

TNF-α reverse (mouse): 5'-TTG GTG GTT TGC TAC GAC GTG – 3'

TNF-α probe (mouse): 5'-TGG CCC AGA CCC TCA CAC TCA GAT CAT C-3'

ICAM-1 forward (mouse): 5'-CTG CTG CTT TTG AAC AGA ATG G-3'

ICAM-1 reverse (mouse): 5'-TCT GTG ACA GCC AGA GGA AGT G-3'

ICAM-1 probe (mouse): 5'-AGA CAG CAT TTA CCC TCA G-3'

HPRT forward (mouse): 5'-GTT AAG CAG TAC AGC CCC AAA ATG-3'

HPRT reverse (mouse): 5'-AAA TCC AAC AAA GTC TGG CCT GTA-3'

HPRT probe (mouse): 5'-AGC TTG CTG GTG AAA AGG ACC TCT CGA AGT-3'

3.8.2 Reverse transcription

Reverse Transcription was performed using the DyNAmo™ Probe 2-Step qRT-PCR Kit (Finnzymes, Espoo, Finland) according to the manufacturers instructions. An amount of 600 ng of total RNA was subjected to reverse transcription using M-MuLV RNase H^- reverse transcriptase. There was no need for separate RNase treatment because the RNase H activity in the enzyme degrades RNA in the RNA-cDNA hybrid.

3.8.3 Real time PCR

Real-time PCR was performed using the DyNAmo™ Probe 2-Step qRT-PCR Kit (Finnzymes, Espoo, Finland) according to the manufacturers instructions. For real time detection the Taqman® Assay system was used.

3.9 Microscopy

3.9.1 Antibodies

Primary antibodies	*Diluted in*	*Dilution*	*manufacturer*
Anti F4/80 rat anti mouse	0.2 % BSA in PBS	1:100	Serotec, Düsseldorf, Germany
Anti TNF-α Rabbit anti mouse	0.2 % BSA in PBS	1:100	Endogen, Rockford, USA
Anti p65 polyclonal Rabbit anti mouse	0.2 % BSA in PBS	1:100	Santa Cruz, Heidelberg, Germany
Secondary antibodies	*Diluted in*	*Dilution*	*manufacturer*
Alexa Fluor® 488 (H+L) goat anti-rat IgG	0.2 % BSA in PBS	1:400	Molecular Probes, MoBiTec, Göttingen, Germany
Alexa Fluor® 647 (H+L) chicken anti-rabbit IgG	0.2 % BSA in PBS	1:400	Molecular Probes, MoBiTec, Göttingen, Germany
HOECHST dye 33342	0.2 % BSA in PBS	5 µg per slice	Sigma, Deisenhofen, Germany

table 2: Antibodies for tissue and cell staining

3.9.2 Staining of A549 cells

A549 cells were grown until confluence on glass coverslips (∅ 12 mm) in 24-well plates and treated as indicated in the respective figure legend. Afterwards cells were washed with PBS and fixed using a phosphate buffered formaldehyde solution (3 %) for 15 min. Cells were washed three times with PBS and permeabilized with Triton X-100 (0.2 %) for 2 min. After washing another three times with PBS, cells were treated with 0.2 % BSA solution for 20 min

in order to prevent unspecific binding of the antibodies. Subsequently cells were incubated with the primary antibody for 1 h (see ***table 2***), washed three times with PBS, and thereafter incubated with the secondary antibody (see ***table 2***) and HOECHST dye for 45 min. Cells were again washed three times with PBS, embedded in mounting medium (DakoCytomation, Hamburg, Germany) and placed onto glass objective slides.

3.9.3 Staining of lung tissue

For analysis of certain proteins in lung tissue, organs were snap-frozen in liquid nitrogen at the indicated times and cut into 10-12 µm sections. For staining of alveolar macrophages and TNF-α, slices were dried overnight at RT and subsequently fixed in 3 % formaldehyde for 15 min. In order to stain alveolar macrohages, an antibody against the murine F4/80 antigen was used. This 160kD glycoprotein is expressed by murine macrophages. Slices were washed three times with PBS and blocked with 1 % BSA for 20 min. This was followed by incubation with 100 µl oft the primary antibody (see ***table 2***) for 1 h at RT. After three washing steps, Slices were incubated with the corresponding secondary antibody (see ***table 2***) for 1h and again were washed three times with PBS. Finally, lung sections were covered with mounting medium (DakoCytomation GmbH, Hamburg, Germany) and dried overnight.

3.9.4 Confocal laser scanning microscopy

The major difference between conventional microscopy and CLSM is the confocal arrangement of an illumination pinhole and a conjugated detector pinhole which ensures that only information from the focal plane reaches the detector. Therefore, an up to 1.4x time's higher resolution can be obtained by using CLSM in comparison to conventional microscopy. Various lasers with different excitation wavelengths facilitate the colocalisation of different

fluorochromes. For analysis of lung tissue and A549 cells an LSM 510 Meta (Zeiss, Oberkochen, Germany) was used.

3.9.5 Staining for leukocyte infiltration

Hematoxylin and eosin (HE) staining was performed by Dr. Herbert Meissner (Institute for Pathology, University of Munich, Germany) as described previously (Gerwig et al., 2003). Leukocyte infiltration was investigated according to morphological characteristics of leukocytes.

3.10 Enzyme-linked immunosorbent assay (ELISA)

3.10.1 TNF-α measurement in mouse blood

Blood samples were centrifuged for 2 min at 4℃ at 13,000 rpm to separate the plasma from the cellular fraction. Measurement of TNF-α by ELISA was performed as described previously (Bohlinger et al., 1996) with an OptEIA Mouse TNF-α Elisa Set (Mono/Mono) (BD Biosciences, Heidelberg, Germany).

3.10.2 TNF-α measurement in whole lung lysates

TNF-*α* was determined on supernatants of lung homogenates by ELISA as described in 3.10.1. Supernatants of lung homogenates were obtained as described previously (Mueller et al., 2004).

3.11 Flow cytometry

Flow cytometry is a technique to analyze suspended individual cells in order to detect fluorescent stains. Cells flow through a focused laser beam in a laminar fluid stream. According to their size, granularity and stain intensity the incident laser beam is scattered and fluorescence can be measured.

3.11.1 Solutions

FACS buffer pH 7.37

NaCl	138.95 mM
KH_2PO_4	1.91 mM
Na_2HPO_4	16.55 mM
KCl	3.76 mM
LiCl	10.14 mM
NaN_3	3.08 mM
Na_2EDTA	0.967 mM

3.11.2 Preparation and staining of cells

Cells were grown until 90 % confluence in 24-well plates and were treated as indicated in the respective figure legend. Afterwards A549 where incubated with 100 µl T/E solution per well for 2 min at 37℃. The cells where gradually detached and the digestion of trypsin was stopped by transferring the cells into FACS tubes containing phosphate buffered formaldehyd solution (10 %) and incubating them for 15 min. Subsequently, cells were washed with PBS and incubated with a flurescent dye-labelled antibody against ICAM-1 (FITC-labelled mouse anti human CD54 IgG1, Biozol, Eching, Germany) in the dark for

20 min. After washing, cells were resuspended in PBS for flow cytometric analysis (FACSCalibur, BD Biosciences, Heidelberg, Germany).

3.12 Statistics

All experiments were performed at least three times unless indicated otherwise in the respective figure legend. Results were expressed as mean ± SEM. Statistical analysis was performed with GraphPad Prism 3.03 (GraphPad Software Inc. San Diego, USA). ANOVA with Bonferroni multiple comparison post-test for comparison of three or more groups or unpaired two-tailed Student t-test for comparison of two groups were used.

4 Results

4.1 Alveolar epithelial cells

4.1.1 A549 alveolar epithelial cells express NPR-A and NPR-C

Expression of natriuretic peptide receptors in alveolar epithelial cells *in vivo* and in primary isolated alveolar epithelial cells has been described previously (Tharaux et al., 1998). However, there existed no data concerning the expression of these receptors in the human alveolar epithelial cell line A549 which we meant to use for these initial experiments. Therefore, we carried out RT-PCR for NPR-A and NPR-C, the major receptors ANP binds to. Due to the fact that expression of NPR-A and NPR-C in endothelial cells has been described previously (Inagami et al., 1995), human umbilical vein endothelial cells (HUVECs) were used as positive control. RT- PCR experiments revealed the presence of both NPR-A and NPR-C mRNA in A549 alveolar epithelial cells (see **figure 12**).

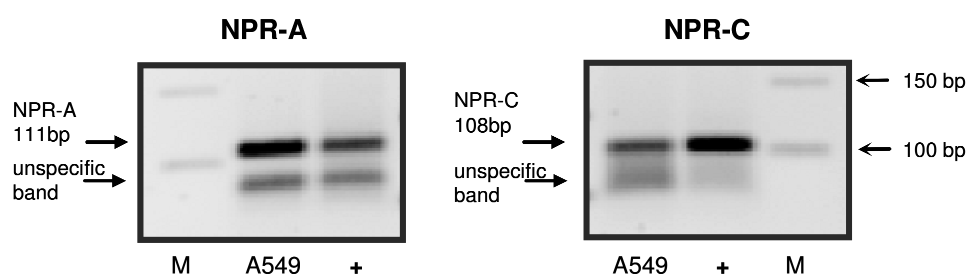

figure 12 ***A549 alveolar epithelial cells express NPR-A and NPR-C***
RNA from A549 alveolar epithelial cells and from HUVECs, used as positive control (+), was isolated as described in 3.5. Afterwards, 1 µg of total RNA was subjected to RT-PCR and subsequent agarose gel electrophoresis (M = marker) (see 3.7 for details).

4.1.2 Influence of ANP on TNF-α induced NF-κB activation

ANP was shown to diminish TNF-α induced activation of pro-inflammatory transcription factors in endothelial cells (Kiemer et al., 2002e). NF-κB is one of the most important mediators regarding TNF-α mediated signalling. In order to determine a possible anti-inflammatory activity of ANP on alveolar epithelial cells, we examined the effects of ANP on TNF-α induced DNA binding activity of NF-κB with EMSA.

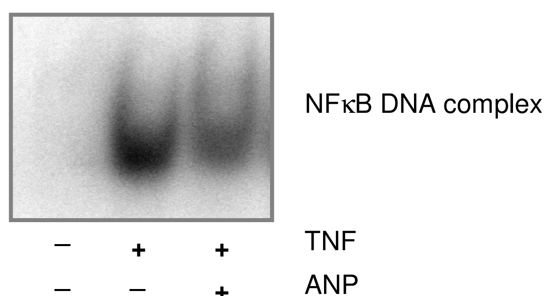

figure 13 ***ANP pretreatment inhibits TNF-α induced NF-κB binding activity***
Cells were either left untreated or were pretreated with ANP (10^{-6}M) for 15 min. Where indicated, cells were treated with TNF-α (10 ng/ml) for 15 min. NF-κB DNA binding activity was measured by EMSA as described in 3.4.

TNF-α already caused increasing levels of NF-κB DNA complex as soon as 15 min. Pretreatment of epithelial cells with 10^{-6} M ANP markedly reduced this NF-κB DNA binding activity as shown in ***figure 13***. Sole treatment with ANP had no effect on NF-κB activation (data not shown). Specificity of DNA-complex was confirmed by competition with a 100-fold excess of unlabelled NF-κB (positive control) and AP-1(negative control) binding sequences (data not shown).

Next we performed immunohistochemistry of the p65 subunit of NF-κB in order examine whether the diminished NF-κB DNA binding activity observed by EMSA is associated with a reduced translocation of NF-κB into the nucleus. A549 cells were investigated by staining of

the p65 subunit in TNF-α treated and ANP preconditioned cells and analyzing them by confocal microscopy.

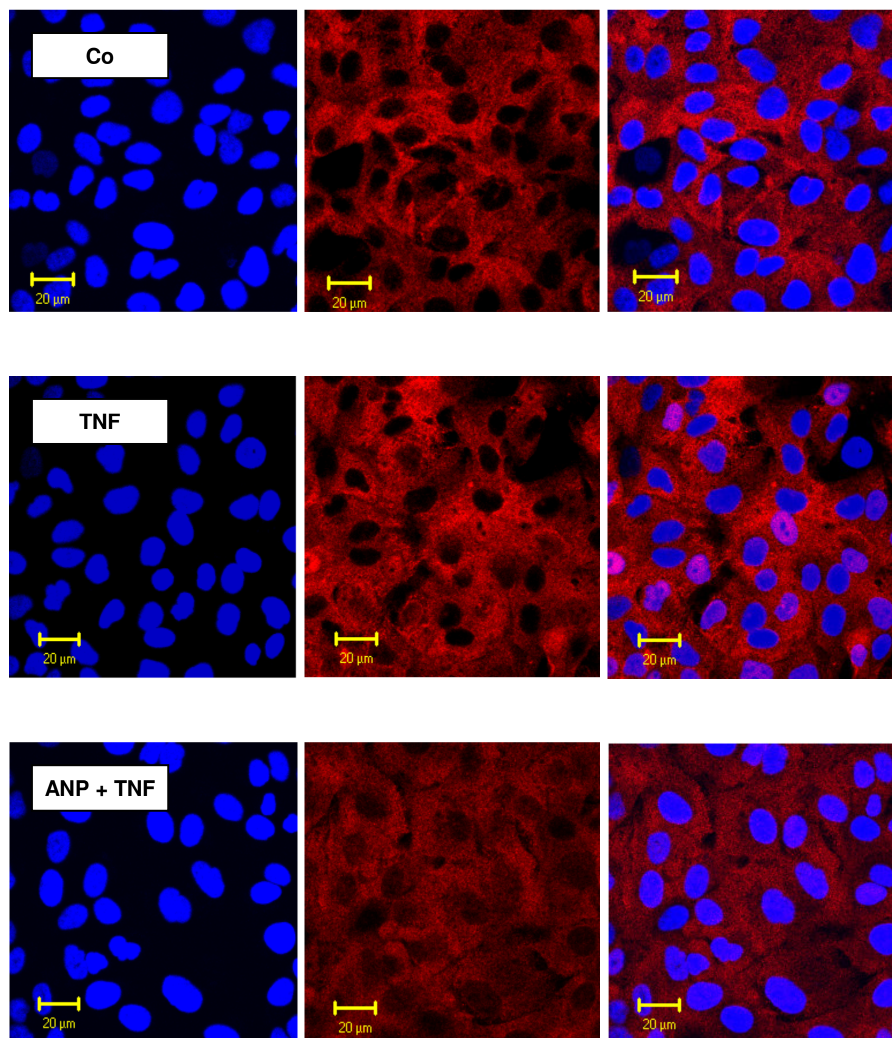

figure 14 ***Influence of ANP on TNF-α induced translocation of p65***
A549 cells were treated with TNF-α (10 ng/ml) for 30 min or left untreated (Co). Where indicated, cells received pretreatment with ANP (10^{-6} M) for 15 min. Subsequently cells were stained for p65 and nuclei as described in 3.9.2 and CLSM was performed as described in 3.9.4.
Blue: nuclei, red: p65

TNF-α treatment (10 ng/ml) leads to p65 translocation into the nuclei of alveolar epithelial cells compared to untreated cells, were p65 remained in the cytoplasm. Preconditioning with ANP (10^{-6} M) resulted in a reduced number of cells with translocated p65.

4.1.3 Influence of ANP on TNF-α induced AP-1 activation

Another major transcription factor mediating TNF-α induced pro-inflammatory events is the activator protein-1. Because AP-1 is activated by TNF-α in our cell model, we investigated the property of ANP pretreatment to reduce AP-1 binding to DNA in the nucleus by EMSA.

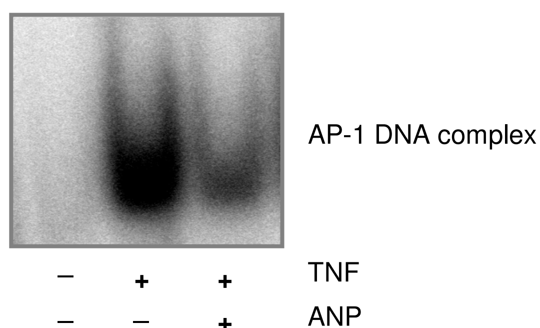

figure 15 ***Preconditioning with ANP reduces TNF-α induced AP-1 induction***
Cells were either left untreated or were pre-treated with ANP (10^{-6}M) for 15 min. Where indicated, cells were treated with TNF-α (10 ng/ml) for 15 min. AP-1 DNA binding activity was measured by EMSA as described in 3.4

Treatment of A549 cells with TNF-α (10 ng/ml) markedly induced AP-1 DNA binding activity after 15 min. Administration of ANP (10^{-6} M) 15 min prior to TNF-α treatment resulted in reduced levels of AP-1 DNA complex detected by EMSA. Specificity of DNA-complex was confirmed by competition with a 100-fold excess of unlabelled AP-1 (positive control) and NF-κB (negative control) binding sequences (data not shown).

4.1.4 Effects of ANP on TNF-α induced ICAM-1 expression

The adhesion molecule ICAM-1 has a prominent role in orchestrating epithelial inflammation. NF-κB and AP-1 both strongly participate in regulation of TNF-α dependent ICAM-1 transcription. Therefore, we examined the influence of ANP pretreatment on TNF-α mediated ICAM-1 expression by flow cytometry.

ICAM-1 surface expression was significantly induced by TNF-α (10 ng/ml and 2 ng/ml) as shown in ***figure 16***. Protein expression was already detectable after 2 h of TNF-α treatment and increased steadily up to 24 h. ANP pretreatment did not effect TNF-α induced ICAM-1 expression

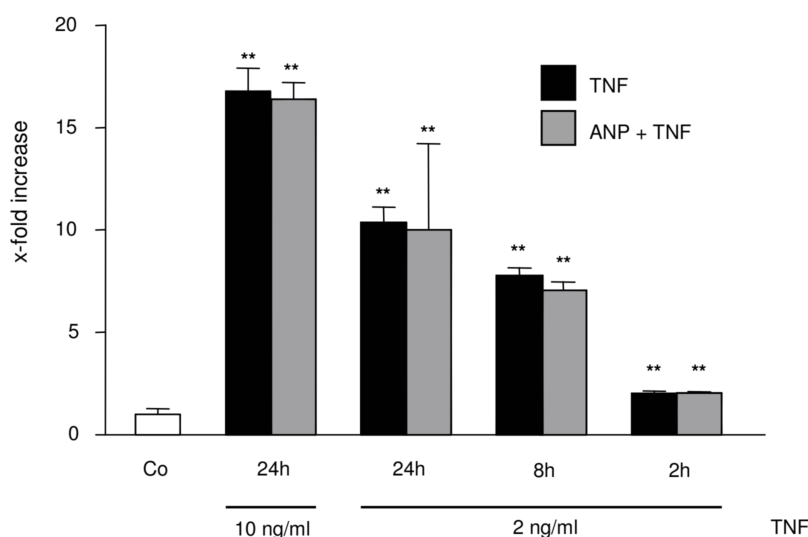

figure 16 No effect of ANP on TNF-α induced ICAM-1 expression
*Cells were treated with TNF-α (10 ng/ml or 2 ng/ml) with or without 15 min preconditioning with ANP (10^{-6} M) for 24 h, 8 h or 2 h. After the indicated times cells were harvested and stained afterwards with FITC-labelled ICAM-1 antibody for flow cytometry (see 3.11). **p ≤0.01 vs. Co*

4.2 Effects of ANP during LPS-induced septic shock in the murine lung

In order to investigate the potential in vivo relevance of our findings, we investigated the effects of ANP in a murine model of septic shock with special focus on the lung.

After treatment of mice and lethal anaesthesia as described in 3.2.3.2, we examined in the lung two major transcription factors NF-κB and AP-1, which are known to have a major impact on inflammatory processes.

4.2.1 Effects of ANP preconditioning on NF-κB binding activity

NF-κB has great importance in LPS mediated signalling (see2.5) Therefore, we investigated the DNA binding activity of this transcription factor in the lung. In the NaCl treated control group only basal levels of NF-κB binding activity were detectable. Treatment with ANP alone had also no effect on this transcription factor. As shown in ***figure 17***, LPS treatment caused a marked increase in NF-κB DNA binding activity after 15 min and 30 min LPS challenge. Interestingly, preconditioning with ANP was able to protect the lung from this increase and caused a remarkable lowering of binding activity. This decline was detectable 15 min after LPS challenge and increased further 30 min after LPS administration. Specificity of DNA-complex was confirmed by competition with a 100-fold excess of unlabelled AP-1(positive control) and NF-κB (negative control) binding sequences (data not shown).

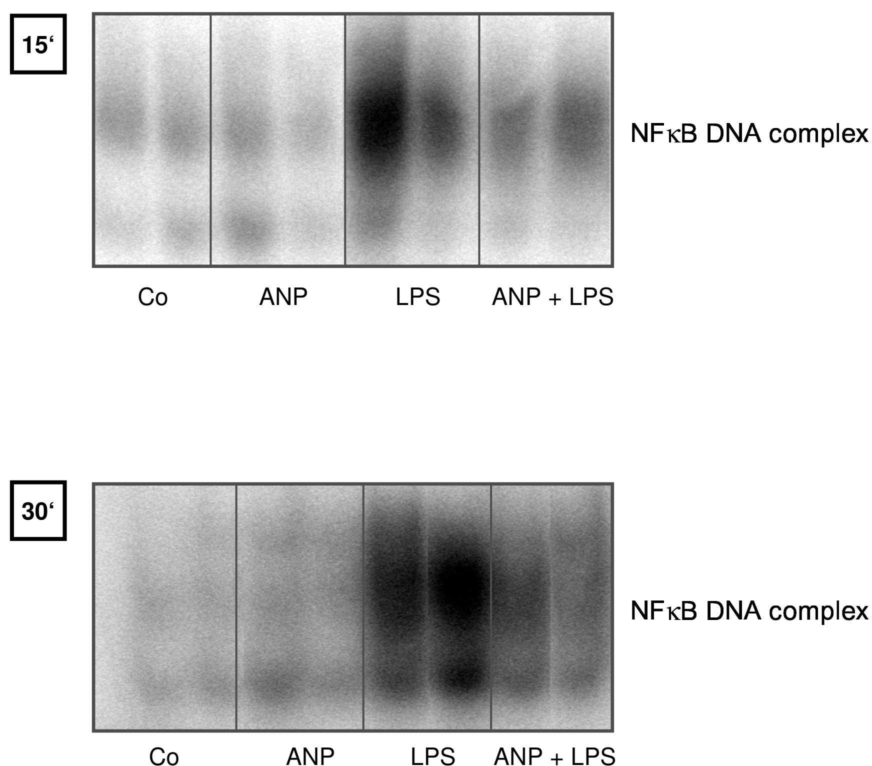

figure 17 ***Preconditioning with ANP leads to a reduction of LPS-induced NF-κB binding activity after 15 and 30 min.*** *Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.). or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 15 min or 30 min after LPS injection, snap-frozen in liquid nitrogen, homogenized and investigated by EMSA as described in 3.4.. Results show one representative EMSA out of two experiments (four lungs in each treatment group).*

4.2.1.1 Effects of ANP on phosphorylation and degradation of IκBα

Having observed an influence of ANP on LPS-induced NF-κB translocation, we were now interested in the possible upstream mechanism responsible for ANP-mediated inhibition of NF-κB. Phosphorylation and subsequent proteasomal degradation if the cytoplasmatic inhibitor of NF-κB protein α (IκBα) is a deciding event in LPS-induced NF-κB activation (see ***figure 7***). In order to determine a possible effect of ANP on phosphorylation and degradation

of IκBα we performed Western Blot analysis as described in 3.3.3 for phosphorylated and non-phosphorylated forms of IκBα.

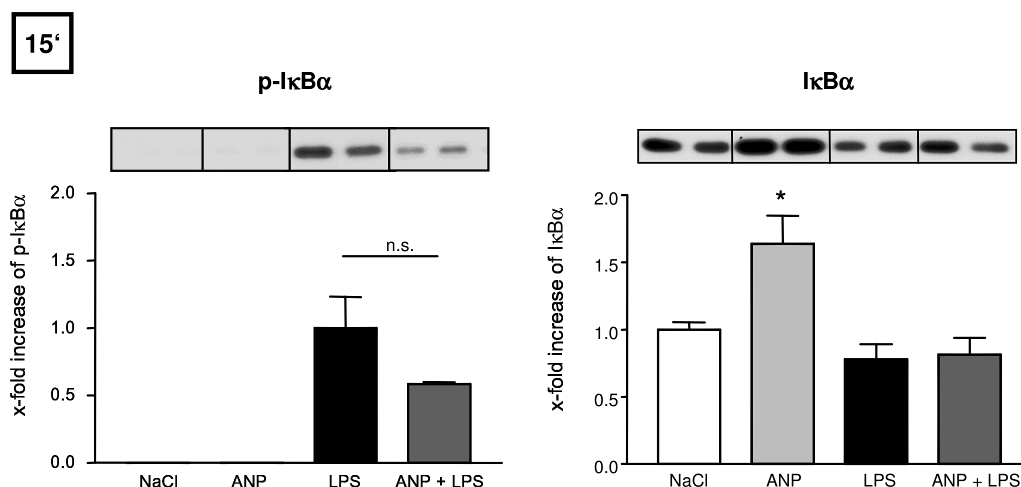

figure 18 ***ANP pre-treatment reduces LPS-induced IκBα phosphorylation and induces total IκBα protein levels.*** *Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1mg/kg b.w.). or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 15 min 30 after LPS or NaCl injection, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in3.3. Representative Western Blots with two lungs out of five are shown. * $p \leq 0.05$ vs. NaCl.*

There were no detectable levels of phosphorylated IκBα protein in the control and ANP treated group of animals (**figure 18**). Phosphorylated IκBα protein occurred after 15 min in LPS treated mice. This phosphorylation was slightly reduced in ANP preconditioned mice. Degradation of IκBα was not yet detectable after 15 min. Astonishingly elevated levels of total IκBα protein could be detected in animals who received only ANP. Degradation of IκBα occurred 30 min after LPS administration as shown in **figure 19**. In contrast, administration of ANP prior to LPS caused only a slight reduction of IκBα protein levels.

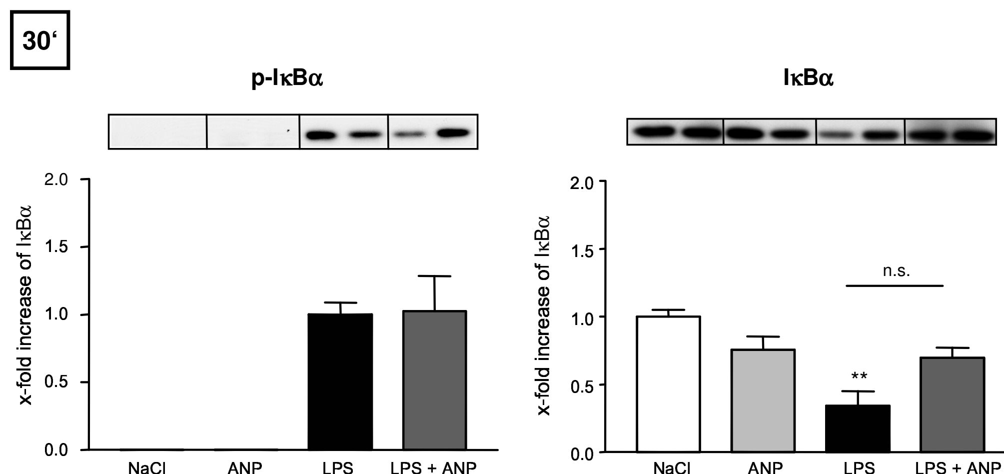

figure 19 Preconditioning with ANP leads to a reduction of LPS-induced degradation of IκBα 30 min after LPS injection. *Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 30 min after LPS injection, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in 3.3. Representative Western Blots with two lungs out of five are shown. * $p \leq 0.05$ vs. NaCl.*

4.2.2 ANP effects on AP-1 DNA binding activity

Another major transcription factor, which is involved in LPS-induced pro-inflammatory pathways, is the activator protein-1. Our previous experiments with alveolar epithelial cells (see 4.1.3) revealed a commanding potency of ANP to influence this pathway. On this account, we investigated the AP-1 DNA binding activity in mouse lung at different time-points.

Basal AP-1 DNA binding activity was low in control animals. 15 min after LPS administration, no detectable levels of AP-1 were found in the lung (data not shown). DNA binding activity increased after 30 min in LPS treated animals vs. animals in the control group as illustrated

in ***figure 20***. ANP alone did not alter AP-1 DNA complex in the nucleus. Preconditioned animals showed a remarkable reduction in AP-1 DNA binding activity at this point in time.

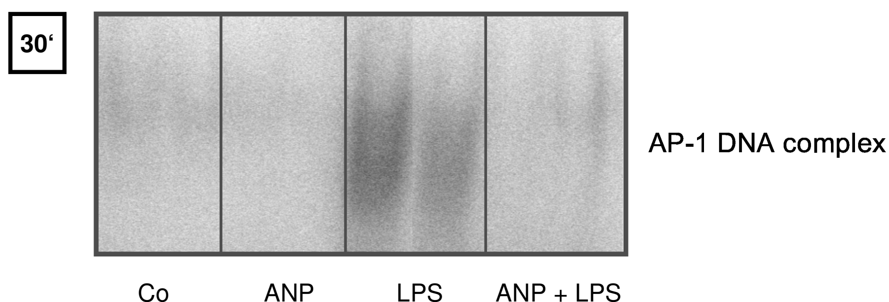

figure 20 ***ANP pretreatment leads to a reduction of LPS-induced AP-1 binding activity after 30 min.*** *Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 30 min after LPS injection, snap-frozen in liquid nitrogen, homogenized and investigated by EMSA as described in 3.4.Results show one representative EMSA out of two experiments (four lungs in each treatment group).*

4.2.3 Influence of ANP on p38 MAPK in LPS treated lung

In order to clarify the possible mechanism by which ANP impairs LPS-induced lung inflammation, we investigated two possible pathways. Several mitogen activated kinases (MAPK) have been described to participate in LPS mediated signalling. As summarized in ***figure 7*** and 2.6, the p38 MAPK is involved in the signal transduction of LPS leading to activation of the transcription factors NF-κB and AP-1. On this account, we examined a possible role of p38 MAPK in ANP-mediated inhibition of this transcription factors in LPS-induced lung injury first.

4.2.3.1 Activation of p38 MAPK in LPS-induced lung inflammation

The time course shown in ***figure 21*** demonstrates increasing p38 MAPK activation in response to LPS in murine lung. MAPK activation was detected by immunoblotting with phospho-specific antibodies (see 3.3.3). This activation occurred very fast peaking already at 15 min after LPS administration and is still detectable after 90 min.

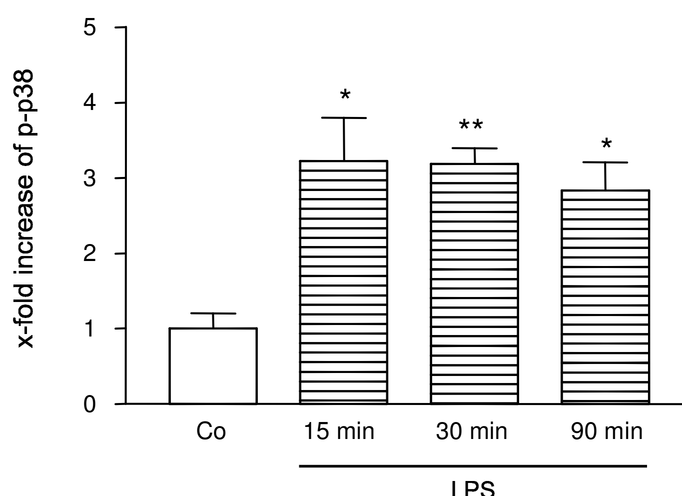

figure 21 ***LPS treatment provokes p38 MAPK phosphorylation in the lung***
*Animals received an i.v. injection of NaCl 0.9 % (Co) or i.p. injection of LPS (1 mg/kg b.w.). Lungs were excised at the indicated time points, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in 3.3. ** $p \leq 0.01$ vs. Co and * $p \leq 0.05$ vs Co.*

4.2.3.2 ANP effects on LPS-induced p38 MAPK activation

Basal levels of phosphorylated p38 MAPK are low in NaCl and ANP treated animals. Pretreatment with ANP was able to reduce significantly LPS-induced activation of p38 MAPK in lung injury as shown by Western Blot (***figure 22***). Interestingly, ANP alone seemed to slightly elevate p38 MAPK phosphorylation in comparison to the NaCl group.

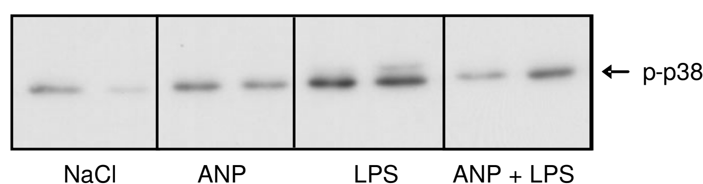

figure 22 **_ANP pretreatment minors LPS-induced phosphorylation of p38 MAPK_**
Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 15 min after LPS injection, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in 3.3. One representative Western Blot out of two with four lungs in each treatment group is shown.

4.2.3.3 Influence of ANP treatment on p38 MAPK activation

First data indicated a possible influence of ANP treatment on basal p38 MAPK activation. Furthermore, protective effects of ANP via activation of p38 MAPK have been described previously in ischemia reperfusion injury in the liver. Therefore, we aimed to investigate the effect of ANP treatment on p38 MAPK activation in the lung.

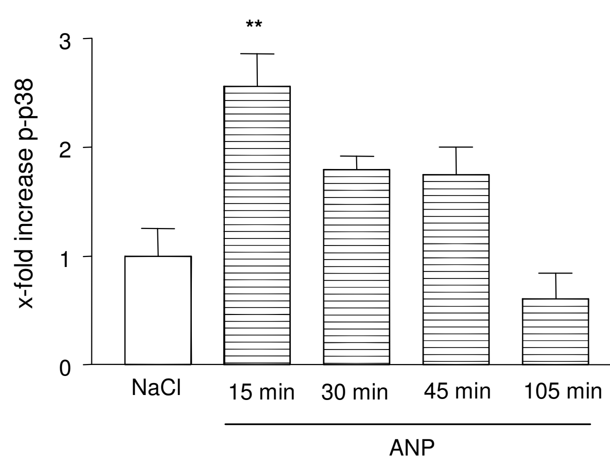

figure 23 **_Effect of ANP treatment on p38 MAPK phosphorylation in the lung_**
*Animals received an i.v. injection of NaCl 0.9 % (Co) or i.v. injection of ANP (5 µg/kg b.w.). Lungs were excised at the indicated time points, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in 3.3. ** $p \leq 0.01$ vs NaCl.*

As shown in ***figure 23***, Western Blot analysis of dual phosphorylated p38 MAPK revealed a distinct increase in p38 MAPK phosphorylation after 15 min ANP treatment. This effect is still noticeable 30 min and 45 min after ANP administration and is abrogated after 105 min. In order to corroborate this effect we examined the in vitro phosphorylation activity by p38 MAPK in ANP treated lungs. ***figure 24*** shows that ANP treatment of mice is able to increase the phosphorylation activity of ANP in the lung.

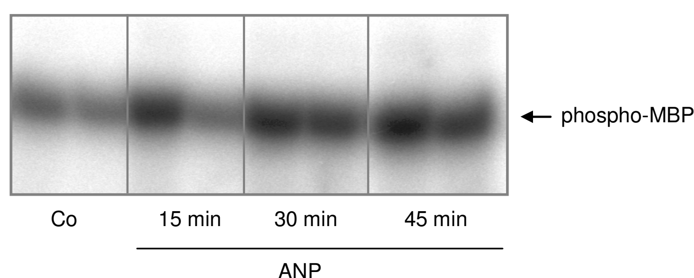

figure 24 ***Effect of ANP on in vitro phosphorylation activity by p38 MAPK***
Animals received an i.v. injection of NaCl 0.9 % (Co) or i.v. injection of ANP (5 µg/kg b.w.). Lungs were excised at the indicated time points, snap-frozen in liquid nitrogen, homogenized and investigated by in vitro phosphorylation assay by p38 MAPK as described in 3.5.

4.2.4 Influence of Akt kinase in LPS treated lung

As a second possible pathway involved in ANP-mediated reduction of NF-κB and AP-1 activation in lung inflammation, we led our interest on the protein kinase Akt, also known as protein kinase B (PKB). New insights in LPS-mediated signal transduction revealed a major role in regulating the response to pro-inflammatory stimuli.

4.2.4.1 Activation of Akt in LPS-induced lung inflammation

First, we wanted to examine potential effects of LPS on Akt activation during murine sepsis in the lung. Therefore, immunoblotting was performed for the phosphorylated form of Akt as described in 3.3.3. ***figure 25*** demonstrates that activation of Akt occurred after 15 min in LPS-induced lung injury and lasted until 90 min after LPS administration.

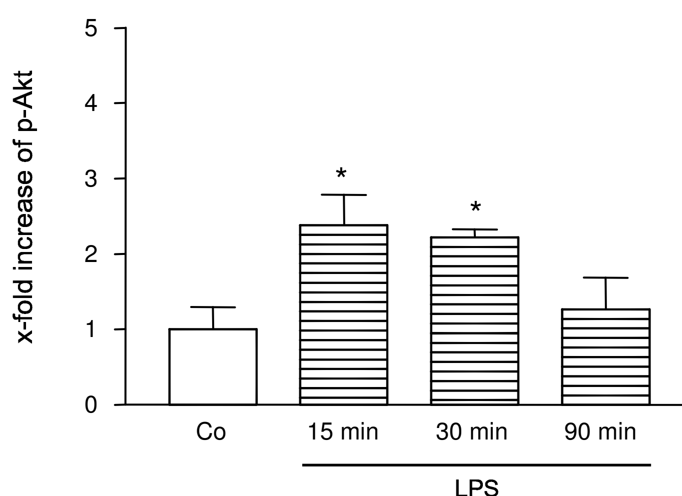

figure 25 ***Akt phosphorylation occurs in the lung after LPS challenge***
*Animals received an i.v. injection of NaCl 0.9 % (Co) or i.p. injection of LPS (1 mg/kg b.w.). Lungs were excised at the indicated time points, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in 3.3. * $p \leq 0.05$ vs. Co*

4.2.4.2 ANP effects on Akt activation

Now we were interested in possible effects of ANP regarding LPS-induced Akt activation.

As illustrated in ***figure 26***, ANP pretreatment was able to reduce LPS-induced Akt phosphorylation. ANP administration alone also seemed to increase Akt activation in the lung.

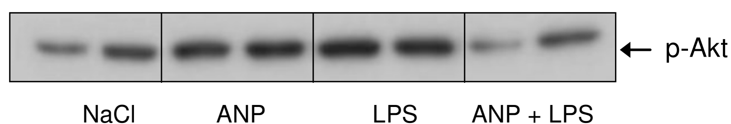

figure 26 ***Preconditioning with ANP leads to a reduction of LPS-induced phosphorylation of Akt after 15 min.*** *Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; ANP: ANP i.v. after 15 min NaCl i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 30 min after LPS injection, snap-frozen in liquid nitrogen, homogenized and investigated by Western Blot as described in 3.3. One representative Western Blot out of two with four lungs in each treatment group is shown.*

4.2.5 Expression of ICAM-1

ICAM-1 is upregulated in response to LPS, TNF-α and other inflammatory mediators occurring in the lung during infection. NF-κB and AP-1, which were shown to be influenced by ANP, have major impact on regulating ICAM-1 expression. On this account, we investigated expression of ICAM-1 in the lung of LPS treated mice and the influence of ANP preconditioning on this important initial process. LPS treatment increases ICAM-1 mRNA expression in mouse lung, beginning after 30 min and accelerating after 90 min. As shown in ***figure 27***, ANP preconditioning was able to reduce ICAM-1 mRNA expression at early points in time, but no difference concerning mRNA expression could be observed 90 min after LPS treatment.

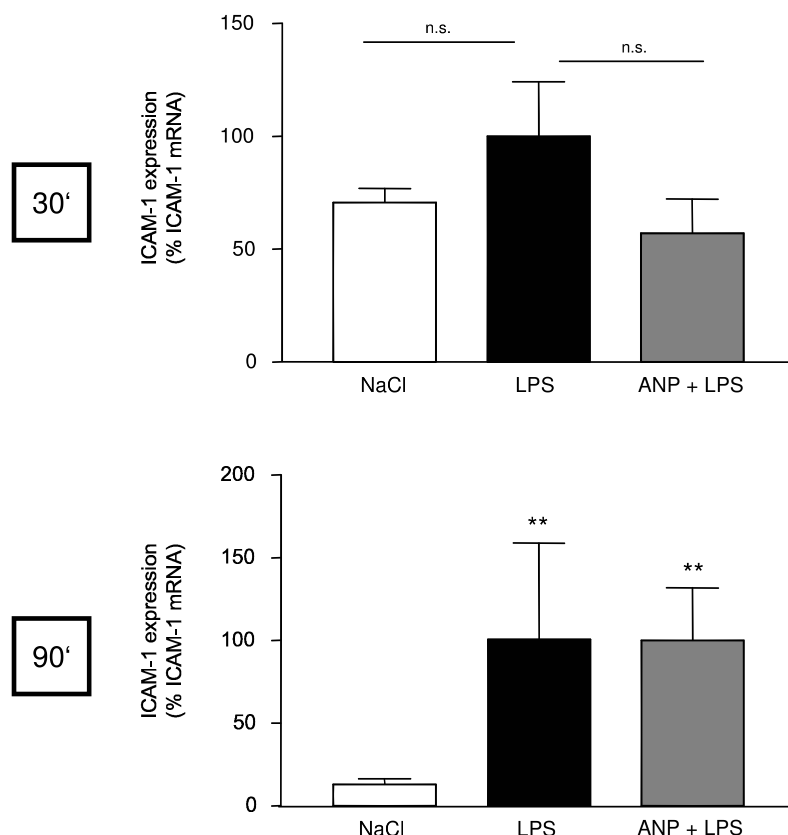

figure 27 ***ANP pretreatment mildly decreases ICAM-1 expression during endotoxaemia after 30min. No difference in expression levels can be observed after 90 min*** *Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 30 min and 90 min after LPS injection and snap-frozen in liquid nitrogen. RNA was extracted and Real-time PCR was performed as described in 3.7.*

4.2.5.1 Leukocyte infiltration

Infiltration of leukocytes into the alveolar space is an important process regarding inflammatory processes in the lung. A major requirement for this event is the expression of adhesion molecules like ICAM-1 in vascular endothelium and respiratory epithelium, in which ANP has shown to be able to interfere. Therefore, we investigated lungs after 30 min and

90 min LPS treatment. At the indicated time points, we were not able to detect any signs of leukocyte infiltration into the alveolar space.

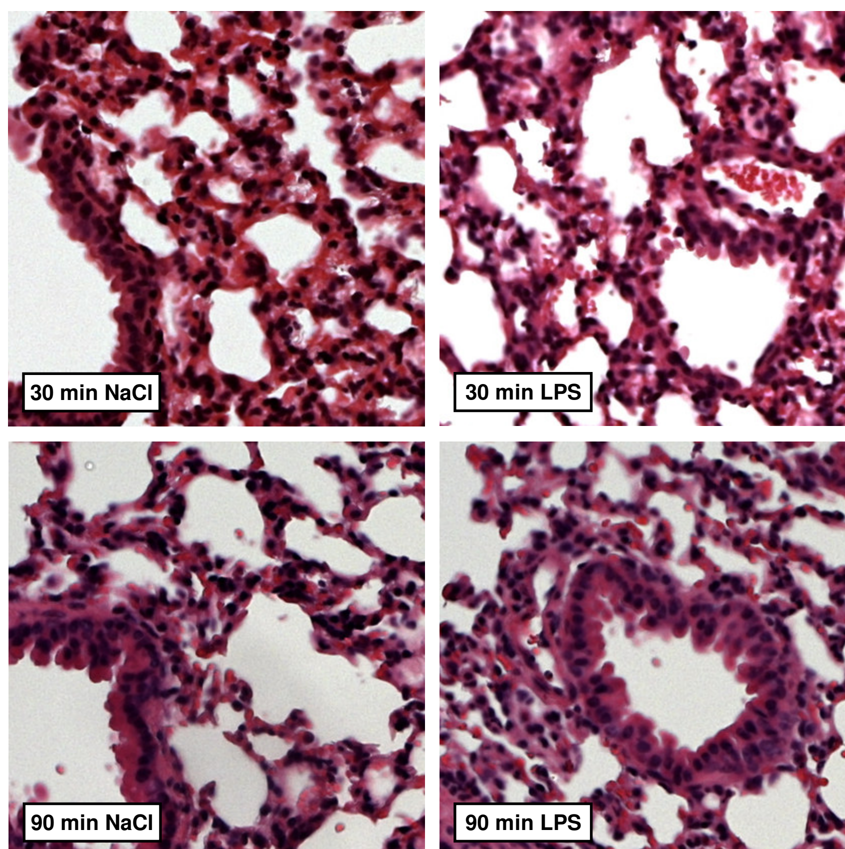

figure 28: ***Leukocyte infiltration 30 und 90 min (100x)*** *Animals were injected intravenously with either NaCl (Co) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.). or NaCl (0.9 %) injection. Co: NaCl treated i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; Lungs were excised 30 min and 90 min after LPS injection, stored in formalin and embedded in paraffin. Slices were stained with haematoxylin and eosin (for details see 3.9.5).*

4.2.6 TNF-α in LPS-induced lung inflammation

TNF-α is an important pro-inflammatory cytokine produced by various cells types, which has an outstanding role in the onset of sepsis. Because the NF-κB pathway is the predominant pathway in regulating the transcription of TNF-α, we were now interested in the effects of ANP regarding LPS-induced TNF-α expression in our model of sepsis.

4.2.6.1 Influence of ANP on serum levels and whole lung expression of TNF-α

The following animal experiments were kindly performed by Dr. Martin Lehner (Biochemical Pharmacology, University of Konstanz).

In order to investigate a potential effect of ANP on serum TNF-α levels, mice were pretreated with different doses of ANP 15 min prior to LPS administration (see ***figure 29***) and plasma TNF-α levels of TNF-α were obtained by ELISA as described in 3.10. As illustrated in ***figure 29**,* ANP preconditioning dramatically reduced LPS-induced TNF-α serum levels in each administered concentration.

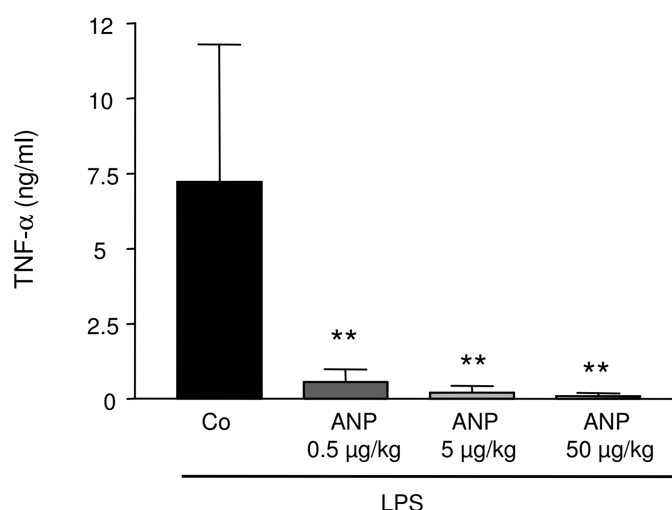

figure 29 ***ANP preconditioning perspicuously diminishes TNF-α serum levels in a murine model of sepsis.*** *Animals were injected i.p. with either NaCl (Co) or ANP (50 µg/kg b.w., 5 µg/kg b.w. or 0.5 µg/kg b.w.) prior to i.p. LPS administration (300 µg/kg b.w.). After 120 min heart blood was obtained and ELISA was performed as described in 3.2.3.1 and 3.10.1 .** $p \leq 0.01$ vs. Co*

Next we wanted to examine whether ANP has also an effect on TNF-α protein levels in the lung. Therefore, lungs were excised after the indicated times (for detail see ***figure 30***) and ELISA was performed in whole lung lysates (3.10.2). ANP pretreatment showed only a minor reduction of LPS-induced TNF-α expression in the whole murine lung as shown in ***figure 30***.

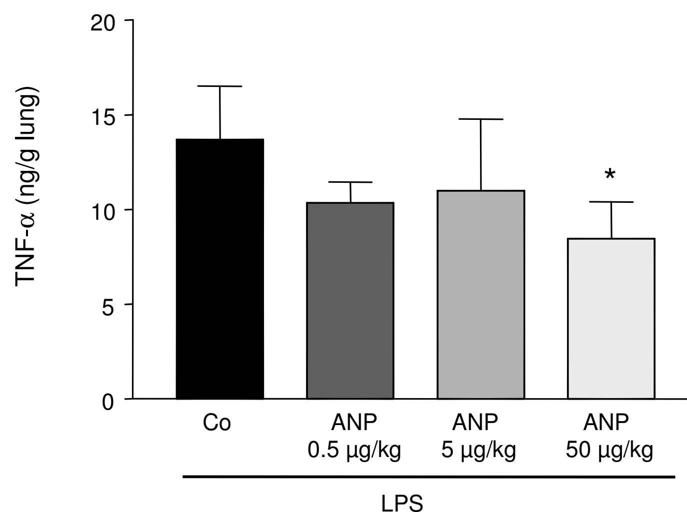

figure 30 ***TNF-α levels in whole lung lysates of mice being pre-treated with ANP are mildly reduced in comparison to lung lysates of mice which only received LPS.*** *Animals were injected i.v. with either NaCl (Co) or ANP (50 µg/kg b.w., 5 µg/kg b.w. or 0.5 µg/kg b.w.) prior to i.p. LPS administration (300 µg/kg b.w.). After 120 min lungs were excised and ELISA was performed as described in 3.10.2 . * $p \leq 0.05$ vs. Co*

4.2.6.2 Localisation of TNF-α in LPS-induced lung inflammation

Several cell types in the lung have been described being able to produce pro-inflammatory mediators like cytokines. In order to determine, which cell type in the lung is involved in TNF-α expression in our model, and if ANP may have a effect on TNF-α expression in these cells, lung tissue was stained for TNF-α and for the F4/80 antigen expressed by macrophages. As shown in ***figure 31***, intracellular TNF-α could be detected after 90 min in LPS treated lungs. Co-staining of macrophages revealed that TNF-α seems to be expressed primarily in alveolar macrophages. There was no detectable difference in TNF-α expression between LPS treated lungs and lungs, which received ANP preconditioning.

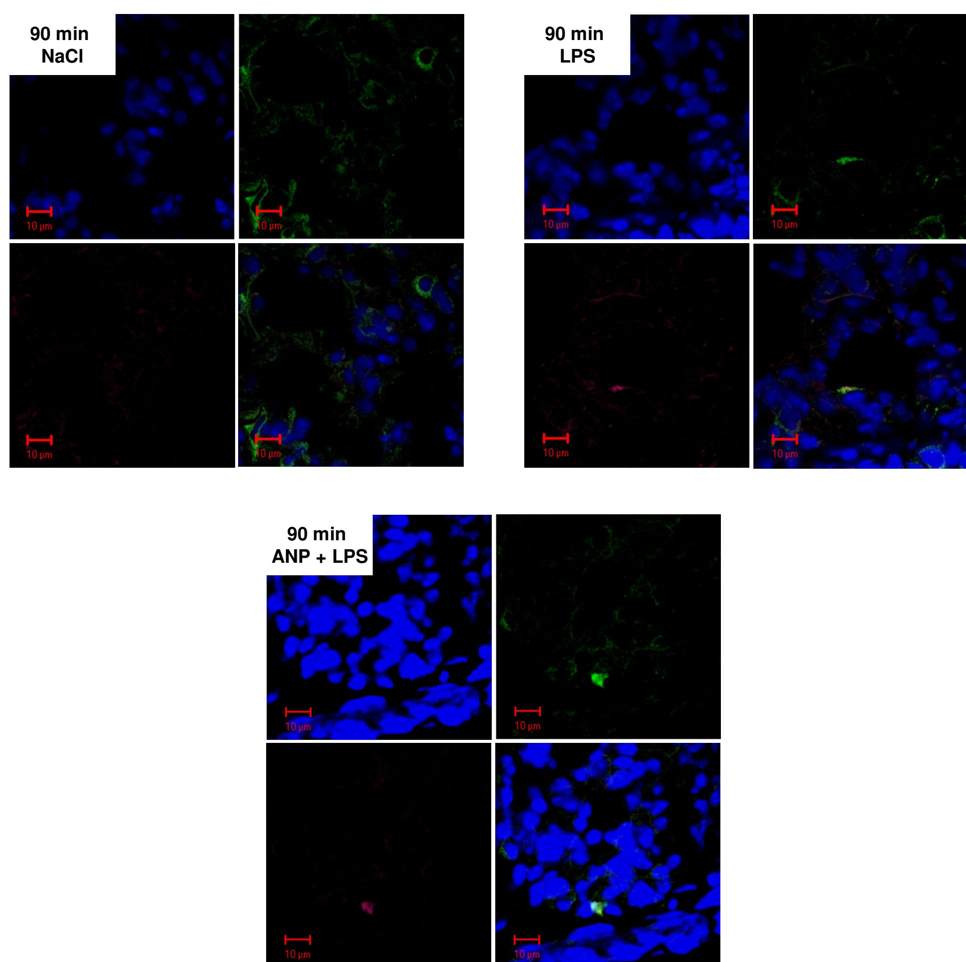

figure 31 ***TNF-α expressing cells in murine lung during LPS-induced sepsis*** *Animals were injected intravenously with either NaCl (Co) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; Lungs were excised 30 min and 90 min after LPS injection and snap-frozen in liquid nitrogen. Slices were stained and CLSM was performed as described in3.9.3 and 3.9.4.*
Blue: nuclei, green: alveolar macrophages, magenta: TNF-α

4.2.6.3 Effects of ANP on LPS-induced TNF-α expression

In previous experiments (see 4.2.6.1), ANP slightly decreased TNF-α measured in whole lung lysates. In order to investigate this observation in our model of murine sepsis, lung tissue was investigated with real-time PCR for TNF-α expression.

Basal levels of TNF-α were extremely low in control animals. LPS administration rapidly induced TNF-α mRNA expression in the lung after 30 min and is still there 90 min after LPS administration as shown in ***figure 32***. ANP showed no significant effect on TNF-α mRNA expression in our model of murine sepsis. After 30 min, a slight but not significant reduction of TNF-α mRNA could be observed. This minor decrease could not be detected after 90 min of LPS treatment.

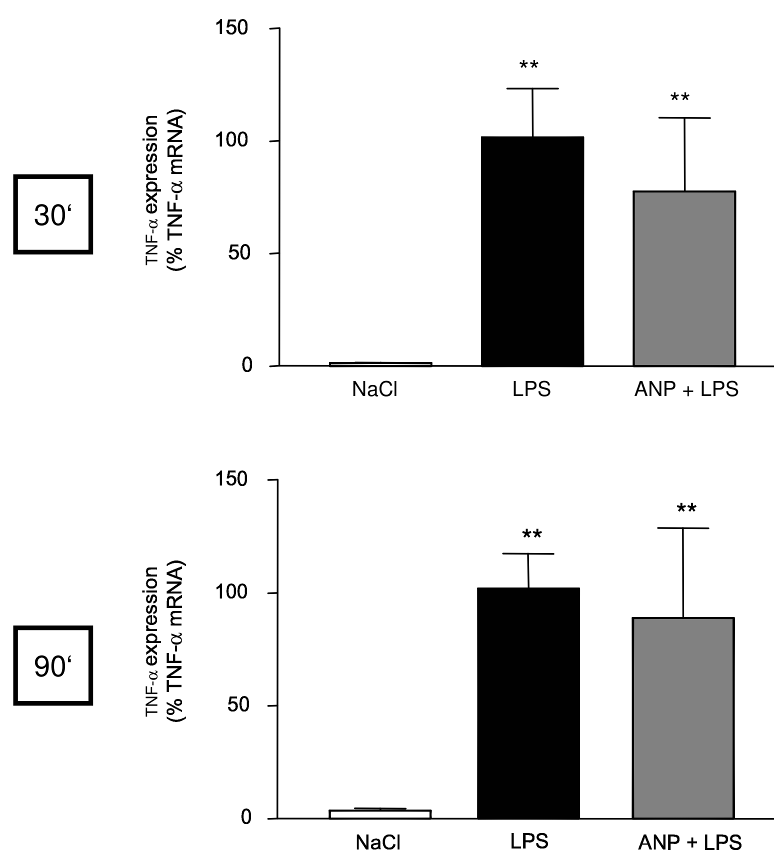

figure 32 ANP has no significant effect on TNF-α expression in endotoxaemia
Animals were injected intravenously with either NaCl (Co) or ANP (5 µg/kg b.w.) 15 min prior to i.p. LPS challenge (1 mg/kg b.w.) or NaCl (0.9 %) injection. Co: NaCl treated i.v.; LPS: NaCl i.v., after 15 min LPS i.p.; ANP + LPS: ANP i.v., 15 min later LPS i.p.. Lungs were excised 30 and 90 min after LPS injection and snap-frozen in liquid nitrogen. RNA was extracted and Real-time PCR was performed as described in 3.7.
** $p \leq 0.01$ vs. NaCl

5 Discussion

5.1 Alveolar epithelial cells

5.1.1 A549 alveolar epithelial cells express NPR-A and NPR-C

In the year 1977, Mason and Williams postulated the concept of alveolar type II cells being a defender of the alveolus (Mason and Williams, 1977). Today it is well known, that synthesis, secretion and recycling of pulmonary surfactant, and thereby regulation of pulmonary tension, is not the only important function of these cells. These cells have also important functions in maintaining alveolar fluid balance, coagulation, fibrinolysis and host defence. Additionally, they crucially contribute to epithelial tissue repair by their ability to differentiate to alveolar type I cells, which form the epithelial component of the thin-air-blood barrier (reviewed in (Fehrenbach, 2001)). Nowadays it is well established, that the lung is not only an extra-atrial source of ANP, but that it also expresses all three natriuretic peptide receptors (Perreault and Gutkowska, 1995). Radioautographic localisation of ^{125}I-ANP after infusion revealed high-end-labelling in the lung particularly in alveolar epithelial cells (Geary et al., 1993). In addition, activation of particulate GC could be observed morphologically with electron microscopy in rat alveolar type II cells (Rambotti and Spreca, 1991). These studies indicate the presence of NPR-A and NPR-B receptors in these cells. Recent studies showed the presence of functional NPR-A und NPR-B, but not the clearance receptor NPR-C in cultured rat alveolar type II cells (Tharaux et al., 1998). Interestingly, in another study ANP was shown to induce a dose dependent accumulation of cGMP, reduced ligand stimulated adenylyl cyclase activity and prevented cAMP accumulation in isolated rat alveolar type II cells. Moreover, ANP inhibited surfactant secretion in these experiments (Panchenko et al., 1998). The observed reduction of cAMP accumulation suggests the presence of a functional NPR-C receptor. This observation disagrees wtih the findings of Tharaux and co-workers. The human cell line A549 is one of the best characterised alveolar epithelial type II cell lines

and is routinely used for many experiments concerning the alveolar epithelium in response to inflammatory stimuli. Stimulation of these cells with ANP lead to a significant increase in intracellular cGMP and subsequent transforming growth factor β (TGF-β) release (Bellocq et al., 1999), but up to now no reports have been published verifying the presence of natriuretic peptide receptors in these cells. Investigating isolated mRNA from these cells, we were able to reveal the expression of natriuretic peptide receptor A and natriuretic peptide receptor C in A549 cells (*figure 12*). Because we focused our interest on possible effects of one natriuretic peptide, ANP, natriuretic peptide receptor B (NPR-B), which preferably binds CNP, was not investigated.

5.1.2 ANP reduces TNF-α induced NF-κB activation

In the last thirty years increasing evidence pictures the alveolar epithelium as a dynamic barrier, which plays an important role in the regulation of inflammatory and metabolic responses to oxidative stress, sepsis and other critical illnesses in the lung (Matthay et al., 2005). The respiratory epithelium is a primary target of inflammatory processes at the blood-epithelial interface, and in addition is able to modulate and amplify inflammatory signals by producing inflammatory mediators such as IL-8, MCP-1 or ICAM-1 (Fehrenbach, 2001; dos Santos et al., 2004). The inflammatory cytokine TNF-α was shown to induce a potent inflammatory answer in alveolar epithelial cells, leading to activation of several important transcription factors in the inflammatory response including NF-κB. This pro-inflammatory transcription factor is rapidly activated after stimulation and regulates the expression of a variety of genes encoding crucial mediators in inflammation. NF-κB is a heterodimer, which consists of two subunits, p50 and p65. In unstimulated cells it is bound to its inhibitory protein IκBα, which keeps NF-κB in the cytoplasm and inhibits its translocation into the nucleus by masking the nuclear localisation sequence (NLS) (Baud and Karin, 2001).

The result of the present work shows, that ANP is able to reduce TNF-α induced NF-κB DNA binding activity in alveolar epithelial cells (*figure 13)*. Moreover, we investigated the translocation of the p65 subunit of NF-κB and demonstrated that this effect is caused by a decreased translocation of p65 into the nucleus in response to TNF-α stimulation (*figure 14*). Previous experiments in our laboratory revealed that ANP is able to inhibit TNF-α induced activation of NF-κB in human umbilical vein endothelial cells and several macrophages as well (Kiemer et al., 2000a; Kiemer et al., 2002a; Kiemer et al., 2002e), therefore supporting our findings. Additionally, reduced NF-κB DNA binding activity was also accompanied by a decreased p65 nuclear translocation in those experiments. By affecting an essential transcription factor in TNF-α signalling, this is the first report that ANP may have direct anti-inflammatory properties on lung epithelium. Interestingly, Hellermann and coworkers reported recently that a novel, plasmid encoded unphysiological C-terminal natriuretic peptide which consists of aa 73-102, inhibited TNF-α induced NF-κB activation in alveolar epithelial cells as well.

5.1.3 ANP inhibits TNF-α induced AP-1 activity

Activator protein-1 (AP-1) is an important transcription factor acting as an environmental biosensor to various external stimuli and regulates gene expression in a variety of biological processes such as proliferation, differentiation and inflammatory processes. Being a homo- or heterodimer, it is mainly composed of Jun-Jun or Jun-Fos proteins. AP-1 activity in response to external stimuli can be influenced by both regulating the transcription of *jun* and *fos* genes, and posttranslational modifications, such as the phosphorylation of cJun (Shaulian and Karin, 2002). The posttranslational impact on AP-1 activation in response to pro-inflammatory cytokines is mainly mediated by two MAPK cascades, JNK and p38 MAPK (Chang and Karin, 2001). Alveolar epithelial cells have been described to show an increased DNA-binding activity of AP-1 in response to TNF-α. Furthermore, recent studies propose an

involvement of glutathione oxidation and subsequent histone acetylation in TNF-α induced AP-1 activation in this special cell type (Rahman et al., 2002).

Being a major transcription factor involved in inflammation of lung epithelium (Rahman, 2000; Rahman et al., 2002), we investigated possible effects of ANP on TNF-α induced AP-1 DNA binding activity. As demonstrated in the present work, ANP is able to inhibit TNF-α induced AP-1 activity in human alveolar epithelial cells (*figure 15*). Former investigations showed varying results regarding inhibitory actions of ANP on this transcription factor. In LPS activated mouse bone marrow macrophages as well as in ischemia reperfusion injury in the rat liver ANP was capable to reduce AP-1 DNA binding activity, and thereby mediating protective effects such as minor TNF-α expression (Kiemer et al., 2000b; Kiemer et al., 2000a). In HUVECs, however, ANP had no influence on TNF-α induced AP-1 activation (Kiemer et al., 2002e). These data point out, that ANP is able to influence AP-1 activation in several species, but this property seems to be a cell type specific event.

5.1.4 ANP does not alter TNF-α induced ICAM-1 expression

Inflammatory cytokines such as TNF-α are able to induce expression of adhesion molecules in alveolar epithelium. This can be regarded as a crucial step in the orchestration of lung inflammation, because an augmented expression of adhesion molecules is indispensable for recruitment of leukocytes to sites of infection (Beck-Schimmer et al., 2004). In addition, recent studies revealed, that ICAM-1 is able to co-stimulate target cells to facilitate antigen presentation (Lebedeva et al., 2005), and that the cross-talk between alveolar epithelial cells and leukocytes, which is mediated by ICAM-1 leads to enhanced TNF-α production (Lee et al., 2004). ICAM-1 transcription is regulated by several transcriptions factors including NF-κB and AP-1. Due to the fact that we demonstrated an inhibitory effect of ANP on these transcription factors, we investigated whether ANP administration possibly leads to a reduced ICAM-1 expression on alveolar epithelial cells. TNF-α increases ICAM-1 surface

expression dependent on dosage and time of TNF-α administration, but interestingly, ANP does not alter this expression (*figure 16)*. This is astonishing, because NF-κB is described as the predominant transcription factor responsible for ICAM-1 expression in these cells (Holden et al., 2004). In contrast, ANP has shown its ability to decrease TNF-α induced ICAM-1 expression in HUVECs in previous experiments done in our laboratory (Kiemer et al., 2002e). The promoter of ICAM-1 contains various transcription factor binding sites besides NF-κB, including AP-1, AP-2, Ets-1 and Sp-1 (Roebuck and Finnegan, 1999). Although ANP has an inhibitory effect on DNA binding activity of NF-κB and AP-1, this reduction does not seem to be sufficient enough to influence ICAM-1 surface expression in the alveolar epithelium.

5.2 Effects of ANP during LPS-induced septic shock in the murine lung

In this part of the work we investigated the *in vivo* relevance of our previous findings and examined potential protective effects of ANP on inflammatory processes in the lung.
On this account, we used a mouse model of LPS-induced septic shock and investigated several important mediators in endotoxin-induced lung injury.

5.2.1 ANP preconditioning reduces LPS-induced NF-κB activation

The transcription factor NF-κB is a central participant in coordinating the transcription of many important immunregulatory mediators involved in sepsis, such as TNF-α, IL-8, ICAM-1 or cyclooxygenase-2 (COX-2). Many genes regulated by NF-κB in response to infection can induce further activation of this transcription factor. This can lead to potentiation of inflammatory responses in the host and to subsequent organ dysfunction and death (Abraham, 2003).
In this work we demonstrate that ANP is able to protect the lung against LPS-induced NF-κB activation (*figure 17).* The inhibitory effect of ANP on NF-κB activation has been shown previously in LPS or IFN-γ treated macrophages (Tsukagoshi et al., 2001; Kiemer et al., 2000a), in TNF-α stimulated or hypoxia treated endothelial cells (Irwin et al., 2005) and in ischemia reperfusion injury in the rat liver (Kiemer et al., 2000b). We were able to demonstrate a reduced NF-κB activity in TNF-α stimulated lung epithelial cells in this work as well. These findings indicate that inhibition of NF-κB by ANP occurs in a variety of different cell types and tissues. In addition, NPR-A deficient mice have been reported to

show increased NF-κB activation in hypertrophic hearts, which is caused by an impaired cGMP signalling, therefore supporting our results (Vellaichamy et al., 2005).

Pulmonary activation of NF-κB is described to play a pivotal role in various acute and chronic lung disorders, like asthma, COPD (chronic obstructive pulmonary disease) and endotoxin-induced lung injury. Several studies exist concerning the effects of ANP on the genesis of asthma in humans (Hulks et al., 1989; Fluge et al., 1995; Chanez et al., 1990; Almirall and Hedenstierna, 1991). On the one hand ANP seems to promote allergic inflammation by acting on both immune and non-immune cells, but on the other hand, airway hyperreactivity is reduced by intravenous or inhalative administration of ANP (Mohapatra et al., 2004). Recent reports revealed protective effects in endotoxin-induced lung injury through administration of NF-κB decoys (Matsuda et al., 2005). On account of this, inhibition of NF-κB activity seems to be an important step in protecting lungs from LPS-induced injury.

5.2.1.1 Effects of ANP on phosphorylation and degradation of IκBα

Activation of NF-κB is regulated by its inhibitory protein IκB, which masks the nuclear localisation sequence of the p65 subunit and retains the p65/p50/IκBα complex in the cytoplasm, thereby inhibiting its function as transcription factor. IκB exists in several isoforms, IκB-α/β/ε and IκBγ, from which IκBα is the best characterised. Hence, the phosphorylation, ubiquitylation and subsequent degradation by 26S proteasome of IκBα is a crucial step in NF-κB activation (Karin and Ben Neriah, 2000). Most of the previous studies investigating effects of ANP on NF-κB didn't examine the influence of ANP on phosphorylation and degradation of IκB-α in response to pro-inflammatory stimuli. (Irwin et al., 2005; Kiemer et al., 2000b; Tsukagoshi et al., 2001) In HUVECs, ANP did not influence TNF-α induced degradation of IκB-α, whereas it delayed the degradation of IκBε (Kiemer et al., 2002e). Interestingly, the results of the present work indicate that ANP affects the degradation of IκBα in response to LPS (*figure 19*). These findings were accompanied by the

observation, that in addition the phosphorylation of IκBα, which is essential for subsequent ubiquitylation and degradation, is delayed in lungs of ANP treated mice (*figure 18*). On account of this, ANP is suggested to affect signalling pathways upstream of IκBα, to inhibit and therefore to lead to a weakened NF-κB activation in response to inflammatory stimuli. This work is the first report which leads to the assumption that ANP inhibits IκBα phosphorylation and may interfere with inflammatory signals leading to IκBα phosphorylation. Further studies are now required to investigate which pathways upstream of IκB-α are modulated by ANP.

In addition, we observed an increase in total IκBα protein levels in ANP treated mice 30 min after ANP administration. Elevated protein levels of IκB-α might contribute to a decreased NF-κB activation in response to LPS by inhibiting the translocation of its subunits into the cytoplasma. The ability of ANP to elevate the expression of IκBα protein has been described previously by our research group in endothelial cells (Kiemer et al., 2002e). These data support our findings. ANP causes transcriptional up-regulation of both IκBα and IκBε protein in HUVECs. A possible influence of ANP on IκBε protein levels in endotoxin-induced lung injury was not investigated in this work. Limitation of NF-κB signalling via up-regulation of IκBα protein levels has also been described for other substances, including glucorticoids (Costas et al., 2000) and TGF-β (Azuma et al., 1999). In addition, up-regulation of IκB by NF-κB itself is known as part of a central feedback loop pathway controlling NF-κB activation. In our model of LPS-induced lung injury it is likely, that up regulation of IκBα by ANP contributes to the weakened NF-κB activation, but considering the minor phosphorylation of IκBα in response to LPS, ANP seems to modulate also upstream targets of IκBa leading to the phosphorylation and thereby influences this pathway at least through two different mechanisms.

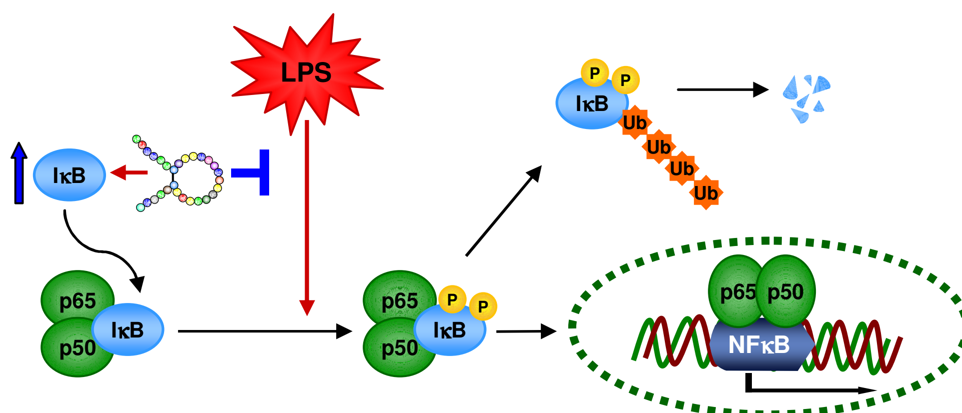

figure 33 Influence of ANP on NF-κB activation in the lung

5.2.2 ANP inhibits AP-1 binding activity in septic mice

Activation of the transcription factor AP-1 occurs in response to a number of diverse stimuli including oxidative stress, DNA damage or exposure to pro-inflammatory cytokines (Shaulian and Karin, 2002). Besides its well known functions in cell proliferation, differentiation, transformation and apoptosis, growing evidence occurs that AP-1 also has crucial functions in the inflammatory response of the lung (Guo et al., 2002; Bozinovski et al., 2002). The role of AP-1 in TLR mediated and cytokine induced signalling was investigated in an extensive way *in vitro* (Yuksel et al., 2002; Guha and Mackman, 2002; Gertzberg et al., 2000; Janssens et al., 2003), but data concerning AP-1 activation during acute lung injury *in vivo* are rare. Recently it has been demonstrated that activation of AP-1 occurs in a model of IgG immunocomplex-induced acute lung injury (Guo et al., 2002). Bozinovski et al. described an AP-1 activation after transnasally instillation of LPS (Bozinovski et al., 2002). On account of this, and due to the fact, that ANP has shown to attenuate TNF-α induced AP-1 activity in alveolar epithelial cells, we investigated the effects of ANP on AP-1 in the lung during endotoxaemia. In the present work an increased AP-1 activation is demonstrated 30 min after LPS administration and ANP was able to completely prevent this event (*figure 20*). As pointed out in 5.1.3, ANP showed varying results regarding inhibitory actions on AP-1 in

previous studies. Activation of this transcription factor can be regulated by two distinct mechanisms, the transcription of *jun* and *fos* genes and the activation of these subunits via phosphorylation by MAPK. Up to now, it is still not clear which of these pathways involved in AP-1 activation is affected by ANP. In recent studies, ANP has been shown to inhibit endothelin-1 induced activation of AP-1 in glomerular mesangial cells *via* inhibition of ERK and JNK, but did not have any effect on IL-1β induced AP-1 activation (Isono et al., 1998). Inhibition of JNK activity by ANP in response to VEGF stimulation has also been shown in bovine aortic endothelial cells (Pedram et al., 2002). Activation of ERK is described to be a critical event in LPS-induced AP-1 activation in the lung (Bozinovski et al., 2002) *via* phosphorylation and stabilizing of c-Jun and thereby enhancing the trans-activation and DNA binding of AP-1 (Shaulian and Karin, 2001).

Our investigations concerning possible effects of ANP on this MAPK revealed very heterogeneous results, because activation of ERK differed enormously even within the NaCl treated group and no explicit difference between control and LPS-treated mice could be observed (data not shown). On this account, we are not able to specify the pathways leading to the reduction of LPS-induced AP-1 activation.

Previous studies in our research group done by Dr. Nicole Bildner revealed that ANP alone is able to induce AP-1 activity in HUVECs. This induction is mediated by a heightened activation of JNK and ERK. In other systems including LPS-treated murine macrophages (Kiemer et al., 2000a) or ischemia reperfusion injury in the rat liver (Kiemer et al., 2000b), ANP had no effect on basal AP-1 activation. The data presented in this work do not reveal an influence of ANP on basal AP-1 activity in the lung.

5.2.3 Influence of ANP on p38 MAPK in LPS-treated lung

The p38 MAPK pathway is very important in the regulation of various stress-induced cellular functions and it is critically involved in the signal transduction of LPS leading to expression of

pro-inflammatory cytokines such as TNF-α, IL-2 and IL-12 (Dong et al., 2002). Recent studies showed, that suppression of p38 MAPK could be a useful therapy for attenuating the inflammatory response (Adcock and Caramori, 2004; Kumar et al., 2003). Furthermore, activation of p38 MAPK is more and more regarded to play a crucial role in the development of acute respiratory distress syndrome (Obata et al., 2000).

5.2.3.1 p38 MAPK is activated in LPS-induced lung inflammation

The p38 MAPK contributes to the development of ARDS by influencing several cell types. On the one hand p38 MAPK suppresses the cytokine production in alveolar macrophages; on the other hand p38 MAPK is required for binding of neutrophils to vascular endothelium being a major step in the extensive pulmonary neutrophil sequestration seen in the course of ARDS (Schnyder-Candrian et al., 2005). Interestingly, controversial reports exist concerning p38 MAPK activation during lung injury *in vivo*. In models of complement-induced lung injury (Nash and Heuertz, 2005) and in studies working with cecal ligation and puncture (CLP) (Singleton et al., 2005), p38 MAPK activation could be observed in lung tissue during the onset of lung inflammation. However, two studies using intranasally instilled LPS obtained contradictory results. Schnyder-Candrien and co-workers observed a rapid increase of p38 activation in LPS-treated mice detected by in vitro phosphorylation assay (Schnyder-Candrian et al., 2005), while Bozinovski did not observe increased p38 MAPK activation in mice detected by Western Blot (Bozinovski et al., 2002). The results presented in this work show a marked increase in p38 MAPK phosphorylation investigated by immunoblotting in the lung after intraperitonial LPS administration *(figure 21)*.

5.2.3.2 ANP decreases LPS-induced p38 MAPK activation in the lung

Being a prominent target for anti-inflammatory therapy and having an important function in LPS-mediated AP-1 and NF-κB activation and subsequent cytokine production, we focused on potential effects of ANP on p38 MAPK activation next. The results of the present work provide evidence, that ANP pretreatment is able to lessen p38 MAPK activation in the lung occurring in the onset of endotoxic shock (*figure 22*). ANP was described previously to influence p38 MAPK activation *in vitro* by our laboratory and by other research groups. TNF-α production was reduced by ANP treatment in IFN-γ activated macrophages *via* an attenuation of p38 MAPK activation (Tsukagoshi et al., 2001). Moreover, we were able to describe that ANP exerts protective effects on TNF-α induced endothelial permeability by inhibition of p38 MAPK activation (Kiemer et al., 2002d). ANP was shown to cause this p38 MAPK inhibition by induction of MAPK phosphatase-1 (MKP-1) *via* Rac1 and NAD(P)H oxidase (Nox2) activation (Furst et al., 2005). In addition, Irwin and co-workers described an inhibitory effect of ANP on hypoxia and TNF-α induced p38 MAPK activation in pulmonary endothelial cells (Irwin et al., 2005). These papers support our finding of ANP inhibiting p38 MAPK and examining possible effects of ANP treatment on MKP-1 would be an interesting target for further investigations. Up to now, all studies investigating inhibitory effects of ANP on p38 MAPK phosphorylation have been done *in vitro*. This work shows for the first time an inhibitory effect of ANP on p38 MAPK activation in inflammatory conditions *in vivo*. Targeting p38 MAPK for anti-inflammatory treatment is an attractive approach and many studies have already been made in which low molecular p38 MAPK inhibitors were investigated in various inflammatory diseases (Kumar et al., 2003). In the literature, a protective effect of p38 inhibition in acute lung injury is described. For example, compound 37, which is a specific p38α,β MAPK inhibitor, was able to prevent LPS-induced bronchoconstriction and neutrophil recruitment into the lungs and bronchoalveolar space in mice (Schnyder-Candrian et al., 2005). Neutrophil influx and

protein leak was also inhibited by oral administration of the p38 MAPK inhibitor SB203580 in a murine model of complement-induced acute lung injury (Nash and Heuertz, 2005). Even though older reports proposed no central role for p38 MAPK in acute lung injury (Arcaroli et al., 2001) and actually reported reduced bacterial clearance and increased cytokine production in the lungs of p38 MAPK-inhibited mice (van den et al., 2001), newer data clearly indicate a protective role of p38 MAPK inhibition in inflammatory processes in the lung. On account of this, we propose a protective role of ANP-mediated p38 MAPK inhibition in the lung of LPS treated mice. As discussed in 5.2.5 and 5.2.6, we aimed to clarify potential effects of this reduction on hallmarks of acute lung inflammation in later steps in our investigations.

5.2.3.3 ANP leads to enhanced p38 MAPK activation in lung tissue

Investigation of p38 MAPK in our model of LPS-induced lung injury revealed astonishing results regarding activation of p38 MAPK in ANP treated mice. Surprisingly, we were able to detect an increase of phosphorylated p38 MAPK in lungs of ANP-treated mice (*figure 23*), even though a decrease of LPS- induced p38 MAPK activation was observed in ANP preconditioned mice as discussed in 5.2.3.2. Up to now, the only report recording an activation of basal p38 MAPK activation by ANP comes from our laboratory and was

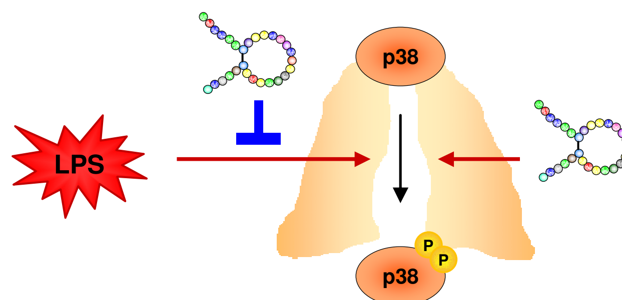

figure 34 Dual effect of ANP on p38 MAPK activation

performed in a model of ischemia reperfusion injury (Kiemer et al., 2002b). In addition, data concerning an effect of the second messenger cGMP generated by NPR-A and NPR-B on this MAPK is rare. An influence of cGMP on p38 MAPK was described in isolated mouse platelets, where cGMP induced PKG activation caused p38 MAPK activation (Li et al., 2006). Similar observations were made in isolated cardiac myocytes, where sodium nitroprussid leads to cGMP generation and subsequent p38 MAPK activation (Kim et al., 2000). In order to confirm the data obtained by immunoblotting we investigated p38 MAPK activity by an in vitro phosphorylation assay and corroborated ANP-mediated increase in activity. This is the first report that ANP is able to increase p38 MAPK activity in the lung. Cellular responses following p38 activation are multifarious and highly stimulus and cell-type dependent. For instance, p38 MAPK is known to stimulate AP-1 activity through phosphorylation of the transcription factors ATF-2, Elk-1 and CCAAT enhancer binding proteins (C/EBPs), which then bind to the promoter elements of *jun* and *fos* and regulate their transcription (Reddy and Mossman, 2002). In our study, AP-1 is not activated in response to ANP alone, indicating other effects of p38 MAPK in response to ANP. Furthermore, many studies have been published concerning the relationship between p38 MAPK and NF-κB activation. In brief, contradictory data exist referring to this topic. On the one hand p38 MAPK has been implicated in contributing to NF-κB activity in response to inflammatory stimuli (Craig et al., 2000; Carter et al., 1999a), on the other hand several reports exist, that rapid p38 MAPK activation can have inhibitory effects on NF-κB activation, even though they can cause p38 MAPK activation themselves (Alpert et al., 1999; Bowie and O'Neill, 2000). In account of this, we suppose that rapid ANP-mediated activation of p38 MAPK might contribute to its inhibitory effect on NF-κB activation.

5.2.4 Influence of ANP on Akt kinase in LPS-treated lung

The protein kinase Akt is involved in various cellular responses including survival, proliferation and gene expression (Song et al., 2005; Neri et al., 2002). In the last years, evidence increased that Akt also participates in the regulation of cellular inflammatory responses. Since several reports have been published proposing an influence of ANP on PI3K/Akt pathway, we focused in the following on this protein kinase.

5.2.4.1 Activation of Akt in LPS-induced lung inflammation

In our model of endotoxaemia, we observed increasing Akt activation *(figure 25).* This event has been demonstrated to contribute to pulmonary neutrophil accumulation and the development of acute respiratory failure in preclinical models of sepsis (Yum et al., 2001). On account of this, we were now interested whether ANP has any effect on LPS-induced Akt activation in the lung.

5.2.4.2 ANP reduces LPS-induced Akt activation in the lung

In our model of LPS-induced septic shock, ANP treatment was able to decrease Akt activation occurring in the lung after LPS administration (*figure 26*). Up to now, several studies have been made investigating potential influence of ANP on Akt signalling in the promotion of anti-apoptotic effects. For instance, Kato and co-workers reported recently, that ANP is able to promote cardiomyocyte survival by cGMP-dependent nuclear accumulation of zyxin and Akt (Kato et al., 2005). In addition, recent results of our laboratory indicate, that ANP treatment mediates anti-apoptotic effects in hepatic ischemia reperfusion injury *via* the

PI3K/Akt pathway (Grutzner et al., in press). To our knowledge, no data exist investigating a potential influence of ANP on Akt pathways activated in inflammatory response.

Data concerning the role of Akt activation during inflammatory processes and their impact on severity and outcome of injury are very controversial. Guha and co-workers showed that the PI3K pathway negatively regulated LPS induction of TNF-α and tissue factor expression. Inhibition of PI3K was described to increase LPS induced activation of MAPK pathways, AP-1 dependent transcription of NF-κB in mouse macrophages (Guha and Mackman, 2002). Inhibition of PI3K by using the inhibitor Wortmannin strongly enhanced LPS-induced cytokine expression and reduced the survival time dramatically in a model of LPS-induced septic shock (Williams et al., 2004). Taken together, recent data suggest that the PI3K/Akt pathway may be a feedback mechanism that prevents excessive innate immune response as proposed by Fukao and co-workers (Fukao and Koyasu, 2003). In contrast, PI3K/Akt pathway was reported to be required for LPS activation of NF-κB in macrophages and endothelial cells (Ojaniemi et al., 2003; Li et al., 2003). Furthermore, Akt can stimulate the transactivation potential of the RelA/p65 subunit of NF-κB *via* utilization of IKK and activation of p38 MAPK (Madrid et al., 2001). Therefore, a decreased Akt activation in ANP preconditioned lungs during endotoxaemia might contribute to the other effects on inflammatory signalling including observed in our studies, including decreased p38MAPK activation and impaired NF-κB activation.

In addition, we observed a slight increase in basal Akt activation in ANP treated lungs. Due to the fact, that ANP mediated Akt activation has been reported previously (Kook et al., 2003), which support our findings, we suppose that Akt activation by ANP might influence LPS-induced pro-inflammatory signalling in a negative way as described by Guha and co-workers (Guha and Mackman, 2002). Whether the observed reduction of LPS-induced Akt activation by ANP entails positive or negative effects in the lung and which pathways are involved in this inhibition, remains to be elucidated.

5.2.5 Impact of ANP on expression of ICAM-1 and leukocyte infiltration

One of the fundamental mechanisms in the development of ARDS is the recruitment of leukocytes, especially neutrophils, into the alveolar space (Yang et al., 2003). Therefore, expression of adhesion molecules like ICAM-1 is a crucial step in the development of lung inflammation. The expression of ICAM-1 in response to LPS is regulated by several kinase pathways and transcription factors including NF-κB, AP-1 and p38 MAPK (Roebuck and Finnegan, 1999; Aplin et al., 1998). Due to the fact, that we observed an ANP-mediated inhibition of these signalling pathways, we further investigated whether ANP can exert influence on pulmonary ICAM-1 expression and subsequent leukocyte infiltration. As presented in this work, our experiments reveal a slight initial inhibition of ICAM-1 expression in the lung after 30 min which was completely abolished 90 min after LPS administration (*figure 27*).

In former experiments ANP has shown inhibitory effects on ICAM-1 expression in response to pro-inflammatory stimuli (Kiemer et al., 2002e). Studies from our own laboratory clearly showed, that ANP pretreatment can inhibit TNF-α induced expression of several adhesion molecules including ICAM-1 and E-selectin in HUVECs (Kiemer et al., 2002e). Additionally, other reports demonstrate the inhibition of ICAM-1 expression via activation of cGMP in endothelial cells (Moon et al., 2005). In contrast, this work provides first evidence that ANP treatment is not able to exert influence on TNF-α induced ICAM-1 expression in alveolar epithelial cells as discussed in 5.1.4. These data leads us to the suggestion, that the slight inhibition of ICAM-1 expression observed after 30 min might be caused by an effect of ANP on endothelial cells, but ANP has no effect on the cardinal ICAM-1 expression in the lung occurring in response to endotoxaemia.

In addition, we investigated leukocyte infiltration in the lung of LPS-treated mice, because this event is a hallmark of lung inflammation and is causally linked to the expression of adhesion molecules (Beck-Schimmer et al., 2004). Seeing that ANP hardly had any effect in

ICAM-1 expression, we wanted to check if infiltration of leukocytes is not altered, too. The results presented in this work revealed, that both 30 and 90 min might be too early points in time for an investigation of leukocyte infiltration (*figure 28*). On account of this, we are not able to make a proposition whether ANP pretreatment may have an influence on leukocyte infiltration or not.

5.2.6 TNF-α in LPS induced lung inflammation

The importance of TNF-α in the pathogenesis of septic shock has been well documented (Cavaillon et al., 2003; Hanada and Yoshimura, 2002). In response to LPS administration, TNF-α is produced rapidly and plasma level peak after 90 min (Taveira da Silva et al., 1993). Even though the TNF-α promoter contains also binding sites for other transcription factors including AP-1, Egr-1 and NF-AT, the expression of TNF-α seems to be predominantly regulated by NF-κB (Yao et al., 1997; Shakhov et al., 1990). Since we have demonstrated an inhibitory action of ANP on LPS-induced NF-κB activity in this work, we further investigated possible effects of ANP on TNF-α expression in lung and serum of LPS challenged mice.

5.2.6.1 ANP treatment alters TNF-α serum levels and protein levels in the lung

TNF-α has been shown to have a major impact on the immune response in endotoxaemia. Being one of the first mediators to occur in the bloodstream after LPS challenge, it leads to subsequent expression and liberation of other cytokines, chemokines, other mediators such as adhesion molecules and even accelerates its own expression (Karima et al., 1999; Hopkins, 2003; Descoteaux and Matlashewski, 1990). Previous works of our laboratory revealed, that ANP reduces LPS-induced TNF-α expression both on mRNA and protein

levels in murine macrophages and minors TNF-α production in whole human blood (Kiemer et al., 2000a). The results presented in this work clearly present, that ANP dose-dependently reduces TNF-α serum levels in a murine model of septic shock (*figure 29*). The impact of TNF-α expression on the outcome in sepsis varies regarding different species (Lorente and Marshall, 2005). TNFR1 deficient mice are resistant to endotoxic shock (Pfeffer et al., 1993) and an anti-TNF-α treatment is highly efficient in reducing mortality in LPS-induced sepsis in this species (Beutler et al., 1985; Tracey et al., 1987). In human studies, however, anti-TNF-α strategies did not come up their expectations and were only partially effective in patients with sepsis (Reinhart and Karzai, 2001). As Melanie Keller, a co-worker in our laboratory demonstrated in her doctoral thesis, ANP pretreatment actually rescues mice from LPS-induced septic shock. The role of ANP-mediated inhibition of serum TNF-α levels in a murine model of endotoxic shock will be further characterized by Kathrin Ladetzki-Baehs doctoral thesis. The lung has been also described as a potential source of TNF-α production during endotoxaemia. Therefore we were interested if ANP pretreatment has an influence on TNF-α protein levels in the lung during endotoxaemia. The results presented in this work show, that TNF-α protein levels in lungs of ANP pre-treated mice are mildly decreased in a dose-dependent manner (*figure 30*).

5.2.6.2 TNF-α is predominantly located in alveolar macrophages in LPS-induced lung inflammation

Especially alveolar macrophages, but also alveolar epithelial cells, vascular smooth muscle cells and endothelial cells have been described to express TNF-α in response to LPS (Ermert et al., 2003). For this reason we were interested in the cell type which is responsible for TNF-α synthesis in our model of LPS-induced lung inflammation and whether an influence of ANP can be observed.

As presented in this work, counterstaining of alveolar macrophages and TNF-α revealed, that TNF-α can be primarily detected in alveolar macrophages in our model of LPS-induced lung inflammation *(figure 31).* This observation coincides with several reports in the literature describing TNF-α production after LPS stimulation(Xing et al., 1993; Ohkawara et al., 1992). As already mentioned above, alveolar epithelial cells and endothelial cells have been also described as potential sources of TNF-α in the lung (Jimenez et al., 2002; Haddad et al., 2002; Burvall et al., 2005), but staining revealed no presence of TNF-α in these cell types 90 min after LPS administration in this work. However, it has to be taken into consideration that we were not able to detect soluble TNF-α, because released TNF-α would have been washed away during preparation of the slices. Interestingly, no difference could be determined in TNF-α staining in alveolar macrophages comparing LPS treated lungs with ANP pre-treated LPS lungs. By reason that alveolar macrophages were rare in stained slices, a quantification of this observation was not possible with this method.

5.2.6.3 ANP has no effect on TNF-α mRNA expression

On account of this, our next experiments focussed on mRNA expression of TNF-α in the lung, in order to elucidate whether the impaired TNF-α protein observed in the lung by ELISA (see 5.2.6.1) is down regulated on transcriptional level. The results of the present work demonstrate that no significant difference between LPS and ANP+LPS treated mice could be detected (*figure 32*). Taken into consideration, that the transcription of TNF-α is predominantly regulated by NF-κB (Shakhov et al., 1990), it is remarkable that the reduced NF-κB activity found in ANP pretreated lungs after LPS challenge doesn't seem to lead to a reduced TNF-α synthesis. Activation of p38 MAPK kinase is described to lead to stabilization of TNF-α mRNA *via* activation MAPK activated kinase-2 (MAPKAP-2) and subsequent phosphorylation of tristetraprolin, a zinc finger protein important for TNF-α mRNA (Mahtani et

al., 2001). Due to the fact that ANP has been shown to impair LPS-induced p38 MAPK activation as described in 4.2.3.2 in this work, ANP might influence TNF-α protein expression on a posttranslational level by modulating TNF-α mRNA stability. This could possibly lead to reduced TNF-α protein levels, although TNF-α mRNA level were not impaired.

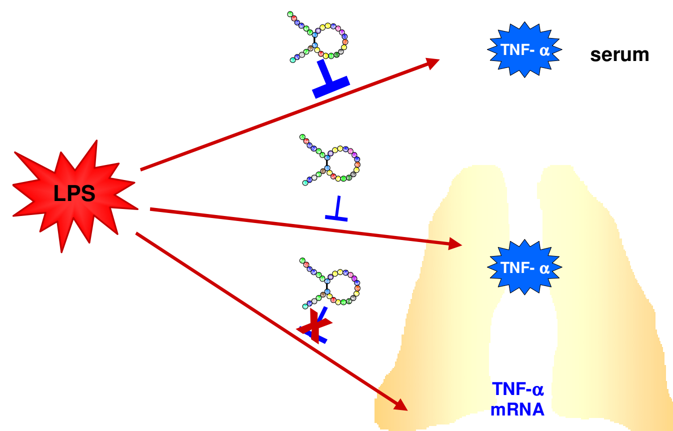

figure 35 Effects of ANP on TNF-α

5.2.7 Outlook

No other possible downstream-targets of NF-κB signalling in response to LPS have been investigated so far. Indeed, various other genes associated with inflammatory processes in the lung contain putative NF-κB and AP-1 binding sites within their promoters, including inducible nitric oxide synthase (iNOS), COX-2 and several matrix metallo proteinases (MMPs), thus highlighting the importance of NF-κB as a key regulator of inflammatory gene activation (Christman et al., 1998). On this account, further studies will focuse on the investigation of outcome parameters influenced by an impaired NF-κB and AP-1 activation, in order to characterize anti-inflammatory actions of ANP in the lung in a more detailed way.

6 Summary

The cardiovascular hormone ANP is known to exert anti-inflammatory properties in macrophages and endothelial cells. This work provides new insight into the inflammatory signalling pathways influenced by the ANP in the lung. For these purposes, the effects of ANP on both alveolar epithelial cells and a model of LPS-induced lung inflammation were characterized.

In alveolar epithelial cells, ANP was shown to inhibit the activation of two major transcription factors, NF-κB and AP-1, in response to TNF-α. Astonishingly, this did not result in a reduced expression of the adhesion molecule ICAM-1.

ANP was also capable to diminish the activation of AP-1 and NF-κB in lung tissue *in vivo* using a mouse model of LPS-induced septic shock. The inhibition of NF-κB activation was caused by a delayed phosphorylation and subsequent degradation of IκB-α as summarized in *figure 36*. In addition, ANP treatment elevated total protein levels of IκB-α.

p38 MAPK and Akt are important mediators in LPS-induced signalling. We demonstrated an activation of these kinases in lung tissue in response to i.p. LPS challenge. ANP treatment was able to lessen this activation. Furthermore, exclusive ANP treatment resulted in an increased p38 MAPK activation, which might contribute to the observed impact on other pathways.

ICAM-1 expression was not impaired in whole lung tissue. ANP strongly decreased TNF-α serum levels dose-dependently, but had only a slight effect on TNF-α tissue levels. Interestingly, TNF-α mRNA expression was not significantly reduced.

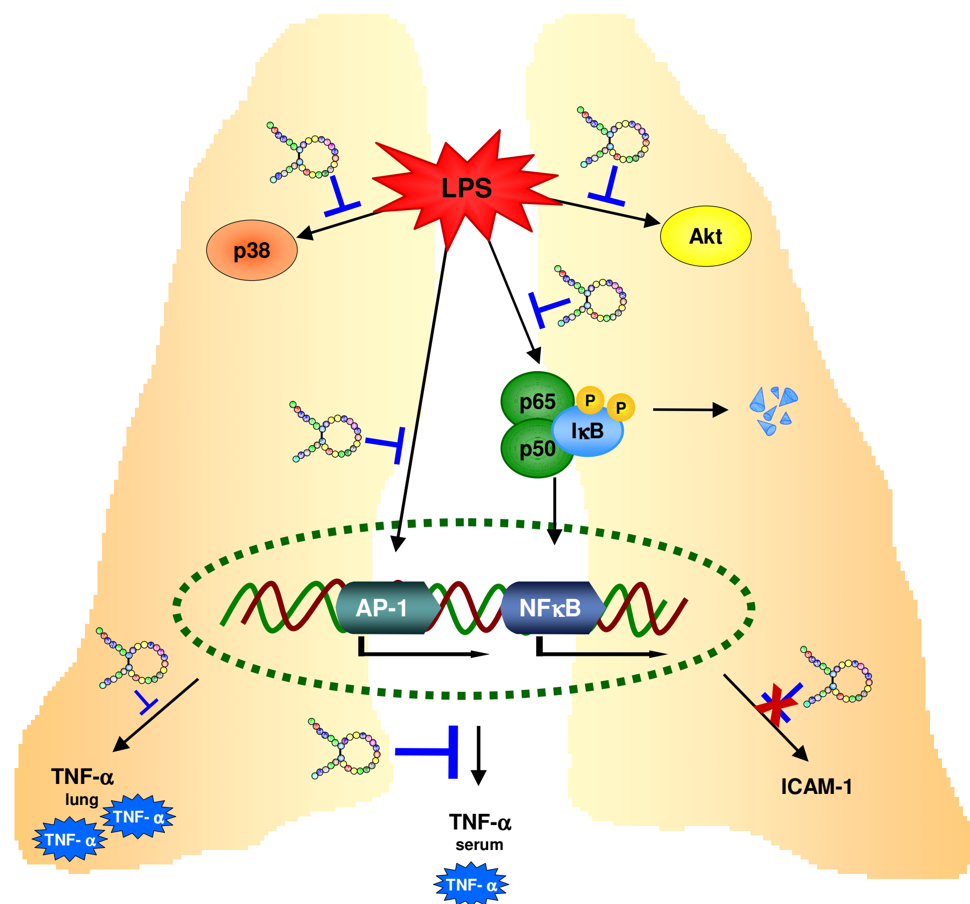

figure 36 ***Schematic diagram of the signalling transduction pathways influenced by ANP pretreatment in LPS-induced lung inflammation***

Taken together this work demonstrates that ANP is able to diminish several important inflammatory pathways which are involved in the development of acute respiratory distress syndrome in LPS-induced sepsis.

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8 Appendix

8.1 Abbreviations

aa	amino acid
ATP	adenosine-5'-triphosphate
AECCC	American-European Consensus Conference Committee
AMV	avian myeloblastosis virus
ANP	atrial natriuretic peptide
AP-1	activator protein-1
APS	ammonium persulfate
ARDS	acute respiratory distress syndrome
ATF-2	activating transcription factor-2
BNP	brain natriuretic peptide
bp	basepair
BSA	bovine serum albumin
°C	degree celsius
cAMP	cyclic adenosin-5'-monophosphate
CAM	cellular adhesion molecule
CAPS	cyclohexylamino-1-propane sulfonic acid
CD	cluster of differentiation
cDNA	copy deoxyribonucleic acid
cGMP	cyclic guanosin-5'-monophosphate
Ci	Curie (1 Ci=$3.7x10^{-7}$ Bequerel)
CLSM	confocal laser scanning microscopy
CNP	C-type natriuretic peptide
Co	control
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
DMSO	dimethylsulfoxid
DNP	dendroaspis natriuretic peptide
dATP	2'-desoxyadenosine-5'-triphosphate
dCTP	2'-desoxycytosine-5'-triphosphate
dGTP	2'-desoxyguanosine-5'-triphosphate
dNTP	dATP, dCTP, dGTP, dTTP
DD	death domain
DTT	dithiothreitol

dTTP	2'-desoxythymidine-5'-triphosphate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene-glycol-O,O'-bis-(2-amino-ethyl)-N,N,N,N,-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular-regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescine isothiocyanate
h	hour
HE	hemotoxyline eosine
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HUVEC	human umbilical vein endothelial cell
IκB	inhibitor of nuclear factor-κB
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
IgG1	immunoglobulin G1
IL	interleukin
IKK	IκB kinase
iNOS	inducible nitric oxide synthase
i.p.	intraperitonial
i.v.	intravenous
IRAK	Interleukin-1 associated kinase
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
l	liter
LFA-1	leukocyte function associated antigen-1
LPS	lipopolysaccharide
m	milli (10^{-3})
M	molar
µ	micro (10^{-6})
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1

MD-2	myeloid differential protein-2
MHC	major histocompatibility complex
min	minute
MKK	MAPK kinase
MMP	matrix metallo proteinase
mRNA	messenger RNA
MyD88	myeloid differentiation primary response gene (88)
n	nano (10^{-9})
NF-κB	nuclear factor κB
NLS	nuclear localisation sequence
NO	nitric oxide
NP	natriuretic peptide
NPR	natriuretic peptide receptor
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK	phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKG	protein kinase B
PMSF	phenylmethylsulfonylfluoride
RT-PCR	reverse transcription PCR
RT	room temperature
PVDF	polyvinylidenfluoride
RIPA	radio-Immunoprecipitation assay
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	rotations per minute
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
sec	second
SEM	standard error of mean
SIRS	systemic inflammatory response syndrome
STE	sodium chloride, Tris, EDTA buffer

T/E	trypsin EDTA buffer
TAB	TAK1-binding protein
TAK	transforming-growth-factor-β-activated kinase
TBE	Tris, borate, EDTA buffer
TBS-T	phosphate buffered saline with Tween
TEMED	N,N,N’,N’,-tetramethylethylendiamine
Tfl	thermus flavus
TGF-β	transforming-growth-factor-β
TIM	TRAF-interacting motif
TLR	Toll-like-receptor
TNF-α	tumour necrosis factor α
TNFR	tumour necrosis factor receptor
TRADD	TNFR1-associated death domain
TRAF	TNF-receptor associated factor
TRAM	TRIF-related Adaptor Molecule
TRIF	TIR(Toll/IL-1 receptor) domain containing adaptor inducing IFN-γ
Tris	Tris-hydroxymethyl-aminomethan
U	unit
V	Volt
W	Watt
VEGF	Vascular endothelial growth factor
VCAM	vascular cell adhesion molecule

8.2 Alphabetical order of companies

AGFA	Cologne, Germany
Amersham	Freiburg, Germany
Bachem	Heidelberg, Germany
BC assay reagents Interdim	Montulocon, France
BD Biosciences	Heidelberg, Germany
Biochrom	Berlin, Germany
BIOCLOT	Aidenbach, Germany
Biomers.net	Ulm Germany
Bio-Rad	Munich, Germany
BioWhittacker	Rockland USA
Biozol	Eching, Germany
Canberra-Packard	Dreieich, Germany
Carl-Roth GmbH	Karlsruhe, Germany
Cell signaling/NEB	Frankfurt/Main, Germany
DakoCytomation	Hamburg, Germany
Dianova	Hamburg, Germany
Endogen	Rockford, USA
Finnzymes	Espoo, Finland
Fuji	Düsseldorf, Germany
Heraeus	Hanau, Germany
Kinematics	Luzern, Switzerland
Kodak	Rochester, USA
Millipore	Bedford, USA
Minerva Biolabs	Berlin, Germany
Molecular Probes/MoBiTec,	Göttingen, Germany
MWG Biotech AG	Ebersberg, Germany
Owl Seperation systems,	Portsmouth USA
PAN Biotech AG	Aidenbach, Germany
PE Applied Biosystems	Hamburg, Germany
Perkin-Elmer	Überlingen, Germany
Promega	Heidelberg, Germany
Quiagen	Hilden, Germany
Roche	Mannheim, Germany

Santa Cruz	Heidelberg, Germany
Sanovi-Cefa	Hannover, Germany
Serotec	Düsseldorf Germany
Stratagene	La Jolla, USA
Sigma	Deisenhofen, Germany
Ssniff	Soest Germany
TECAN	Crailsheim, Germany
Tocris	Westwood, Ellisville, USA
USB	Cleveland, USA
VWR International	Munich, Germany
Zeiss	Oberkochen, Germany

8.3 Publications

8.3.1 Poster presentations

E. Koch, K. Ladetzki-Baehs, M. Keller, A.K. Kiemer, A. Wendel, A.M. Vollmar
„ Preconditioning with atrial natriuretic peptide modulates LPS-induced NF-κB activation *in vivo* “
Naunyn-Schmiedeberg's Arch of Pharmacol 2003, 367 (Supplement1)
45. Frühjahrstagung der Deutschen Gesellschaft für Pharmakologie und Toxikologie in Mainz, 2004

K. Ladetzki-Baehs, M. Keller, E. Koch, S. Zahler, A.K. Kiemer, A. Wendel, A.M. Vollmar
„Preconditioning with the cardiovascular hormone ANP rescues mice from LPS-induced sepsis"
47. Frühjahrstagung der Deutschen Gesellschaft für Pharmakologie und Toxikologie in Mainz, 2006

8.3.2 Oral presentations

E. Koch, M. Keller, A.K. Kiemer, A. Wendel, A.M. Vollmar
„Präkonditionierung mit ANP reduziert die LPS-induzierte NF-κB Aktivierung in der Mäuselunge",
Doktorandentagung der Deutschen Pharmazeutischen Gesellschaft,
Freudenstadt-Lauterbad 2004

8.3.3 Original publications

C. Mueller, F. Duenschede, E. Koch, A.M. Vollmar, A.K. Kiemer
„ Alpha-lipoic acid preconditioning reduces ischemia-reperfusion injury of the rat liver *via* the PI3-kinase/Akt pathway "
Am J Physiol Gastrointest Liver Physiol 2003 Oct; 285(4):G769-78

8.4 Curriculum vitae

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