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Cytoprotective and Anti-Inflammatory Properties of the Atrial Natriuretic Peptide during Ischemia/Reperfusion and Endotoxemia

Melanie Keller

aus Karlsruhe

2005

dedicated with love to my family

In loving remembrance of my brother -
I will never forget about you.

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Frau Prof. Dr. Alexandra K. Kiemer und Frau Prof. Dr. Angelika M. Vollmar betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

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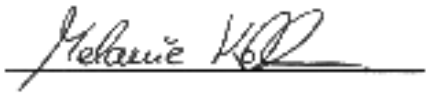

(Melanie Keller)

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1. Contents

2. Introduction

2.1 BACKGROUND AND AIM OF THE STUDY

Ischemia-reperfusion injury (IRI), which is unavoidable in liver transplantation, is a multifactorial process that can cause non-function or dysfunction of the graft (Bilzer,M. et al., 2000a, Clavien,P.A. et al., 1992b, Jaeschke,H., 1996). These complications are the major cause for retransplantation and mortality. An understanding of the mechanisms involved in IRI is essential for the design of therapeutic strategies to prevent IRI and thus improve the outcome of liver transplantation.

In recent years, the Atrial Natriuretic Peptide (ANP) has been demonstrated to posses potential in protection against IRI (Bilzer,M. et al., 1994d, Carini,R. et al., 2003b, Cottart,C.H. et al., 2003a, Gerbes,A.L. et al., 1998e, Sangawa,K. et al., 2004, Vollmar,A.M. et al., 2001). Nevertheless, many questions remain to be answered about signaling pathways involved in ANP-mediated effects and the processes responsible for its protective properties.

The isolated perfused rat liver is a well-investigated model for examination of treatment-mediated effects during ischemia and reperfusion (IR) in the liver (Bilzer,M. et al., 1994c, Bilzer,M. et al., 2000b, Gerwig,T. et al., 2003h). Using this setting, Kiemer *et al.* demonstrated that ANP-preconditioning increases the activity of p38 MAPK (Kiemer,A.K. et al., 2002l) in isolated perfused livers during IR. Functional consequences of an activation of p38 MAPK are widely unknown. This protein kinase has been shown to participate in the regulation of cytoskeletal structures in various cells (Landry,J. et al., 1995b). Thus, our field of interest was to find a causal connection between ANP-mediated effects and possible cytoskeletal changes during IR in this model. In respect of IRI in the liver, ANP was evidenced to exhibit protective effects also *in vivo* (Cottart,C.H. et al., 2003b), but so far nothing is known about signaling pathways responsible for this action. Apototic cell death in the liver during IR is discussed controversial (Gujral,J.S. et al., 2001c, Kohli,V. et al., 1999d, Rosser,B.G. et al., 1995a) and ANP was demonstrated to mediate anti-apoptotic effects in the isolated perfuse rat liver (Gerwig,T. et al., 2003g, Kulhanek-Heinze,S. et al., 2004c). We therefore aimed to determine whether ANP mediates effects in the liver *in vivo* regarding apoptotic death during IR.

ANP has not only been shown to have protective properties in IRI but also anti-inflammatory effects *in vitro* (Tozawa,Y. et al., 2002c, Vollmar,A.M. et al., 2001). In previous studies we were able to show that ANP prevents TNF-α production in murine macrophages and whole human blood after LPS-

stimulation (Kiemer,A.K. et al., 2000a). Moreover, we demonstrated that ANP also inhibits TNF-α-induced activation of NF-κB in human endothelial cells and reduces LPS-induced TNF-α secretion in Kupffer cells (KC) (Kiemer,A.K. et al., 2002w, Kiemer,A.K. et al., 2002a). With regard to inflammatory processes during LPS-induced endotoxemia TNF-α has been demonstrated to be one of the prototypic pro-inflammatory cytokines mediating many of the immunopathological features of this disease (Dinarello,C.A., 1997). During sepsis endogenous ANP is suggested to be a regulatory mediator, as the level of its precursor Pro-ANP in blood from septic patients has been demonstrated to be an important prognostic marker for the outcome of sepsis (Morgenthaler,N.G. et al., 2005a). Another topic discussed in this work is therefore the disease pattern of sepsis. This life-threatening disorder results from a harmful host response to infection and is the leading cause of death for patients in intensive care units (Angus,D.C. et al., 2001a, Cohen,J., 2002f). Despite intensive research on the mechanisms involved in the fatal outcome of this disease, few is known about potential therapies preventing death of patients suffering from sepsis.

Based on the knowledge of ANP-mediated protective effects during deleterious processes in IRI and its anti-inflammatory properties we therefore aimed to answer the following questions:

1. Does pretreatment with ANP cause cytoskeletal changes in the liver during IR in the isolated perfused rat liver?
   Which cell type is affected?
   What are the underlying signaling mechanisms for these changes?

2. Is apoptosis of liver cells during liver transplantation influenced by ANP?
   Which anti-apoptotic pathway is involved?

3. Are anti-inflammatory properties of ANP able to maintain survival after LPS-induced septic shock?
   How does ANP influence inflammatory processes during endotoxaemia?

2.2 ISCHEMIA/REPERFUSION INJURY (IRI)

2.2.1 General aspects

Although substantial progress has been made in surgical techniques, preservation of organs and optimization of pretreatment procedures, dysfunction or non-function of the graft after transplantation is still a severe problem causing complications and mortality after liver transplantation. IRI causes up to 10 % of early organ failure and can lead to acute and chronic rejection in transplanted patients (Fellstrom,B. et al., 1998, Howard,T.K. et al., 1990). Consequently, minimizing the damaging effects of IRI could significantly increase the number of patients that may undergo a successful liver transplantation. For this reason it is important to understand the mechanisms underlying IRI in order to design therapeutic strategies.

IRI in the liver can be divided into two phases: First, deprivation of oxygen during ischemia activates deleterious processes in the cells. The resulting damage is even amplified in the second phase, the following restoration of oxygen supply during reperfusion (Carini,R. et al., 2003a).

Hypoxic conditions occurring during ischemia lead to a disruption of the mitochondrial respiratory chain followed by depletion of ATP and breakdown of energy metabolism in the cell (Nishimura,Y. et al., 1998, Paxian,M. et al., 2003a). Consequences of ATP deprivation are the disturbance of ion homoeostasis, an increase in intracellular calcium, and an activation of calcium dependent proteases, which in turn cleave proteins essential for cellular survival and function (Kohli,V. et al., 1999b, Sindram,D. et al., 1999).

During reperfusion, when oxygen supply of the tissue is restored, the formation of reactive oxygen species (ROS) plays a crucial role (Teoh,N.C. et al., 2003a). ROS are released by Kupffer cells (KC), the resident liver macrophages, which are rapidly activated after early reperfusion and contribute to tissue damage due to activation of proteases, nitric oxide synthases, phospholipases and endonucleases, which in turn are responsible for oxidation of cellular lipids, cytoskeleton disruption, membrane damage, and DNA degradation. ROS are therefore suggested to act as direct cytotoxin to

epithelial cells (EC) and hepatocytes (Bilzer,M. et al., 1999b, Clavien,P.A. et al., 1992a).

KC also produce pro-inflammatory mediators including TNF-α, interleukins, platelet-activating factor (PAF), and chemokines attracting neutrophiles to migrate to the injured tissue. In a vicious circle, interleukins further promote the production of ROS, the release of TNF-α stimulates KC to produce even more TNF-α, and attraction of ROS releasing neutrophiles causes an additional increase in ROS levels and, moreover, disturbances in microcirculation. These disturbances again induce vascular congestion, edema, and further infiltration of inflammatory cells (for review see (Selzner,N. et al., 2003, Teoh,N.C. et al., 2003b)). IRI is therefore characterized by inflammatory processes, deterioration of cell structure and disturbances of microcirculation in the affected tissue – events that cause death of hepatic cells subsequently leading to loss of function of the organ.

2.2.2 Tissue damage and IRI: apoptosis or necrosis?

The relevance of apoptotic versus necrotic cell death in ischemia/reperfusion is still controversial. When liver cells are irreversibly damaged during ischemia they can undergo necrotic or apoptotic cell death, whereas the switch to the respective kind of cell death is suggested to depend on the severity of the death-inducing stimulus. Thus, moderate hypoxia may induce apoptosis and severe hypoxia may induce necrosis (Kaplowitz,N., 2000). Nevertheless, some groups consider apoptosis the major type of cell death during IR: Several studies demonstrate that 50-70 % of endothelial cells and 40-60 % of hepatocytes undergo apoptosis during reperfusion (Gao,W. et al., 1998a, Kohli,V. et al., 1999c, Rudiger,H.A. et al., 2003). Moreover, inhibition of apoptotic cell death, for example by treatment with caspase inhibitors (Cursio,R. et al., 1999c, Natori,S. et al., 2003), suppression of expression of pro-apoptotic genes *via* small interference RNA (Contreras,J.L. et al., 2004a), or overexpression of anti-apoptotic genes (Bilbao,G. et al., 1999, Selzner,M. et al., 2002) prevents cell death and improves survival after prolonged periods of ischemia.

However, the results of other groups indicating hepatocellular necrosis to be responsible for IRI yielded a controversy over the past years about the type of cell death occurring during reperfusion (Clavien,P.A. et al., 2001, Gujral,J.S. et al., 2001b, Jaeschke,H. et al., 2004a, Redaelli,C.A. et al.,

2002, Teoh,N.C. et al., 2003c).

A possible solution of this conflict might be the presence of both types of cell death during reperfusion, which was described by Lemasters *et al.* as “necrapoptosis” (Lemasters,J.J., 1999). The author suggests that necrosis and apoptosis can be induced by the same stimulus, with the progression to one of theses forms of cell death depending on the presence or absence of ATP. In this context the onset of mitochondrial permeability transition (MPT) which is followed by mitochondrial uncoupling and ATP depletion plays the key role in electing the necrotic or apoptotic pathway. On the one hand, ATP depletion after MPT onset has been demonstrated to cause necrotic cell death (Qian,T. et al., 1997). On the other hand, it leads to mitochondrial swelling followed by release of cytochrome c, activation of the caspase cascade, and subsequent apoptotic cell death (Liu,X. et al., 1996). This theory is confirmed by data of other groups demonstrating that in case of a prevention of ATP-depletion necrotic cell death does not occur (Leist,M. et al., 1997, Nicotera,P. et al., 1999, Paxian,M. et al., 2003b).

With respect to IRI in the liver, ANP pretreatment has been shown to cause prevention of apoptosis and necrosis *ex vivo* (Gerwig,T. et al., 2003f). Therefore, aim of the study was to investigate the *in vivo* effect of ANP on apoptosis during liver transplantation and its signaling pathway during orthotopic rat liver transplantation.

2.3 ROLE OF THE PI3-KINASE PATHWAY IN APOPTOTIC CELL DEATH

2.3.1 Apoptosis and necrosis

Apoptosis is a highly regulated process, which was first described by Kerr *et al.* in 1972 and is responsible for elimination of excess cells during development and damaged cells during regular tissue turnover (Kerr,J.F. et al., 1972b). It is characterized morphologically by chromatin condensation,

nucleosomal DNA degradation, cell shrinkage, and blebbing of the plasma membrane, which results in the separation of the cell into small vesicles called “apoptotic bodies” (Kerr,J.F. et al., 1972a, Walker,N.I. et al., 1988). Structural alterations of the cell surface ensure that apoptotic bodies are recognized and phagocytized either by macrophages or parenchymal cells and subsequently degraded within lysosomes (Duvall,E. et al., 1985). Degradation of apoptotic cells is a result of activation of caspases and endonucleases, which induce cleavage of structural proteins, repair enzymes, and DNA. Caspases, which represent a family of cystein-dependent aspartate-specific proteases, play a crucial role in the progression of apoptosis (Nicholson,D.W. et al., 1997). They are expressed constitutively as inactive proenzymes composed of an N-terminal prodomain and two subunits, and are activated through proteolytic cleavage of the zymogen. Apoptotic cell death is mostly limited to a single cell or a small group of cells antagonising inflammatory processes by maintenance of an intact plasma membrane, thus preventing the release of potentially inflammatory cell content (Walker,N.I. et al., 1988). However, previous findings suggest that apoptotic cell death can be equally effective as necrosis in inducing hepatic inflammation by triggering neutrophil accumulation in the liver tissue (Faouzi,S. et al., 2001, Jaeschke,H., 2002, Yin,X.M. et al., 2003b).

In contrast to apoptosis, necrosis is a passive, barely regulated process occurring in response to a variety of harmful conditions and cytotoxic substances, such as hyperthermia, cellular trauma, metabolic poisons, etc.. This from of cell death is characterized by cell swelling and lysis, and typically induces local inflammation by release of cytoplasmatic components, which in turn attract phagocytes (Raffray,M. et al., 1997, Walker,N.I. et al., 1988).

As demonstrated in various studies, dysregulation of apoptosis pathways in the liver contributes to diseases like hepatocellular carcinoma, viral hepatitis, autoimmune hepatitis, ischaemia-reperfusion injury, toxic liver damage and acute liver failure (Jaeschke,H. et al., 2004b, Nakamoto,Y. et al., 2003, Yin,X.M. et al., 2003a). Therefore, it is essential to find new strategies for a successful intervention in apoptotic pathways for protection against tissue damage in the liver. The influence of ANP on apoptotic signaling in the liver is thus an attractive target for investigation of protective strategies against IRI.

2.3.2 Involvement of PI3-kinase in anti-apoptotic signaling

2.3.2.1 PI3-kinases

Phosphatidylinositol 3-kinases are heterodimers composed of a catalytic subunit (p110) and a regulatory subunit (p85), the latter being activated by receptors with protein tyrosine kinase activity as well as by G-protein-coupled receptors (Datta,S.R. et al., 1999f, Osaki,M. et al., 2004a). They are characterized by their ability to phosphorylate inositol ring 3`-OH groups in inositol phospholipids. Binding of the ligand (for example growth factors such as IGF-1) to its receptor results in association of the SH-domain of PI3-K with the receptor, followed by allosteric activation of the catalytic subunit of PI3-K (**figure 1**). Consequently, active PI3-K causes immediate generation of the second messenger PIP_3 (phosphatidylinositol-3,4,5-triphosphate), which is responsible for recruitment of other signaling proteins containing a pleckstrin homology (PH)-domain to the plasma membrane.

2.3.2.2 Akt kinase

The 57 kDa serine/threonine kinase Akt, also termed PKB due to sequence homologies with PKA and PKC, is the cellular homologue of the transforming oncogene of AKT8 onciovirus (Datta,S.R. et al., 1999e, Scheid,M.P. et al., 2001). To date, three members of the Akt family have been identified, namely Akt1, Akt2, and Akt3 (PKB α,β,γ), which are closely related to each other, with up to 80 % of amino acid homology. The amino-terminus of each Akt isoform contains a PH-domain, while its kinase domain is similar to that of PKA and PKC. As described in 2.3.2.1, PIP_3 recruits proteins with PH-domains to the plasma membrane, thus causing translocation of Akt towards the membrane (**figure 1**). This translocation brings Akt into proximity with other PH-domain-containing enzymes such as PDK-1 (3-phosphoinositide-dependent kinase 1). PDK-1 then phosphorylates Akt at threonine 308 in the activation loop, thereby enabling binding of ATP to its substrates. For full kinase activation, additional phosphorylation at serin 473 is necessary, but the kinase responsible, termed PDK-2, has not been identified yet. Possible candidates are PDK-1, the protein kinase ILK (integrin-linked kinase), and Akt itself (Brazil,D.P. et al., 2001, Persad,S. et al., 2001, Toker,A. et al., 2000).

2.3.2.3 Bad

Bad is a member of the family of Bcl-2 proteins functioning as an apoptosis-regulating factor (Chao,D.T. et al., 1998, Downward,J., 2004c). Several members of the Bcl-family promote survival (including Bcl-2, Bcl-X_L, BAG, A1, MCL-1), while others induce apoptotic cell death (including Bad, Bcl-X_s, Bax, Bak). In viable cells anti-apoptotic proteins are localized in membranes such as the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane, anchored through an additional carboxy-terminal hydrophobic domain. The pro-apoptotic proteins, in contrast, are mainly found in the cytosol (Krajewski,S. et al., 1993, Wolter,K.G. et al., 1997). Interaction of the proteins and formation of homo- and heterodimers play a crucial role in prevention or activation of apoptotic processes (Datta,S.R. et al., 1999d). Thus, Bad in its unphosphorylated form binds and inactivates anti-apoptotic proteins such as Bcl-2 or Bcl-X_L leading to proapoptotic effects (**figure 1**). Phosphorylation of Bad at either of the two potential sites (Ser112, Ser136) causes Bad to dissociate from Bcl-2 or Bcl-X_L, respectively, and to associate instead with the cytoplasmic 14-3-3 proteins preventing Bad from dephosphorylation and subsequently promoting cell survival.

Three kinases have been suggested to phosphorylate Bad at three different residues: Erk 1/2 and PKA phosphorylate Bad at Ser 112, Akt at Ser 136, while PKA is additionally able to phosphorylate Bad at Ser 155 (Downward,J., 2004b).

Various studies report an essential role of Bad as a downstream target of Akt in anti-apoptotic effects of this kinase. For example, Akt promotes survival of neurons *via* phosphorylation of Bad at Ser 136 (Datta,S.R. et al., 1997b). Moreover, it has been shown in different tumor cells that Akt inhibits apoptosis by phosphorylating Bad (Datta,S.R. et al., 1997a, del Peso,L. et al., 1997, Fernando,R.I. et al., 2004). Akt also phosphorylates Bad at Ser136 *in vivo* and mutation of Bad Ser 136 to alanine abrogates the blocking effect of Akt in Bad-induced apoptosis (Datta,S.R. et al., 1999c).

In summary, all these studies demonstrate that Bad plays a key role in the Akt survival signaling. However, due to the lack of knowledge about signaling pathways regarding prevention of apoptosis of liver cells after IR, aim of the present study was to elucidate a possible participation of the PI3-kinase pathway and thus Akt-dependent phosphorylation of Bad during this insult.

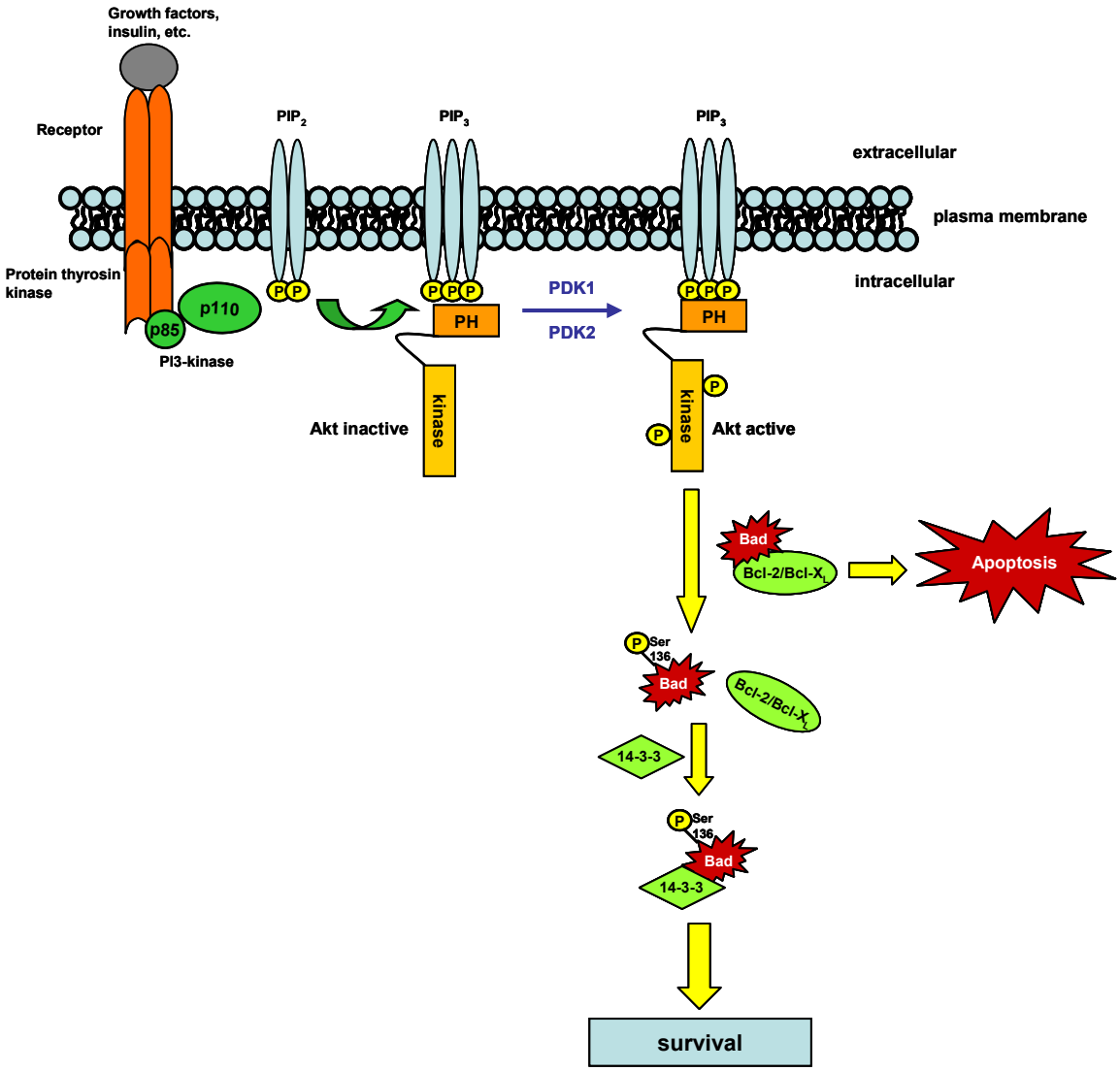

figure 1: Mechanisms of Bad inactivation *via* the PI3-kinase pathway

2.4 HEAT SHOCK PROTEINS AND CYTOSKELETON

2.4.1 Heat shock proteins

Stress or heat shock proteins (Hsp) were first discovered in 1962 as a set of highly conserved proteins with cytoprotective properties, whose expression was induced by different kinds of cellular stress (Ritossa,F., 1996). These proteins were shown to be ubiquitous and abundant in nearly all cellular compartments with their expression and activation dependent on stimuli contributing to cellular stress, like heat shock, oxygen deprivation, ROS, cytokines, etc.. According to their molecular weight they are divided into four major families: Hsp90, Hsp70, Hsp60, and the small Hsp (Hsp27 and α-crystallin) (Georgopoulos,C. et al., 1993). Hsp function – mostly in an energy-dependent manner - as so called "molecular chaperones" with responsibility for the import of proteins into cellular compartments, folding of proteins, degradation of unstable proteins, resolution of protein complexes, refolding of misfolded proteins, and control of regulatory proteins (Santoro,M.G., 2000). Induction of Hsp in response to stress correlates with increased resistance of the cells to subsequent damage. The stress-induced transcription requires the activation of a transcription factor , namely heat shock factor (HSF), which then binds to the heat shock responsive element (HRE) on the DNA, whereas the high specificity of each heat shock protein results from multiple HSFs and a cell type specific allocation of Hsp (Morimoto,R.I., 1998). Previous studies have shown that the induction of the heat shock response requires the activation and translocation of Hsp to the nucleus (Ellis,S. et al., 2000, Ohgitani,E. et al., 1999).

A characteristic feature of small Hsp, namely Hsp27 (which corresponds to Hsp25 in mouse and rat) and α-chrystallin, is their oligomerization of unphosphorylated monomers to large protein aggregates (>500 kDa) (MacRae,T.H., 2000). Hsp27, an ATP-independent chaperone, which was first isolated and described by Miron *et al.* 1988 (Miron,T. et al., 1988), facilitates the refolding of partial denaturated proteins into active conformations *in vitro* (Jakob,U. et al., 1993). Moreover, it can act as a regulator of actin polymerization (Benndorf,R. et al., 1994d). It has been shown in previous studies *in vitro* and *in vivo* that expression of Hsp27 is in accordance to DNA-binding activity of HSF (Ito,H. et

al., 1996, Kato,K. et al., 1998). To date four differents HSFs have been identified and recently, HSF-1 was demonstrated to be responsible for activation of Hsp27 mediated effects in renal cells (Riordan,M. et al., 2004).

2.4.2 Influence of Hsp27 on cytoskeletal structures

Post-translational regulation of Hsp27 *via* phosphorylation is essential for cytoprotective properties of the heat shock protein (Landry,J. et al., 1995a, Lavoie,J.N. et al., 1995). In respective studies a nonphosphorylatable form of Hsp27 was used in Chinese hamster cell lines, which were no longer resistant against heat shock, showed higher sensitivity against cellular stress, and exhibited a significant deceleration in regeneration of cytoskeletal structures. The latter observation, indicating a regulation of actin dynamics actually is the most important function of Hsp27, and has therefore been investigated intensively in various systems (Aufricht,C. et al., 1998b, Dalle-Donne,I. et al., 2001, Gomes,M.D. et al., 2003, Panasenko,O.O. et al., 2003).

Prior studies on the interaction of Hsp27 and cytoskeletal proteins demonstrate that, in its unphosphorylated form, Hsp27 inhibits the polymerization of actin by binding to the capping end of actin filaments, thus preventing actin monomers (G-actin) from binding to actin filaments (F-actin) (Benndorf,R. et al., 1994c, Wieske,M. et al., 2001). Moreover, Miron *et al.* demonstrated that unphosphorylated Hsp27 contributes to the depolymerization of filamentous (F-) actin into monomeric G-actin *in vitro* (Miron,T. et al., 1991).

However, when phosphorylated, Hsp27 dissociates from actin filaments, thereby inducing the conversion of G-actin into actin filaments (Arrigo,A.P. et al., 1994). The phosphorylation of the heat shock protein is regulated *via* the MAPK-signaling cascade. Phosphorylation at Ser 82 and Ser 15, promoting the dissociation of Hsp27 multimers, is achieved by activation of p38 MAPK and subsequent phosphorylation of MAPKAPK-2, which in turn directly phosphorylates Hsp27 (Lambert,H. et al., 1999, Landry,J. et al., 1995c). This activation pathway of the heat shock protein has been described in different cells and organ systems, but has as yet been unknown in the liver (Freshney,N.W. et al., 1994b, Murashov,A.K. et al., 2001b, Nakano,A. et al., 2000a). The function of Hsp27 as a cytoprotective mediator of cytoskeletal stabilization has been thoroughly investigated in

the heart, suggesting an involvement of the heat shock protein in cardioprotection after ischemic preconditioning *in vitro* and *in vivo* (Cohen,M.V. et al., 2000, Sakamoto,K. et al., 2000c, Sanada,S. et al., 2001e).

Sakamoto *et al.* additionally demonstrated that in rat hearts ischemia induces translocation of Hsp27 to the cytoskeleton (Sakamoto,K. et al., 2000b), and Aufricht *et al*. showed the same effect in rat kidneys (Aufricht,C. et al., 1998a). This reallocation of Hsp27 accompanied by cytoskeletal changes was also observed in pancreas tissue of rats (Schäfer,C. et al., 2000), cardiomyocytes of rabbits (Armstrong,S.C. et al., 1999), and human keratinocytes (Wong,J.W. et al., 2000), therefore suggesting an interaction of Hsp27 with the cytoskeleton to be important for its regulatory function in actin polymerization.

2.4.3 Actin polymerization

Actin filaments are dynamic structures which undergo permanent reconstruction. This persistent assembly and disassembly is very important for their functional role inside the organism. Representing 5 % of total cellular protein, actin is available in the cell in equal shares as monomeric form (G-actin) in the cytosol and filamentous chains (F-actin) in the cytoskeleton and cellular membranes. F-actin is composed of two strands of actin polymers, which are twisted in a helical structure. As shown in **figure 2**, the polymerization of actin is an energy-dependent process.

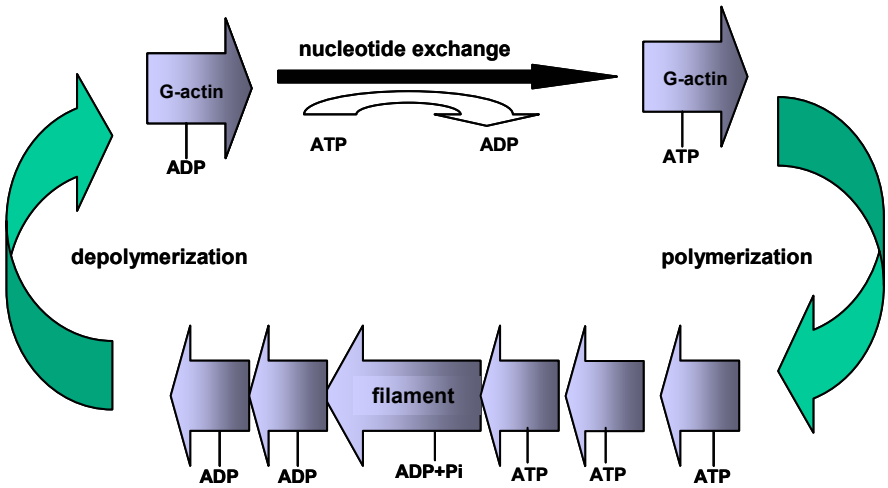

figure 2: Schematic illustration of actin polymerization (see text for details)

Monomeric actin binds to adenosine-triphosphate (ATP), which is hydrolyzed to adenosine-diphosphate (ADP) immediately after attachment of G-actin monomers to the actin filament. Vice versa, as soon as ATP binds to the complex and replaces ADP, which is important for stabilization of the F-actin strand, actin depolymerization occurs, leading to separation of G-actin monomers (Kuhn,T.B. et al., 2000b).

The balance of intracellular F- and G-actin is regulated by actin binding proteins, which are divided into two classes, depending on their regulatory function (McGough,A., 1998): monomer binding proteins, for example thymosine, are responsible for retention of G-actin in the cytosol of cells and instant allocation of actin monomers when actin polymerization is necessary for reparation or stabilization of cellular structures. The second class of actin binding proteins can attach to polymeric structures like F-actin, thus influencing polymerization, but also crosslinking of F-actin chains. The actin depolymerizing factor (ADF) is one example for such a regulator protein (Kuhn,T.B. et al., 2000a). Following dephosphorylation of ADF, this actin binding protein enhances the F-actin turnover by increasing actin depolymerization and cleaving filaments. The capping proteins also belong to the second class of actin binding proteins (Landry,J. et al., 1995d). This proteins bind to the “capping end” of actin filaments and prevent the attachment of additional actin monomers. A representative of these proteins is Hsp27, which regulates F-actin polymerization by means of occupying the G-actin binding end of actin filaments, thus inhibiting polymerization (see 2.4.2).

In the liver actin filaments are present predominantly in the plasma membrane of hepatocytes and sinusoidal cells (Song,J.Y. et al., 1996a). They are responsible for maintenance of cell morphology, intracellular transport processes, exo- and endocytosis, and canalicular motility responsible for bile flow (Denk,H. et al., 1986, Fisher,M.M. et al., 1979, Marceau,N. et al., 2001). In addition cytoskeletal structures are important to ensure stability and mobility of liver cells and play an important role in the regulation of store-operated calcium-channels (SOCs), and in the organization of the endoplasmic reticulum and tight junctions (Marceau,N. et al., 2001, Song,J.Y. et al., 1996c, Wang,Y.J. et al., 2002a) (Arias I.M., 2001b).

2.5 THE ATRIAL NATRIURETIC PEPTIDE

Atrial natriuretic peptide (ANP) is a peptide hormone, which was first described in 1981 by De Bold *et al.* (de Bold,A.J. et al., 1981). Besides ANP, more natriuretic peptides have been discovered: brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and dendroaspis natriuretic peptide (DNP) (Levin,E.R. et al., 1998a, Richards,A.M. et al., 2002). This year, Fry *et al.* even found three additional natriuretic peptides in the venom of the Australian snake *Oxyuranus microlepidotus*, which are structurally different from ANP/BNP, but have not been further characterized yet (Fry,B.G. et al., 2005).

ANP, with its circular structure composed of 28 amino acids (see **figure 3**), is generated by cleavage of its precursor hormone pro-ANP, which has to be released by previous cleavage of Pre-pro-ANP (Suttner,S.W. et al., 2004a, Vesely,D.L., 2003). The structure of ANP (see **figure 3**) shows a ring of 17 amino acids which is characteristic for natriuretic peptides, with a disulfide linkage between two cystein residues being essential for biological activity (Currie,M.G. et al., 1984).

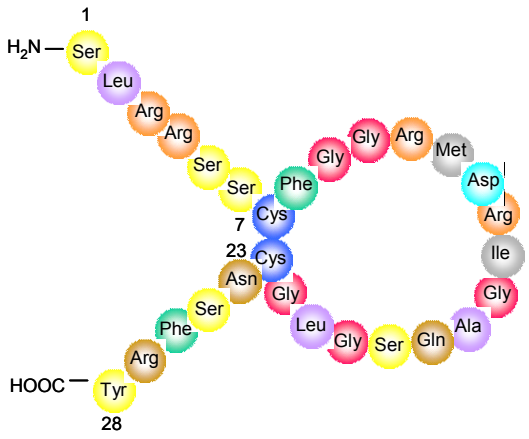

figure 3: Structure of ANP

ANP is secreted predominantly by cardiomyocytes upon atrial distension caused by volume expansion, occurring for example in congestive heart failure and kidney disease, and has been described to possess natriuretic, diuretic and vasorelaxant properties (for review see (Suttner,S.W. et al., 2004b)).

BNP is particularly released by ventricular myocardial cells, while CNP is primarily expressed in the central nervous system and in vascular endothelium (Levin,E.R. et al., 1998b). With respect to biological functions of natriuretic peptides, CNP has been suggested to be a local vascular regulator.

ANP and BNP have an important function in the regulation of blood pressure and volume homeostasis by balancing the renin-angiotensin-aldosterone system, thus exhibiting natriuretic and also diuretic effects predominantly in the kidney. Moreover, ANP has been demonstrated to protect against IRI and to have anti-inflammatory effects (Carini,R. et al., 2003d, Vollmar,A.M. et al., 2001).

2.5.1 Receptors and signaling mechanisms

Natriuretic peptides (NP) mediate their effects *via* interaction with the NP receptor (NPR), with three different types identified to date (Misono,K.S., 2002, Tremblay,J. et al., 2002).

The NPR-A receptor binds ANP with a higher affinity than BNP, whereas CNP exerts its properties *via* NPR-B. Both receptors possess an intracellular kinase-like domain and a catalytic guanylyl cyclase domain, which is activated after binding of NPs to the receptor, and in turn increases the intracellular level of cyclic guanosine monophosphate (cGMP) (see **figure 4**). The third NPR, however, is structurally different, as it does not contain a guanylyl cyclase domain and only a short intracellular domain. NPR-C functions as a clearance receptor responsible for removing excessive NPs from the bloodstream. Moreover, this receptor is suggested to mediate inhibition of adenylyl cyclase activity, to cause activation of phospholipase C, and to be responsible for ANP-mediated inhibition of COX-2 expression in LPS-stimulated macrophages (Hu,R.M. et al., 1992, Kiemer,A.K. et al., 2002m, Palaparti,A. et al., 2000).

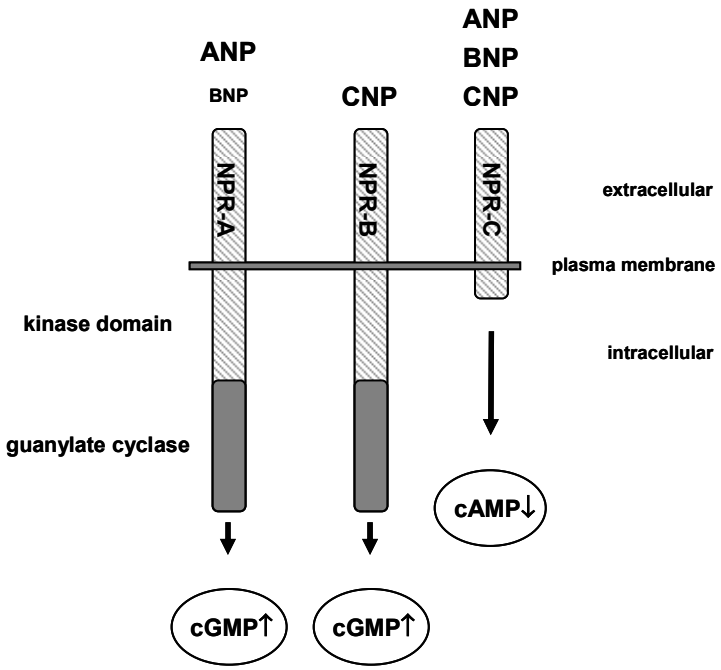

figure 4: Natriuretic peptide receptors (NPR) and their ligands.

Various studies on knockout mice lacking NPR-A proved that this receptor is essential for ANP-mediated effects (Melo,L.G. et al., 2000, Pandey,K.N. et al., 1999). With regard to IRI in the liver, the protective properties of ANP depend on cGMP-mediated signaling pathways, as the cGMP-analogue 8-Bromo-cGMP exactly mimicked ANP-induced effects.

cGMP exerts its regulatory functions by interaction with several cGMP receptor proteins. It has been demonstrated that cGMP-dependent protein kinases (PKG) are responsible for mediation of cGMP dependent actions in different cell types (Lohmann,S.M. et al., 1997, Pfeifer,A. et al., 1999). Interestingly, in higher concentrations, cGMP is also able to activate cAMP-dependent protein kinases (PKA) (Cornwell,T.L. et al., 1994). Another possibility for interaction of cGMP with the cAMP signaling pathway is *via* binding to specific phosphodiesterases (PDE), thus stimulating (PDE type II) or inhibiting (PDE III) the degradation of cAMP (Lincoln,T.M. et al., 1993). Moreover, in heart and kidneys, cGMP has been shown to regulate a cGMP-gated ion channel, therefore contributing to natriuretic effects in these tissues (Biel,M. et al., 1998).

2.5.2 Effects ANP during IR and inflammatory processes

2.5.2.1 ANP and IRI

Besides its vasodilating, hypotensive, and natriuretic activities (Kourie,J.I. et al., 1999b, Kourie,J.I. et al., 1999a, Vesely,D.L., 2001), ANP has been described to protect against IRI *ex vivo* in the liver and in the (Bilzer,M. et al., 1994b, Carini,R. et al., 2003e, Gerbes,A.L. et al., 1998d, Kiemer,A.K. et al., 2002f), and protective effects in the liver have even been demonstrated *in vivo* (Cottart,C.H. et al., 2003c). Perfusion of livers with ANP prior to ischemia and reperfusion markedly reduced cell damaged, apoptosis in hepatocytes, necrosis in hepatocytes and endothelial cell, and furthermore improved bile flow (Bilzer,M. et al., 1994a, Gerwig,T. et al., 2003e).

Investigation of underlying signaling mechanisms suggested that ANP mediates its hepatoprotective actions *via* reduced activation of the redox sensitive transcription factors NF-κB and AP-1 resulting in decreased expression of the cytokine TNF-α (Kiemer,A.K. et al., 2000k, Kiemer,A.K. et al., 2000l). Moreover, Bilzer *et al.* demonstrated an influence of ANP on Kupffer cell (KC) mediated injury of liver tissue. Their results showed that ANP was able to protect liver cells against oxidative stress of activated Kupffer cells without influencing superoxide formation of KC (Bilzer,M. et al., 1999a). Furthermore, ANP activates the heat shock transcription factor (HSF) paralleled by an increase of protein levels of the heat shock protein Hsp70 (Kiemer,A.K. et al., 2002e), which is not only discussed to grant thermotolerance, but also to protect against ROS-induced damage and IRI (Becker,J. et al., 1994, Terajima,H. et al., 2000). As demonstrated by Kiemer *et al.,* ANP increases the activity of p38 mitogen activated kinase (MAPK) in the liver (Kiemer,A.K. et al., 2002k). This MAPK is suggested to possess protective properties in the liver and heart after IR (Sanada,S. et al., 2001d, Schauer,R.J. et al., 2003, Teoh,N. et al., 2002).

Taken together, ANP seems to mediate its protective effects in the liver by either directly preventing the production of mediators of cell damage or by improvement of the cellular resistance to cytotoxic products. In order to evaluate the potential of ANP as a new pharmaceutical agent mediating protective effects during IR, the influence of ANP on signaling pathways involved in IRI of the liver *in vivo* were further investigated.

2.5.2.2 ANP and inflammation

ANP has been demonstrated to possess cytoprotective actions in various cells and organs besides the liver. A growing body of evidence describes the ability of ANP to prevent LPS-induced inflammatory processes. In macrophages, ANP has been shown to inhibit the LPS-mediated induction of inducible nitric-oxide synthase (iNOS), therefore preventing the formation of NO, an important mediator of inflammation (Kiemer,A.K. et al., 1997b, Kiemer,A.K. et al., 1998a, Kiemer,A.K. et al., 2001). Moreover, treatment of macrophages with ANP causes inhibition of LPS-induced cyclo-oxygenase-2 (COX-2) and decreases TNF-α production after treatment with LPS (Kiemer,A.K. et al., 2000b, Kiemer,A.K. et al., 2002p, Tsukagoshi,H. et al., 2001b).

Besides these *in vitro* observations, inhibition of the ANP receptor binding involved inflammation in *vivo* in rats with serotonin ($5\text{-}HT_{2A)}$ receptor- mediated acute hemodynamic changes (Tozawa,Y. et al., 2002b), also pointing to anti-inflammatory properties of ANP. Furthermore, ANP increased phagocytosis in LPS-stimulated KCs in combination with deceleration of TNF-α release by the liver macrophages (Kiemer,A.K. et al., 2002b). In bovine pulmonary microvascular and macrovascular endothelial cells stimulated with TNF-α or bacterial endotoxin (LPS), ANP treatment inhibited NF-kappaB activation and TNF-α synthesis, thus preventing inflammatory processes (Irwin,D.C. et al., 2005b). This anti-inflammatory effect of the peptide was also demonstrated in human umbilical vein endothelial cells (HUVEC), as ANP prevented TNF-α induced NF-κB activity and expression of monocyte chemoattractant protein-1 (MCP-1) (Kiemer,A.K. et al., 2002v, Weber,N.C. et al., 2003b). Finally, ANP has also an important function in regulating the plasma volume during endotoxemia (Qu,X.W. et al., 1998a), and the ANP precursor Pro-ANP is even suggested as a prognostic marker in septic patients (Morgenthaler,N.G. et al., 2005b), emphasizing ANP as a potential protective endogenous mediator in sepsis. To date, however, there are no data explaining the signaling mechanism responsible for ANP-mediated effects during endotoxemia.

In summary, the anti-inflammatory potential of ANP is of high interest making ANP a major target of subsequent studies (for review see (Fürst,R. et al., 2005a, Fürst,R. et al., 2005b, Kiemer,A.K. et al., 2005, Vollmar,A.M. et al., 2001).

2.6 SEPSIS

2.6.1 General aspects

Sepsis, a life-threatening disorder that arises from the body's response to infection, is the leading cause of death for patients in an intensive care unit, with its overall mortality of 30-50 % exceeding that of almost all common cancers (Angus,D.C. et al., 2001b, Cohen,J., 2002e).

The first characterization of this deadly disease reaches back to 1879 when Louis Pasteur for the first time showed bacteria to be present in blood from patients suffering from "puerperal septicaemia". The "concept of endotoxin-induced shock and death" was then described in 1894 by Pfeiffer. However, a definition of sepsis was reached only in 1992 (American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference, 1992): "Sepsis is the response of the host to microbial infection with evidence of systemic inflammation, consisting of two or more of the following: increased or decreased temperature or leukocyte count, tachycardia, and rapid breathing. Septic shock is sepsis with hypotension that persists after resuscitation with intravenous fluid."

During sepsis the most common site of infection are the lungs, abdominal cavity, urinary tract, and primary infections of the bloodstream. This infection is initiated by gram-negative bacteria in 25-30 % of septic patients. Gram-positive and polymicrobial infections account for 30-50 % and 25 % of cases, respectively (Annane,D. et al., 2005b).

As septic patients ultimately die from multiple organ failure, which in early stages of endotoxemia develops from failure of a single organ, maintenance of organ function is an important target of investigation. The kidney is one of the affected organs during endotoxemia (Cohen,J., 2002g, Guijarro,C. et al., 2001a). Due to the knowledge about ANP influencing functional parameters of the kidney during endotoxic shock in rats (Aiura,K. et al., 1995b), aim of the present study was to elucidate potential anti-inflammatory effects of ANP during endotoxemia in this organ.

2.6.2 Pathogenetic networks in sepsis

The pathogenetic network of cellular events causing organ dysfunction in patients suffering from sepsis is very complex and has therefore not been completely elucidated. An important effect occurring during endotoxemia is the activation of coagulant pathways (Cohen,J., 2002b). Following an infection with LPS or other microbial components, the expression of tissue factor (TF) on endothelial cells is induced. TF in turn activates proteolytic cascades, followed by formation of thrombin, which then generates fibrin from fibrinogen. Simultaneously, levels of plasminogen activator inhibitor-1 (PAI-1) are increased resulting in disruption of fibrinolyis (**figure 5**). Subsequently, increased production of fibrin causes inadequate tissue perfusion and organ failure due to aggregation of fibrin clots in small vessels. This coagulant effect is amplified by concomitant downregulation of anti-coagulant proteins such as antithrombin, protein C, and tissue factor pathway inhibitor (Okajima,K., 2001).

Besides this activation of blood coagulation, the release of pro-inflammatory mediators such as TNF-α and IL-1 by endothelial cells, neutrophils, and monocytes increases the production of NO by inducing the expression of iNOS. Consequently, NO provokes further vascular instability, cellular dysoxia by inhibition of the mitochondrial respiratory chain, in combination with disruption of epithelial tight junctions (Annane,D. et al., 2005c). The release of ROS, TNF-α, interferon γ, and high mobility group box 1 (HMGB1) from macrophages also contributes to loss of function of the endothelial structure causing further instabilization of the vascular system (Woltmann,A. et al., 1998). Activation of the complement system, increased expression of adhesion molecules on neutrophils, and subsequent migration of activated leucocytes from the bloodstream into inflammatory tissue leads to additional deleterious effects on organ function (Holub,M. et al., 2003). In addition, a significant increase in lymphocyte apoptosis has been observed in septic patients, consequently provoking an increased susceptibility to hospital-acquired infection (Hotchkiss,R.S. et al., 1999, Hotchkiss,R.S. et al., 2001). In summary these detrimental events occurring during endotoxemia culminate in coagulopathy, fever, vasodilatation, and capillary leak finally causing multiple organ failure and death of patients (see schema in **figure 5**).

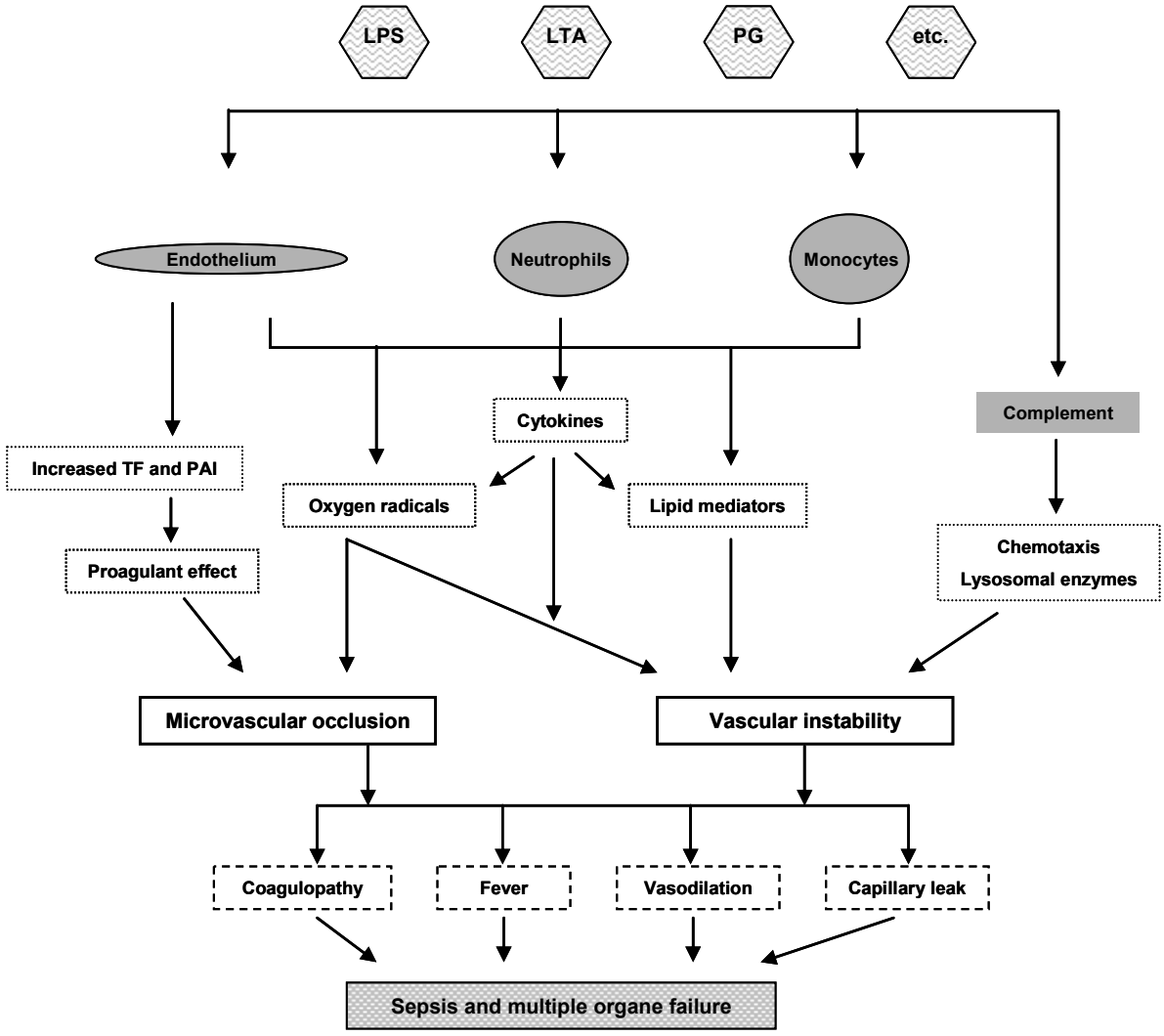

figure 5: The pathogenetic network during sepsis (see text for details). LPS: Lipopolysaccharide, LTA: lipoteichoic acid, PG: peptidoglycan, TF: tissue factor, PAI: plasminogen-activator-inhibitor

The major problem in successful treatment of sepsis is the complexity of challenge and the enormity of problems inside the organism of patients once the challenge has been successfully addressed. Indeed, two new therapies have made it to market, namely Drotecogin (recombinant human activated protein C) (Bernard,G.R. et al., 2001) and low doses of hydrocortisone (Annane,D. et al., 2002), but theses treatments are only effective in the early management of septic shock. Moreover, promising strategies such as inhibition of mediators of the inflammatory cascade (for example TNF-α, interleukin 1, platelet activating factor, or NO synthase) failed to improve survival of patients (Marshall,J.C., 2003). Therefore, it does not astonish that to date a reliable, successful treatment of septic patients is an unrealized request.

2.6.3 Toll-like receptors

In both, insects and vertebrates, the rapid response to pathogens is called innate immunity. In Drosophila the protein Toll is responsible for recognition of pathogens and activation of the innate immune response (Lemaitre,B. et al., 1996). In 1997 Medzhitov *et al.* found the human homologue of this protein, which is a type I transmembrane protein with an extracellular domain consisting of a leucine-rich repeat (LRR) domain and a cytoplasmic domain homologous to the cytoplasmic domain of the human interleukin (IL)-1 receptor (Medzhitov,R. et al., 1997a). This observation gave rise to extensive investigation of this protein, and up to now, ten members of the Toll like receptor (TLR) family have been defined (Cohen,J., 2002a). The ligands of TLRs are incompletely identified, and are apparently not limited to microorganisms, but rather to stimuli that might generally be perceived as danger for the host. Examples for TLR ligands from microorganisms are peptidoglycan from Gram-positive bacteria and bacterial lipoprotein from Gram-negative bacteria, both recognized by TLR2. TLR5 binds flagellin from bacterial flagella, whereas TLR9 binds to conserved motifs in bacterial DNA (Yamamoto,M. et al., 2004a). Important for endotoxemia induced by lipopolysaccharide (LPS), an endotoxin from the cell-wall of Gram-negative bacteria, was the observation of Poltorak *et al.* in 1998 (Poltorak,A. et al., 1998). They showed that mutation in the gene encoding for TLR4 in mice causes defects in LPS-signaling. Thus, they identified TLR4 as the receptor responsible for LPS-mediated signaling.

The engagement of TLRs on macrophages, neutrophiles, and endothelial cells activates multiple signal transduction cascades that lead to alterations in gene expression, particularly nuclear factor κB (NF-κB) dependent gene expression (Yamamoto,M. et al., 2004b). This transcription factor is crucially involved in pro-inflammatory cytokine expression (see 2.6.4). Subsequently, the generated inflammatory mediators can act on the cell in an autocrine fashion, on neighbouring cells in a paracrine, and on distant tissues in an endocrine manner, evoking new signaling cascades, all together being responsible for the consequences of infection in the organism (Beutler,B., 2004).

2.6.4 Signaling pathways during LPS-induced sepsis

After infection of the host, LPS is carried in the blood by a specific carrier protein, namely Lipopolysaccharide-binding protein (LBP) (Cohen,J., 2002c). This interaction causes monomerization of LPS, thus transforming it into the bioactive formation (Schromm,A.B. et al., 1996). Through the surface-bound CD14 receptor the endotoxin interacts with cells of the innate immune system (Wright,S.D. et al., 1990) (see schema **figure 6**). As previously shown, CD14 is not only present as membrane-bound receptor, but also in a soluble form in the circulation (Cohen,J., 2002d). The endotoxin-CD14 complex then engages the LPS-specific receptor TLR4 (2.6.3) and thus initiates intracellular signaling. For activation of TLR4, the cell-surface molecule MD-2 is essential, which is suggested to be responsible for correct positioning of the receptor on the cell surface (Shimazu,R. et al., 1999, Yang,H. et al., 2000).

Each TLR possesses its own TIR (Toll/IL-1 receptor/resistance motif) domain responsible for interaction with adaptor proteins such as the myeloid differentiation protein MyD88 in case of TLR4. These adaptors have been demonstrated to be crucial for the cellular response to LPS-binding (Kawai,T. et al., 1999, Zhang,F.X. et al., 1999a). In addition to MyD88, subsequent elements recruited to the TLR4 activation complex are the IL-1 receptor associated kinase (IRAK) and TNF-α receptor-associated factor (TRAF6) (Medzhitov,R. et al., 1997b, Medzhitov,R. et al., 1998). The essential role of IRAK and TRAF-6 has been demonstrated in mutants *in vitro* and in knock-out studies *in vivo* (Cao,Z. et al., 1996, Lomaga,M.A. et al., 1999, Zhang,F.X. et al., 1999b). However, the mechanism by which TRAF6 activates the IκB kinase (IKK) complex downstream in the course of LPS-mediated signaling is not understood yet. IKKα and IKKβ form the IKK complex together with IKKγ (also named NEMO for NF-κB essential modulator) (DiDonato,J.A. et al., 1997, Rothwarf,D.M. et al., 1998). Upon phosphorylation, IKK rapidly phosphorylates its downstream target, the NF-κB inhibitor proteins IκB at two phosphorylation sites (Ser 32 and Ser 36 in IκBα) (Whiteside,S.T. et al., 1997). Once phosphorylated, IκBs undergo polyubiquitination by a specialized E3 ubiquitin ligase complex known as E3 $^{I\kappa B}$(DiDonato,J. et al., 1996, Winston,J.T. et al., 1999, Yaron,A. et al., 1998). Polyubiquitinated IκBs are then targeted for rapid degradation by the S26 proteasome, thus releasing NF-κB and facilitating translocation of the transcription factor into the nucleus (Karin,M. et al., 2000). The NF-κB

family so far consists of five identified members: p65 (also named RelA), RelB, c-Rel, p50/p105, and p52/p100. In unstimulated cells these proteins exist in homo- or heterodimers in the cytosol, inactivated through binding to their inhibitor IκB (Chen,L.F. et al., 2004b). The classical NF-κB heterodimer, which is p50/p65, is predominately regulated by IκBα (Beg,A.A. et al., 1995b, Klement,J.F. et al., 1996a). After secession of phosphorylated IκB, NF-κB with its two subunits is able to migrate from the cytosol into the nucleus, where it binds to the DNA. Subsequently, the activation of transcription of NF-κB target genes is induced and even further enhanced *via* direct acetylation of histones, which surround these genes, by the p65 subunit of NF-κB (Chen,L. et al., 2001). Among the target genes of NF-κB are those encoding for TNF-α, IL-1, and also IκBα, thus displaying not only a starting point of inflammatory processes, but also a negative regulatory response to NF-κB activation (Brown,K. et al., 1993, Sun,S.C. et al., 1993).

Ultimately, the binding of LPS to TLR4 provokes immense production of inflammatory cytokines, which are then released into the circulation by the LPS-responding cells.

Note: In this study only the LPS-mediated effects on NF-κB activation are further investigated (**figure 6**). Besides this signaling cascade infection with LPS might also lead to the activation of MAPK pathways causing for example activation of the activator protein-1 (AP-1) transcription factors, jun and fos (Irie,T. et al., 2000, Ninomiya-Tsuji,J. et al., 1999), but this signaling pathway is not further specified in this work.

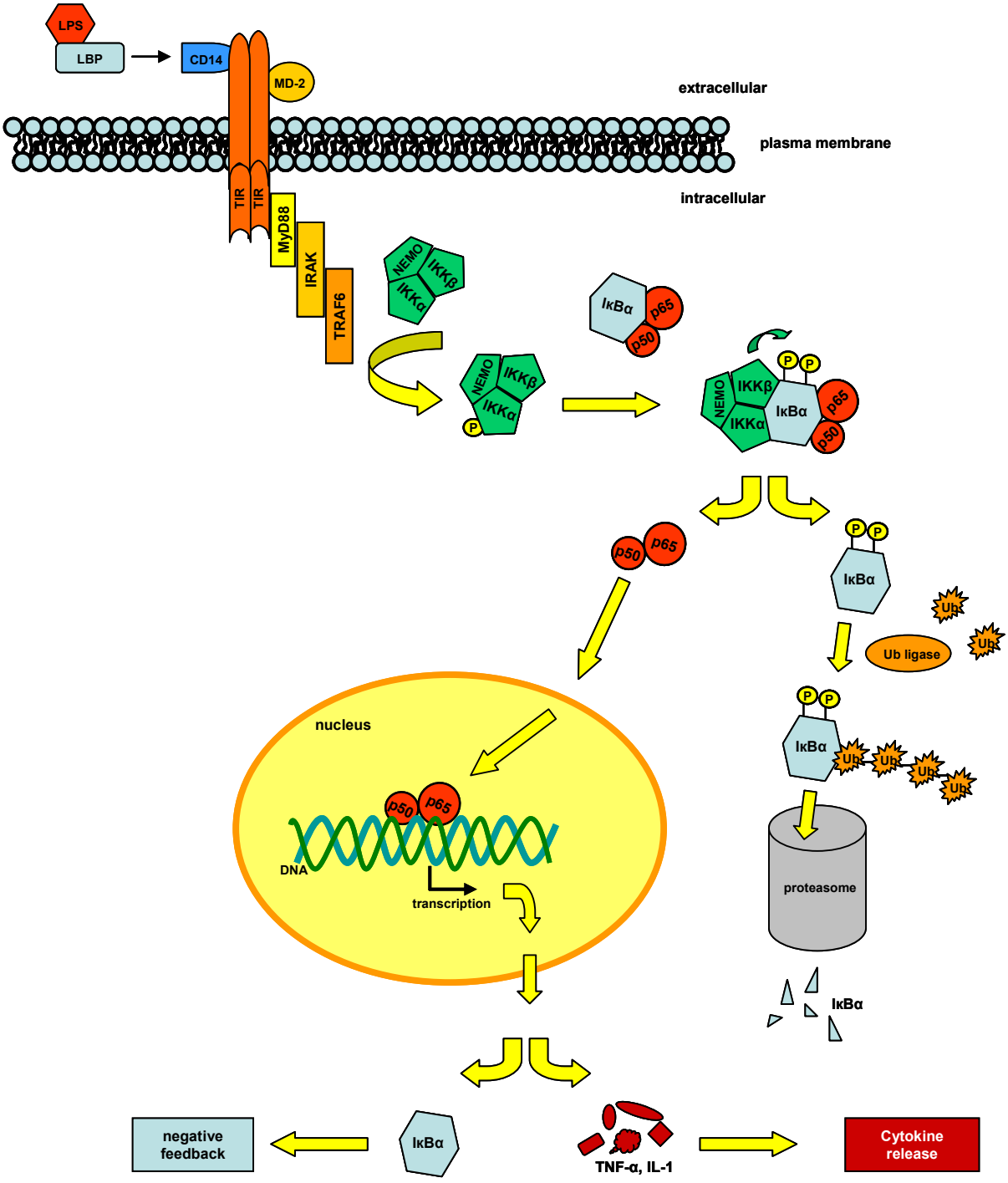

figure 6: Signaling pathways leading to NF-κB activation during LPS-induced sepsis.

3. Materials and Methods

3.1 THE ISOLATED PERFUSED RAT LIVER

3.1.1 Animals

Male Sprague-Dawley rats (weight range: 200-300 g) were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) and housed in a climatized room with a 12 h light-dark cycle. The animals had free access to chow (Ssniff, Soest, Germany) and water and received human care. All studies were performed with the permission of the government authorities and in accordance with the German Legislation on Laboratory Animal Experiments.

3.1.2 Materials and solutions

3.1.2.1 Materials

Atrial Natriuretic Peptide (ANP, rat), SB203580 (SB), and Wortmannin (WM) were purchased from Calbiochem/Novabiochem (Bad Soden, Germany). Narcoren® was from Merial GmbH (Halbergmoos, Germany). All other materials were purchased from either Sigma (Deisenhofen, Germany) or VWR International™ (Munich, Germany).

3.1.2.2 Solutions

Krebs-Henseleit buffer (KH buffer) pH 7.4

NaCl	118 mM
KCl	4.8 mM
KH_2PO_4	1.2 mM
$MgSO_4$	1.2 mM
$CaCl_2$	1.5 mM
$NaHCO_3$	25 mM

3.1.3 Experimental setting

Liver perfusion was performed by Dr. Tobias Gerwig (Department of Pharmacy, University of Munich, Munich, Germany).

As described by *Gerbes et al.* (Gerbes,A.L. et al., 1998c), male Sprague-Dawley rats were anaesthetized with Pentobarbital (Narcoren®, 50 mg/kg body weight, intraperitoneally), 250 IU heparin were administered, the portal vein was cannulated, and the liver was perfused *in situ* with hemoglobin- and albumin-free bicarbonate buffered Krebs-Henseleit (KH) solution (pH 7.4, 37 °C) gassed with 95% O_2 and 5% CO_2. The perfusion medium was pumped through the liver at a constant flow rate in a non-recirculating manner.

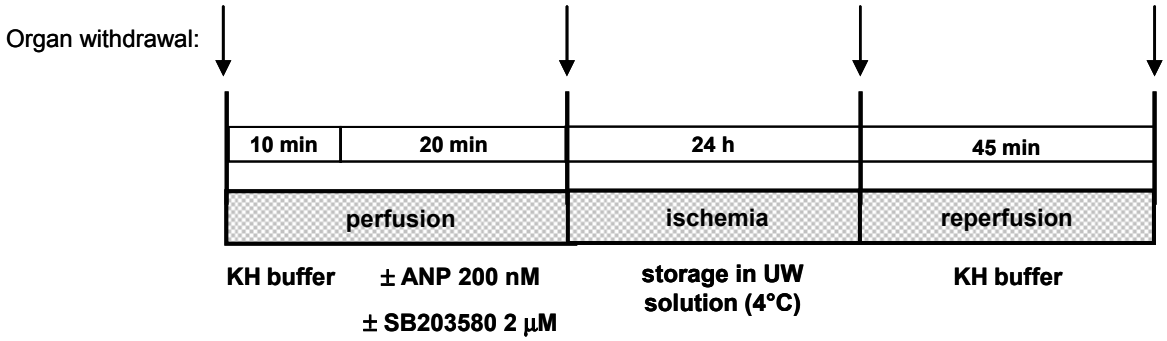

figure 7: Isolated liver perfusion: Livers were perfused with KH buffer before ischemia for 30 min either in the absence or in the presence of 200 nM ANP, ±SB (2 µM) given 20 min prior to ischemia. Livers were kept under ischemic conditions for 24 h in University of Wisconsin solution (UW solution) and reperfusion for 45 min. Organs were excised and snap-frozen at the indicated times (n=6 animals in each treatment group).

After 10 min of perfusion with KH buffer, ANP (200 nM) was added to the perfusion buffer (depending on the treatment group), followed by an additional perfusion for 20 min. When indicated, SB was added to the KH buffer followed by 30 min of perfusion (**figure 7**).

Subsequently, livers were perfused with 30 ml of cold (4 °C) University of Wisconsin (UW) solution (DuPont Pharma GmbH, Bad Homburg, Germany) for 1 min and stored in UW solution for 24 h at 4 °C under ischemic conditions.

Following ischemia, livers were reperfused with KH solution for up to 45 min. The graft's common bile duct was cannulated with a PE-tube and bile was collected and its volume determined. Bile flow was calculated per min and g liver tissue. At the indicated times (after 30 min perfusion, 24 h ischemia, 45 min reperfusion), livers were snap-frozen in liquid nitrogen and stored at -85 °C until further examination (see also **figure 7**). All experiments were performed with n=6 animals for each treatment group.

3.1.4 Homogenization and fractionation of liver tissue for Western Blot analysis

A fractionation protocol was employed according to published methods with some modifications (Carey,D.J. et al., 1987a, Carey,D.J. et al., 1987b, Fox,J.E. et al., 1992). Briefly, 50 mg of liver tissue were homogenized in 1.5 ml of lysis buffer (50 mM Tris-HCl pH 7.0, 5 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 40 µl Complete®) containing 1% Triton® X-100 (Roth, Karlsruhe, Germany) with a dounce homogenizer. After centrifugation at 15,000*xg* (15 min, 4 °C) the cytoskeletal fraction remained in the pellet. Supernatants, representing the cytosolic fraction, were cleared by ultracentrifugation (100,000*xg*, 2.5 h, 4 °C). The pellet was resuspended in SDS-containing sample buffer and the cytosolic fraction was diluted with the same sample buffer. Samples were stored at –20 °C until use for Western Blot analysis.

3.1.5 Quantification of actin content in liver fractions

After detection of F- and G-actin in liver samples by Western Blot analysis, the densitometric intensity of the corresponding bands was used to evaluate the ratio of F-, and G-actin respectively to total actin (F- + G-actin) in the corresponding sample.

3.2 RAT LIVER TRANSPLANTATION

3.2.1 Animals

Syngeneic, male Lewis rats (donors: 207±12 g; recipients: 276±18 g) were purchased from Charles River Wiga (Sulzfeld, Germany) and housed in a temperature- and humidity-controlled room 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 operation. All studies were performed with the permission of the government authorities and in accordance with the German Legislation on Laboratory Animal Experiments.

3.2.2 Materials and solutions

Materials and solutions used for *in vivo* experiments are described in chapter 3.1.2.

3.2.3 Experimental setting

In vivo experiments with ANP were performed by Dr. Uwe Grützner (Institute for Surgical Research, Klinikum Großhadern, University of Munich, Germany).

In transplantation experiments donor animals obtained a 20 min intravenous infusion of either 0.9 % NaCl or ANP (5 μg/kg b.w.) prior to hepatectomy. For investigation of signaling pathways additional rats were treated with Wortmannin (WM, 16 μg/kg b.w., in 0.1 % DMSO), DMSO (0.1%), or a combination of ANP and WM (see **figure 8**).

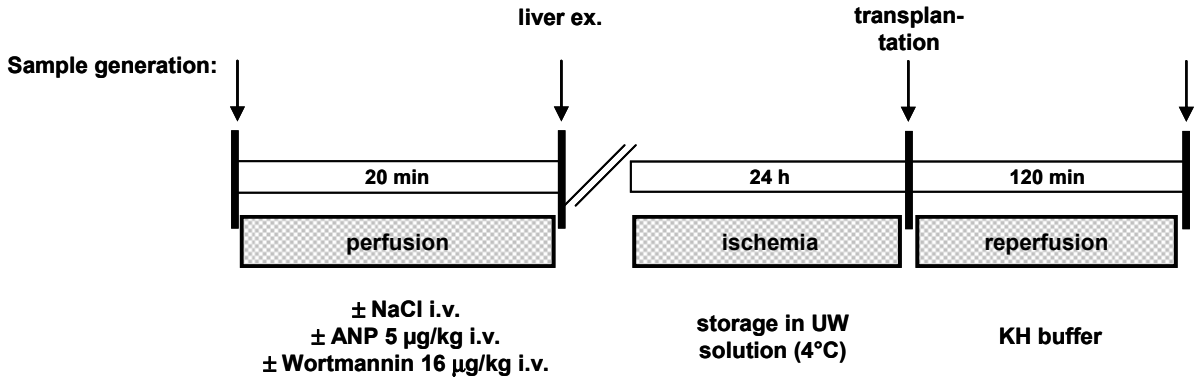

figure 8: ANP pretreatment *in vivo*: Rats received an intravenous infusion of 0.9 % NaCl, ANP (200 nM), Wortmannin (16 µg/kg b.w.), or various combinations for 20 min. The liver was excised, kept for 24 h under ischemic conditions, transplanted into recipient, and reperfused for up to 2 h. Livers were excised at the indicated time points, snap-frozen, and stored at -85 °C until further investigation.

Donor and recipient operations were performed under ether anesthesia after premedication with atropine (0.1 mg/kg b.w.). Blood pressure and heart rate were continuously monitored by a catheter in the carotid artery. A jugular catheter was used to apply substances and substitute plasma volume. Body temperature was kept between 36.5 °C and 37.5 °C by means of a heating pad. Donor livers were preserved by retrograde aortal flush with 10 ml UW-solution and stored at 4 °C for 24 h. Prior to implantation, the livers were rinsed with 10 ml of cold Ringer's solution (B. Braun Medical Inc., Irvine, CA, USA) *via* the portal vein at a hydrostatic pressure of 10 cm H_2O.

Orthotopic liver transplantation was performed using a modified cuff technique as described (Schauer,R.J. *et al.*, 2004). Grafted livers were simultaneously reperfused after completion of the arterial anastomosis. Portal clamping time was less than 20 min in all experiments.

The graft's common bile duct was cannulated with a PE-tube and bile was collected and its volume determined. The bile flow was calculated per min and g liver tissue. Plasma samples (400 µl) were obtained from the recipient before hepatectomy as well as 60 and 120 min after reperfusion of the transplanted liver. The removed blood volume was replaced by saline. After starting reperfusion, rats received 1.0 ml of albumine (5%) and 0.5 – 1.0 ml sodium bicarbonate solution to maintain blood pressure and physiological pH. To avoid fluid loss and drying of the liver, the abdominal cavity was covered with Saran wrap.

After 20 min preconditioning, 24 h cold ischemia, or 120 min reperfusion, a small section of each organ

was placed in 4% paraformaldehyde and embedded in paraffin wax for TUNEL staining. The remaining organ was snap frozen in liquid nitrogen and stored at -85 °C until further analysis (**figure 8**). All experiments were performed using n=4 animals for each treatment group.

3.2.4 Determination of activities of serum aminotransferases and lactate dehydrogenase

Activities of serum aminotransferases (alanine transferase (ALT), aspartate transferase (AST)) and LDH were determined as established markers of hepatic injury. Activities were measured by Dr. Uwe Grützner (Institute for Surgical Research, Klinikum Großhadern, University of Munich, Germany) 2 h after reperfusion using a serum multiple analyzer (Olympus AU 2700, Germany) at 37 °C.

3.2.5 PKA activity assay

This assay was performed with a commercial PKA assay kit obtained from Calbiochem. Tissue samples (100 μg) were homogenized in ice-cold “extraction buffer” and centrifuged as described in the manufacturer’s manual. Supernatants were used for measurement of PKA activity by *in vitro* phosphorylation of the specific peptide substrate kemptide with [γ^{32}P]-ATP.

3.2.6 Caspase-3-like activity assay

Caspase activity was measured by applying a synthetic peptide substrate which is coupled to a fluorophor. Cleavage of the fluorogenic substrate by the activated caspase leads to increased fluorescence.

After homogenization of 100 mg liver tissue in 1 ml of lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA (pH 7.5), Complete®), samples were centrifuged at 14,000 rpm (10 min, 4 °C). In the supernatants, caspase-3-like activity was determined as reported previously (Gerwig,T. et al., 2003c, Gerwig,T. et al., 2003d). Briefly, generation of free fluorescent 7-amino-4-trifluoro-methylcoumarin (AFC) from the substrate DEVD-AFC (Sigma, Deisenhofen, Germany) was measured after incubation at 37 °C with a microplate reader (Spectra Fluor Plus, TECAN Deutschland GmbH, Crailsheim, Germany). Protein concentrations were determined by Pierce Assay (Pierce,R.H. *et al.*, 2000) (Pierce, Rockford, USA).
Control experiments revealed the linear character of activity regarding duration and protein concentration. Thus, specific caspase-3-like activity was calculated per µg protein.

3.3 MURINE MODEL OF LPS-INDUCED SEPSIS

3.3.1 Animals

Specific pathogen-free male BALB/c mice (22±6 g) were supplied by the in house Animal Breeding Facility of the University of Konstanz and housed in a temperature- and humidity-controlled room (22 °C, 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 and in accordance with the German Legislation on Laboratory Animal Experiments and followed the directives of the University of Konstanz Ethical Committee.

3.3.2 Materials and solutions

3.3.2.1 Materials

Rat ANP was purchased from Calbiochem/Novabiochem (Bad Soden, Germany), Lipopolysaccharide (LPS) from *Salmonella abortus equi* S. from BIOCLOT (Aidenbach, Germany). Pentobarbital (Nembutal), which was used for anesthesia of animals, was from Sanofi-Ceva (Hanover, Germany). TNF-α enzyme-linked immunosorbent assay (ELISA) was purchased from Amersham Bioscience (Braunschweig, Germany). All other materials were purchased from either Sigma (Deisenhofen, Germany) or VWR International™ (Munich, Germany).

3.3.2.2 Solutions

ANP and LPS were diluted in a total volume of 300 µl sterile 0.9 % saline solution containing 0.1% human serum albumine (HSA).

3.3.3 Experimental setting

ANP (5 µg/kg b.w.) was injected intravenously (i.v.). Septic shock was induced by intraperitoneal (i.p.) injection of a sublethal LPS dose of 1 mg/kg b.w. for the 90 min experiments, and a lethal dose of 2.5 mg/kg b.w. for survival experiments 20 min after ANP treatment (**figure 9**).

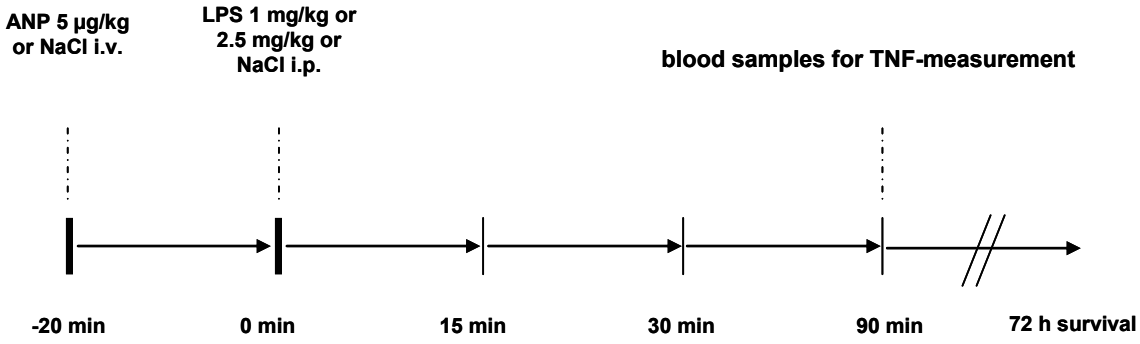

figure 9: Experimental setting for ANP preconditioning *in vivo* before LPS-induced septic shock. Animals were injected i.v. with ANP (5 µg/kg b.w.) or NaCl and after 20 min with LPS or NaCl i.p.. After 90 min blood samples were taken and used for determination of TNF-α level. LPS was injected in a sublethal (1 mg/kg b.w.) or lethal (2.5 mg/kg b.w.) dose. Survival of animals was observed until up to 72 h after LPS injection. For sample generation, organs were excised at indicated times (20 min after ANP injection t = 0 min, after LPS injection at t = 15 min, t = 30 min, and t = 90 min) after lethal intravenous anesthesia. For measurement of TNF-α concentration, blood samples were generated after 90 min.

3.3.3.1 Survival experiment (72 h)

To investigate whether ANP preconditioning has an effect on survival after injection of a lethal LPS dose, ANP (5 µg/kg b.w.) was injected i.v. 20 min prior to i.p. LPS-injection (2.5 mg/kg b.w. in 300 µl saline containing 0.1% HSA). After LPS-injection the animals' state of health was observed continuously for 72 h. Animals of the control group were injected with 0.9 % NaCl at t = -20 min and t = 0 min. Animals of the LPS-group were injected with NaCl instead of ANP at the onset of the survival experiment (see also **figure 9**).

After 90 min, blood samples for TNF-α measurement were obtained by slightly cutting the animals' tail and collecting approximately 50 µl blood into a heparinized microfuge tube. Blood samples were centrifuged (13,000 rpm, 2 min, 4 °C) immediately before TNF-α concentration was determined by ELISA (see 3.3.4).

Four treatment groups were generated, each group with n=8 animals (see **table 1**).

table 1: Treatment groups for survival experiment.

Treatment group	t = -20 min	t = 0 min
Control (Co)	NaCl i.v.	NaCl i.p.
ANP	ANP (5 µg/kg b.w.) i.v.	NaCl i.p.
LPS	NaCl i.v.	LPS (1 mg or 2.5 mg/kg b.w.) i.p.
ANP/LPS	ANP (5 µg/kg b.w.) i.v.	LPS (1 mg or 2.5 mg/kg b.w.) i.p.

3.3.3.2 Tissue sample generation (endotoxemia)

This *in vivo* experiment started with the intravenous injection of NaCl or ANP (5 µg/kg b.w.). Animals received an i.p. injection of NaCl or LPS (1 mg/kg b.w.) after 20 min. At the indicated times (see **figure 9**), blood and tissue samples were obtained after lethal intravenous anesthesia of mice with 150 mg/kg b.w. pentobarbital plus 0.8 mg/kg b.w. heparin.

Blood samples were taken after 90 min following abdominal laparotomy. TNF-α serum concentrations were determined. For this purpose, blood was withdrawn by cardiac puncture, filled into heparinized microfuge tubes, and centrifuged (2 min, 13,000 rpm, 4 °C).

Kidneys were excised at t = 0 min, t = 15 min, and t = 90 min, and snap-frozen immediately in liquid nitrogen. The latest time point for excision of organs was 90 min. Therefore, this kind of experimental setting for investigation of ANP effects on LPS-signaling is termed “endotoxemia”.

Four treatment groups were generated, each group consisting of n=6 animals (see **table 2**).

table 2: Treatment groups for endotoxemia.

Treatment group	t = -20 min	t = 0 min
Control (Co)	NaCl i.v.	NaCl i.p.
ANP	ANP (5 µg/g b.w.) i.v.	NaCl i.p.
LPS	NaCl i.v.	LPS (1 mg/kg b.w.) i.p.
ANP/LPS	ANP (5µg/kg b.w.) i.v.	LPS (1 mg/kg b.w.) i.p.

3.3.4 Determination of TNF-α concentrations in blood samples

Blood samples were centrifuged for 2 min at 4 °C at 13,000 rpm to separate the plasma from the cellular fraction. Measurement of TNF-α by ELISA was performed as described previously (Bohlinger,I. *et al.*, 1996) with an OptEIA Mouse TNF-α Elisa Set (Mono/Mono) (BD Biosciences, Heidelberg, Germany). Briefly, 50 µl of sample were pipetted onto a microtiter plate which was coated with a mouse specific TNF-α antibody. 50 µl of a biotinylated antibody reagent was added, followed by incubation for 2 h at room temperature (RT). After several washing steps, 100 µl of a streptavidine-horseradish peroxidase-conjugate was added. Following incubation for 30 min (RT) and five more washing steps, incubation for 30 min (RT, protected from light) with 100 µl of the substrate solution TMB (3', 5, 5' - tetramethyl-benzidine) caused a color development proportional to the amount of mouse TNF-α in the sample. The reaction was terminated by addition of 100 µl stop solution (0.18 M sulphuric acid) and optical density was determined at 450 nm using the SUNRISE Absorbance Reader from TECAN (TECAN Deutschland GmbH, Crailsheim, Germany).

3.4 HISTOLOGICAL ANALYSIS OF TISSUE

3.4.1 TUNEL staining

TUNEL staining was kindly performed by Dr. Herbert Meissner and Mrs. Andrea Sendlhofer (Institute of Pathology, University of Munich,, Germany).

Liver samples were fixed in 4% formaldehyde, embedded in paraffin, and cut into 6 µM sections. Liver sections were rinsed three times with PBS (pH 7.4, containing calcium and magnesium), before blocking for 20 min with 1% BSA. Apoptotic processes were examined by quantification of DNA fragmentation by the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling method (TUNEL) as described by Gavrieli *et al.* (Gavrieli,Y. *et al.*, 1992). The number of apoptotic liver cells was determined by staining of liver sections with the ApopTaq® Peroxidase *in situ* Apoptosis Detection Kit according to the

manufacturer's instructions (Intergen, Purchase, New York, USA). For counting the apoptotic cells, an area of 1.96 mm^2 containing approximately 4,000 hepatocytes was examined by a Zeiss Axiolab microscope (Zeiss, Jena, Germany).

3.4.2 Hematoxylin staining

Necrotic cell death and morphological changes of tissue were characterized by hematoxylin and eosin (HE) staining. Morphological criteria for necrosis included increased eosinophilia, vacuolization, cell disruption, loss of architecture, and karyolysis. HE staining was performed by Dr. Herbert Meissner and Mrs. Andrea Sendlhofer (Institute of Pathology, University of Munich, Germany).

3.4.3 Immunohistochemistry

For analysis of localization and / or presence of certain proteins in liver or kidney, organs were snap-frozen in liquid nitrogen at the indicated times and cut into 6-10 μm sections.

For staining of phospho-Akt and F-actin, slices were dried overnight at RT and subsequently fixed in 3% formaldehyde for 15 min. For p65 staining, organs were fixed for 3 min in 3% formaldehyde immediately after cutting and were kept in PBS.

Fixed slices of liver or kidney tissue were washed three times with PBS and blocked with 1% BSA for 20 min. This was followed by incubation with 100 µl of the primary antibody (see **table 3**) for 1 h at RT. After three washing steps, slices were incubated with the corresponding secondary antibody (see **table** 3) for 1 h at RT and again were washed three times with PBS. Finally, liver or kidney sections were covered with mounting medium (DakoCytomation GmbH, Hamburg, Germany), dried overnight, and examined by confocal microscopy (LSM 510 Meta, Zeiss, Jena, Germany).

table 3: Antibodies used for tissue staining.

Primary antibodies	**Diluted in**	**Dilution**	**manufacturer**
Mouse anti-phospho-Akt monoclonal (Ser 473)	0.2% BSA	1:200	Cell Signaling, Frankfurt/Main, Germany
Rabbit anti-phospho-Akt polyclonal (Ser 473)	0.2% BSA	1:200	Cell Signaling, Frankfurt/Main, Germany
Rabbit anti p65 polyclonal	0.2% BSA	1:100	Santa Cruz, Heidelberg, Germany
Secondary antibodies			
Alexa Fluor® 488 Goat anti-mouse IgG	0.2% BSA	1:400	Molecular Probes, MoBiTec, Göttingen, Germany
Alexa Fluor® 647 chicken anti-rabbit IgG (H+L)	0.2% BSA	1:400	Molecular Probes, MoBiTec, Göttingen, Germany
Alexa Fluor® 488 goat anti rabbit IgG (H+L)	0.2% BSA	1:400	Molecular Probes, MoBiTec, Göttingen, Germany
Rhodamine conjugated phalloidin	methanol p.a.	1 unit per slice (5 µl stock solution	Molecular Probes, MoBiTec, Göttingen, Germany
HOECHST 33342 dye	0.2% BSA	5 µg per slice	Sigma, Deisenhofen, Germany

3.5 WESTERN BLOT ANALYSIS

3.5.1 Sample preparation – homogenization of tissue

30 mg of kidney or liver tissue were homogenized in 0.6 ml of lysis buffer (50 mM Tris-HCl, 5 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4 pH 7.0, 1x Complete®) containing 1% Triton® X-100 (Roth, Karlsruhe, Germany) with a dounce homogenizer. Care was taken to ensure a homogenous suspension. After centrifugation of samples at 14,000 rpm (10 min, 4 °C), 10 µl of the supernatant were used for determination of protein content (see 3.5.2). The remaining supernatant was diluted with 3x SDS-

containing sample buffer (see 3.5.4.1) and boiled for 5 min at 95 °C. Aliquots were stored at – 80 °C until Western Blot analysis.

3.5.2 Measurement of protein concentrations with Pierce assay

In order to employ equal amounts of protein in all samples analyzed by Western Blot, the protein concentrations were determined using the Pierce assay (BC assay reagents, Interdim, Montulocon, France) as described by Smith *et al.* (Smith,P.K. et al., 1985).

3.5.3 Immunoprecipitation

After homogenization of liver tissue (see 3.5.1) sample protein concentrations were determined by Pierce Assay (3.5.2). 100 µg protein in 100 µl lysis buffer were incubated with 2.5 µl of primary antibody (rabbit anti Hsp25 or rabbit anti-Bad) shaking overnight at 4 °C. Following centrifugation and resolution of the pellet in 500 µl lysis buffer, the antibody-antigen complex was precipitated by incubation with 50 µl of washed Agarose-A-beads (Sigma, Deisenhofen, Germany) for 2 h (shaking, 4 °C), followed by additional centrifugation. The beads were washed three times with cold lysis buffer and resuspended in 40 µl of 3x SDS-containing sample buffer. After addition of 40 µl 1x sample buffer, samples were boiled for 5 min at 95 °C followed by centrifugation in order to remove the beads. 35 µl of the supernatant were used for detection of phospho-Bad by Western Blot analysis.

3.5.4 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

3.5.4.1 Solutions

SDS sample buffer (stock solution)	
Tris-HCl (pH 6.8)	37.5 ml
SDS	6 g
Glycerol	30 ml
Bromphenol blue	15 mg
H_2O	ad 100 ml

SDS sample buffer (3x)	
Stock solution	950 µl
β-mercaptoethanol	50 µl

To prepare 1x sample buffer solution, the appropriate amount of water was added to the 3x solution.

Separation gel 10%	
PAA solution (30%)	5.0 ml
1.5 M Tris-base, pH 8.8	3.75 ml
SDS 10%	150 µl
H_2O	6.1 ml
TEMED	15 µl
APS 10%	75 µl

Stacking gel	
PAA solution (30%)	1.275 ml
1.25 M Tris-HCl, pH 6.8	750 µl
SDS 10%	75 µl
H_2O	5.25 ml
TEMED	15 µl
APS 10%	75 µl

For preparation of separation gels different concentrations of polyacrylamide (PAA, 30% solution of PAA / 0.8% bisacrylamide 37.5 : 1 (v/v), Rotiphorese™ Gel 30, Roth, Karlsruhe, Germany) were used according to the molecular weight of the analyzed protein.

Electrophoresis buffer (5x)	
Tris-base	15.0 g
Glycine	72.0 g
SDS	5.0 g
H_2O	ad 1,000 ml

To prepare 1x Electrophoresis buffer, the appropriate amount of water was added to the 5x buffer.

3.5.4.2 Electrophoresis

Denaturating SDS-PAGE allows the separation of proteins according to their molecular weight. Proteins are solubilized in a sodium dodecylsulfate (SDS) solution, a highly negatively charged detergent. After denaturation of the proteins by addition of dithiothreitol (DTT) and boiling of samples, negatively charged SDS-polypeptide complexes migrate towards the anode. Their migration velocity corresponds to the protein's molecular weight (Laemmli,U.K., 1970).

Equal amounts of protein (70 µg per slot for IκBα and phospho-IκBα, 100 µg for other proteins) were loaded and separated by SDS-PAGE (Mini PROTEAN 3, BioRad Laboratories, Munich, Germany). Electrophoresis was run at 100 V for 21 min for stacking, and 4 min at 200 V for separating the proteins.

3.5.5 Western Blot

3.5.5.1 Solutions

Tris-CAPS (5x), pH 9.6

Tris-base	36.34 g
CAPS	44.26 g
H_2O	ad 1,000 ml

Anode buffer		Cathode buffer	
Tris-CAPS (5x)	20 ml	Tris-CAPS (5x)	20 ml
Methanol	15 ml	SDS 10%	1 ml
H_2O	ad 100 ml	H_2O	ad 100 ml

Tank Blotting buffer (5x)

Tris-base	15.2 g
Glycine	72.9 g
H_2O	ad 1,000 ml

Tank Blotting buffer (1x)

Blotting buffer (5x)	200 ml
Methanol	200 ml
H_2O	ad 1,000 ml

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

Tris-base	3.0 g
NaCl	11.1 g
Tween 20	1.0 ml
H_2O	ad 1,000 ml

Coomassie staining solution

Coomassie brilliant blue G	1.5 g
Acetic acid (100%)	50 ml
Ethanol (96%)	225 ml
H_2O	ad 500 ml

Coomassie destaining solution

Acetic acid (100%)	100 ml
Ethanol (96%)	335 ml
H_2O	ad 1,000 ml

ECL solution

Solution 1:

Luminol (250 mM)	50 µl
p-Coumaric acid (90 mM)	22 µl
1 M Tris, pH 8.5	500 µl
H_2O	4,500 µl

Solution 2:

H_2O_2 (30 %)	3 µl
1 M Tris, pH 8.5	500 µl
H_2O	4,500 µl

Stripping buffer

Tris-HCl	62.5 mM
SDS	2%
β-mercaptoethanol	0.8%

5% Blotto:

5% (m/v) solution of low fat dried milk (Blotto, BioRad, Munich, Germany) in TBS-T pH 8.0.

Antibodies:

table 4: Primary and secondary antibodies used for Western blot analysis. Abbreviations: WB = Western Blot analysis, IP = Immunoprecipitation.

Primary antibodies	**Diluted in**	**Dilution**	**Manufacturer**	**Used for**
Mouse anti-actin	5% Blotto	1:1,000	Biozol, Eching, Germany	WB
Rabbit anti-Akt (tot)	5% BSA	1:1,000	Cell Signaling, Frankfurt/Main, Germany	WB
Rabbit anti-phospho-Akt (Ser 473)	1%BSA	1:1,000	Cell Signaling, Frankfurt/Main, Germany	WB
Rabbit anti-Bad (tot)	5% BSA	1:1,000	Cell Signaling, Frankfurt/Main, Germany	WB, IP
Mouse anti-phospho-Bad (Ser 112)	5% BSA	1:2000	Cell Signaling, Frankfurt/Main, Germany	WB
Rabbit anti-phospho-Bad (Ser 136)	1% Blotto	1:500	Cell Signaling, Frankfurt/Main, Germany	WB
Rabbit anti-Hsp25 (tot)	5% Blotto	1: 1,000	Stress Gen Biotechnologies, Victoria, Canada	WB, IP
Rabbit anti-phospho-Hsp27 (Ser 82)	5% BSA	1:1,000	Cell Signaling, Frankfurt/Main, Germany	WB
Rabbit anti IκB-α (tot)	1% Blotto	1:1,000	Santa Cruz Heidelberg, Germany	WB
Rabbit anti-phospho-IκB-α (Ser 32)	1% Blotto	1:1,000	Cell Signaling, Frankfurt/Main, Germany	WB
Secondary antibodies				
Goat anti-rabbit IgG (H+L)	1% Blotto	1:20,000	Dianova, Hamburg, Germany	WB
Goat anti mouse IgG1	5% Blotto	1:1,000	Biozol, Eching, Germany	WB

3.5.5.2 Semi dry Blotting

For the transfer of proteins to a polyvinylidene fluoride (PVDF) membrane by Semi dry Blotting in a discontinuous buffer system, we used the Fastblot B43 (Biometra, Göttingen, Germany). The membrane

(Immobilon-P, Millipore, Bedford, MA, USA) was soaked in methanol for 5 min and in anode buffer for 30 min. Afterwards, proteins were transferred at 134 mA for 60 min from the gel to the membrane.

3.5.5.3 Tank Blotting

Proteins were transferred to a nitrocellulose membrane (Hybond™ ECL™, Amersham Bioscience, Braunschweig, Germany) at 23 V overnight at 4 °C with a Mini Trans-Blot® (BioRad Laboratories, Munich, Germany), and 1x Blotting buffer was used for Tank Blotting (see 3.5.5.1).

3.5.5.4 Coomassie blue staining

In order to check the equal loading of samples and correct blotting, the remaining proteins in the gels were stained with Coomassie staining solution for 30 min. Afterwards, gels were destained shaking for 60 min in destaining solution before they finally were stored in water.

3.5.5.5 Protein detection

Prior to immunologic detection of the referring proteins, unspecific binding sites were blocked by incubating the membrane for 60 min at RT in a 5% (m/v) solution of low fat dried milk (Blotto, BioRad, Munich, Germany) in TBS-T pH 8.0.

Detection of the protein of interest through binding of a specific primary antibody to the protein was performed by incubation of the membrane overnight at 4 °C with the appropriate solution of the specific primary antibody (see **table 4**). After four washing steps (4 x 5 min in TBS-T), the membrane was incubated with the secondary antibody (conjugated to horseradish peroxidase (HRP), **table 4**) for 60 min, followed by four additional washing steps (2 x 10 min, 2 x 5 min).

For detection of proteins, the membrane was incubated in a mixture (1:1) of the two detection solutions, solution 1 and solution 2 (see 3.5.5.1) for 1 min. Chemoluminescence detection was performed by exposure of the membrane to a medical X-ray film (Fuji, Düsseldorf, Germany) and subsequent development with a Curix 60 developing system (Agfa-Gevaert N.V., Mortsel, Belgium).
Quantification was performed with a Kodak image station (NEN, Cologne, Germany).

3.5.5.6 Reprobing

For removing primary and secondary antibodies from the membrane, blots were incubated in stripping buffer (see 3.5.5.1) shaking for 30 min at 50 °C. After six washing steps (5 min in TBS-T at RT), the membrane was blocked for 1 h with 5% low fat dried milk (in TBS-T) before detection with another primary antibody as described in 3.5.5.5.

3.6 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

3.6.1 Solutions

Buffer A		Buffer B	
HEPES pH 7.9	10 mM	HEPES pH 7.9	20 mM
KCl	10 mM	NaCl	400 mM
EDTA	0.1 mM	EDTA	1 mM
EGTA	0.1 mM	EGTA	0.5 mM
DTT	1 mM	Glycerol	25%
PMSF	0.5 mM	DTT	1 mM
		PMSF	1 mM

DTT and PMSF were added to the Buffer stock solution (A and B) directly before use.

STE buffer pH 7.5	
Tris-HCl	10 mM
NaCl	100 mM
EDTA	1 mM

5x binding buffer pH 7.5	
Glycerol	20%
$MgCl_2$	5 mM
EDTA	2.5 mM
NaCl	250 mM
Tris-HCl	50 mM

Gel loading buffer pH 7.5	
Tris-HCl	250 mM
Bromphenolblue	0.2%
Glycerol	40%

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

10x TBE pH 8.3	
Tris	0.89 M
Boric acid	0.89 M
EDTA	0.02 M

non-denaturating polyacrylamide gel (4.5%)	
10x TBE	5.3%
PAA solution (30%)	15.8%
glycerol	2.6%
TEMED	0.05%
APS	0.08%

0.25x TBE was used as electrophoresis buffer and prepared by adding the appropriate amount of water to the 10x solution.

3.6.2 Extraction of nuclear protein

30 mg of kidney tissue were homogenized in 0.3 ml of Buffer A (see 3.6.1) on ice with a dounce homogenizer. Care was taken to ensure a homogenous suspension. Samples were centrifuged at 1,000 rpm at 4 °C for 10 min. The pellet was resuspended in 300 µl Buffer A, followed by addition of 18 µl Nonidet-40 (NP-40) and careful mixing of samples. After 10 min incubation on ice, samples were centrifuged for 1 min at 14,000 rpm and 4 °C and the pellet was resolved in 40 µl Buffer B. Samples were

incubated by shaking for 30 min at 4 °C. After centrifugation (14,000 rpm, 10 min, 4 °C) undiluted supernatants were used for EMSA, and diluted 1:20 for determination of protein concentrations.

3.6.3 Measurement of protein concentration with Bradford-assay

Protein concentration in isolated nuclear fractions was determined as described by Bradford *et al.* (Bradford,M.M., 1976).

3.6.4 Radioactive labeling of consensus oligonucleotides

Double-stranded oligonucleotides, containing the consensus sequence for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3', Promega, Mannheim, Germany), were labeled with 5'-[γ-^{32}P]triphosphate (3,000 Ci/mmol) by using the T4 polynucleotide kinase (PNK) (USB, Cleveland, USA) which catalyzes the transfer of the terminal phosphate of ATP to the 5'-hydroxyl termini of the DNA. Oligonucleotides were incubated with PNK for 10 min at 37 °C. The reaction was stopped by addition of 0.5 M EDTA solution. The radioactive labeled DNA was separated from unlabeled DNA by using NucTrap probe purification columns (Stratagene, La Jolla, USA). Radiolabeled DNA was eluated from the column with 70 µl of STE buffer and frozen at -20 °C until use for EMSA.

3.6.5 Binding reaction and electrophoretic separation

Equal amounts of nuclear protein (approximately 2 µg) were incubated in a total amount of 14 µl containing 2 µg poly(dIdC) and 3 µl reaction buffer for 10 min at RT. 1 µl of the labeled oligonucleotide probe (approximately 300,000 cpm) was added and incubated with nuclear protein for 30 min at RT. The

nucleoprotein-oligonucleotide complexes were separated by gel electrophoresis (Mini-Protean 3, BioRad, Munich, Germany) at 110 V for 60 min on non-denaturating polyacrylamide gels (see 3.6.1, Rotiphorese™ Gel 30, Roth, Karlsruhe, Germany). Bands were detected by exposure of the gels to Cyclone Storage Phosphor Screens (Canberra-Packard, Dreieich, Germany) for about 24 h (time of exposure depended on radioactivity of the labeled oligonucleotides), followed by analysis with a phosphorimager station (Cyclone Storage Phosphor System, Canberra-Packard, Dreieich, Germany).

3.7 STATISTICAL ANALYSIS

All experiments were performed at least three times per treatment group (3-8 animals per treatment). Data are expressed as mean ± SEM. Statistical significance between groups was determined with one sample or student's t-test using GraphPad Prism® Version 3.03 for Windows (GraphPad Software Inc., San Diego, USA). P values < 0.05 were considered statistically significant.

4. Results

4.1 EFFECTS OF ANP DURING ISCHEMIA AND REPERFUSION IN THE LIVER

4.1.1 Effects of ANP preconditioning in the isolated perfused rat liver

4.1.1.1 Participation of p38 MAPK in ANP-mediated cytoskeletal changes during IR

We have shown previously that ANP pretreatment causes an increase in p38 MAPK activity (Kiemer,A.K. et al., 2002j) and changes in the hepatic content of cytoskeletal F-actin (diploma thesis). Thus, we were now interested in the correlation between p38 MAPK activation and cytoskeletal changes in ANP-preconditioned livers. For this reason, we used the specific p38 MAPK inhibitor SB203580 (SB). Perfusion with 2 μM SB together with ANP completely abolished the enhanced hepatic F-actin content after ANP preconditioning (**figure 10)**.

figure 10: ANP increases F-actin *via* p38 MAPK. Livers were perfused with KH buffer (Co), ANP (200 nM), SB (2 μM), or a combination of ANP and SB for 20 min followed by a 24 h period of ischemia (3.1.3). Cytoskeletal fractions were investigated by Western Blot. (see 3.1.4 and 3.5).

Similarly, ANP pretreatment caused a decrease of G-actin in liver homogenates (**figure 11)**. This effect was abrogated by inhibition of p38 MAPK, so that G-actin content in these samples resembled untreated controls. Again, treatment with SB alone had no influence (**figure 11**).

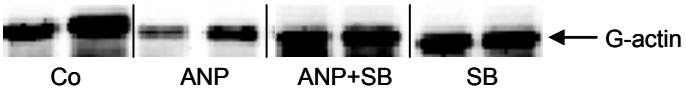

figure 11: Liver treatment with a combination of ANP and SB abrogates the ANP-mediated reduction of G-actin in livers after 24 h ischemia. After perfusion with KH buffer (Co), ANP (200 nM), SB (2 µM), or a combination of ANP and SB for 20 min, livers were kept under ischemic conditions (UW solution, 4 °C) for 24 h. Cytosolic fractions were analyzed *via* Western Blot as described (3.1.4 and 3.5).

Changes in F-actin content mediated by ANP were confirmed by staining of tissue sections. Staining with rhodamine-conjugated phalloidin, which specifically binds to F-actin, showed a low fluorescence in tissue sections of untreated livers. This correlated with a marginal F-actin content (**figure 12 A**). Slices of ANP-pretreated livers showed a very high staining intensity, representing an obvious increase in hepatic F-actin content. This cytoskeletal changes were observed especially at the membranes of hepatocytes (**figure 12, B**).

Again, perfusion of the livers with SB alone had no effect on hepatic F-actin (**figure 12 C**). Preconditioning with a combination of SB and ANP however, abolished the ANP-mediated effect (**figure 12 D**). This links the observed effect to activation of p38 MAPK.

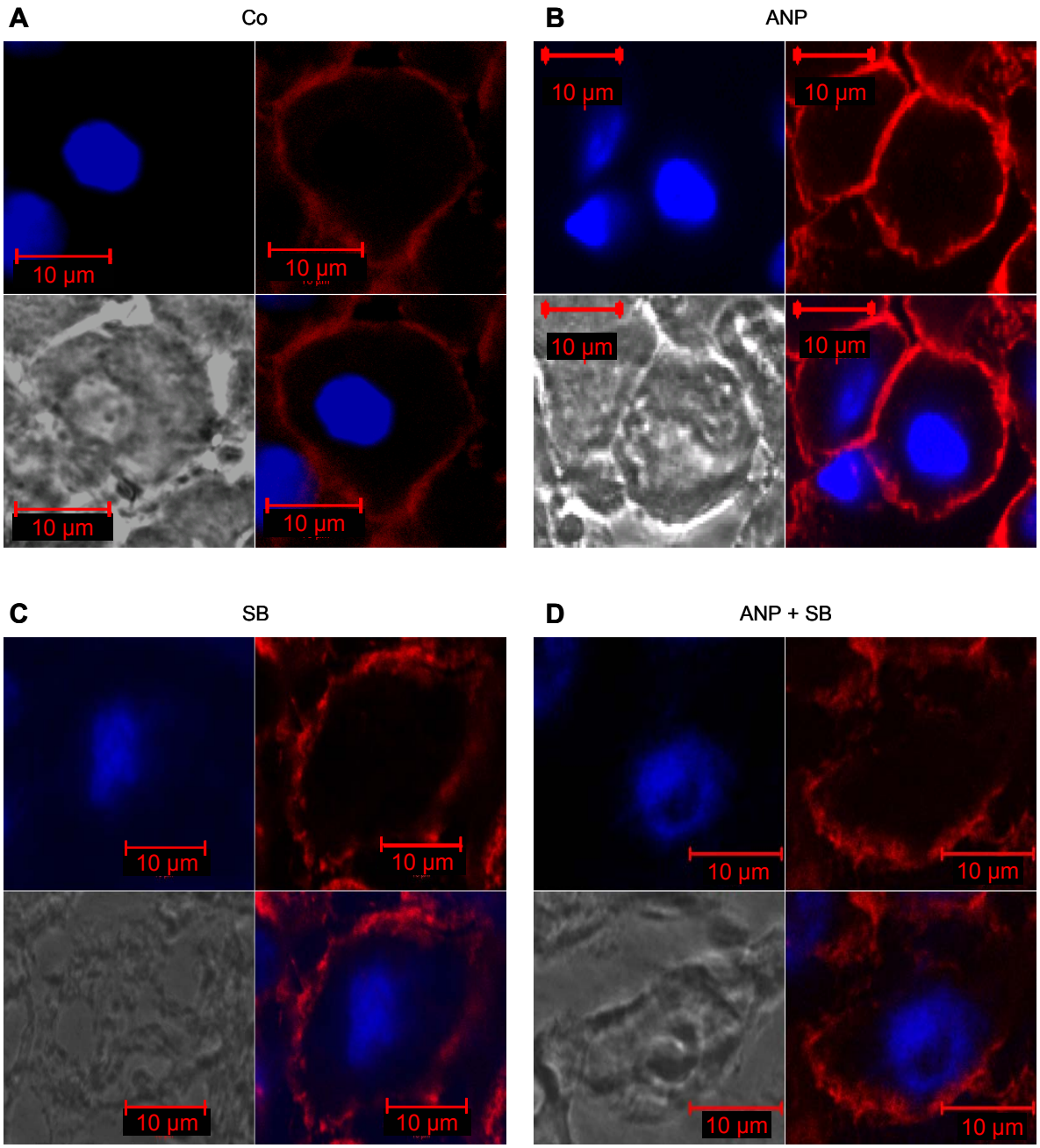

figure 12: ANP increases hepatic F-actin content *via* p38 MAPK. After perfusion with KH buffer (**A**), ANP (200 nM) (**B**), SB (**C**), or a combination of ANP and SB (**D**) for 20 min, livers were excised and kept under ischemic conditions (UW solution, 4 °C) for 24 h. Slices of snap-frozen livers were stained with Hoechst 33342 for nuclei (blue) and Rhodamine conjugated phalloidin for cytoskeletal F-actin (red) (see 3.1.3 and 3.4.3). Liver sections were examined by confocal laser scanning microscopy (63-fold amplification).

4.1.1.2 Role of p38 MAPK in ANP-mediated phosphorylation of Hsp27 during ischemia

We have previously shown that ANP pretreatment leads to an enhanced activation of p38 MAPK (Kiemer,A.K. et al., 2002i) and increases the hepatic content of its downstream target Hsp27. Moreover, we demonstrated a translocation of the heat shock protein towards the cytoskeleton after 24 h ischemia (diploma thesis). Hsp27 has been described to act as an actin capping protein controlling actin polymerization (Landry,J. et al., 1995e), with its function depending on phosphorylation by upstream kinases (Benndorf,R. et al., 1994b).

Based on these results, we were now interested in the phosphorylation status of Hsp27 in untreated and ANP-preconditioned livers, and a possible connection between p38 MAPK activation and phosphorylation of Hsp27 by employing the p38 MAPK inhibitor SB 203580.

As shown in **figure 13**, ANP caused an increase in phosphorylation of the small heat shock protein already at 30 min perfusion, and further increasing after 24 h ischemia.

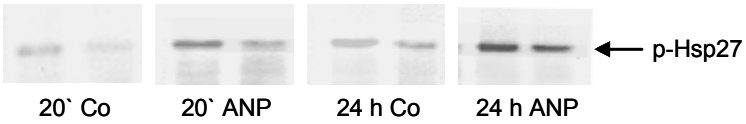

figure 13: Preconditioning with ANP augments phosphorylation of Hsp27. Following perfusion with KH buffer (Co), ANP (200 nM), SB (2 µM), or a combination of ANP and SB for 20 min, livers were kept under ischemic conditions (UW solution, 4 °C) for 24 h. Hepatic phospho-Hsp27 was analyzed by Western Blot after immunoprecipitation (see 3.1.3 and 3.5).

In order to confirm that enhanced phosphorylation of Hsp27 ischemia is mediated *via* p38 MAPK, we perfused the livers with a combination of ANP and SB. In fact, the increase of Hsp27 phosphorylation induced by ANP was completely abrogated (**figure 14**). Pretreatment with SB alone exerted no significant effect on phosphorylation of Hsp27. Thus, activation of p38 MAPK is essential for the phosphorylation of the heat shock protein induced by ANP.

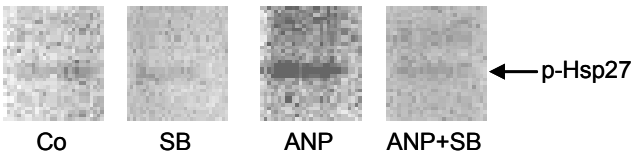

figure 14: Inhibition of p38 MAPK leads to an abrogation of enhanced Hsp27 phosphorylation after ANP pretreatment in ischemic livers. Livers were perfused with KH buffer (Co), ANP (200 nM), SB (2 µM), or a combination of ANP and SB for 20 min. Afterwards, organs were kept under ischemic conditions (UW solution, 4 °C) for 24 h, snap-frozen and homogenized as described. Hepatic Hsp27 was isolated by immunoprecipitation with a specific anti-Hsp27 antibody. Analysis of phospho-Hsp27 (p-Hsp27) levels in the samples by Western Blot was performed as described with an anti-phospho-Hsp27 antibody (see 3.1.3 and 3.5 for details).

4.1.2 Effects of ANP preconditioning in the liver during IR *in vivo*

4.1.2.1 ANP affects apoptosis in ischemic liver

Apoptotic processes were monitored by measuring caspase-3-like activity and TUNEL staining. After 24 h cold ischemia, an increase of hepatic caspase-3-like activity compared to sham operated animals was observed. The effect was still detectable after 2 h of reperfusion (125% vs. Co) (**figure 15 A**). ANP pretreatment completely abolished IR-induced caspase activity as shown in **figure 15 B**.

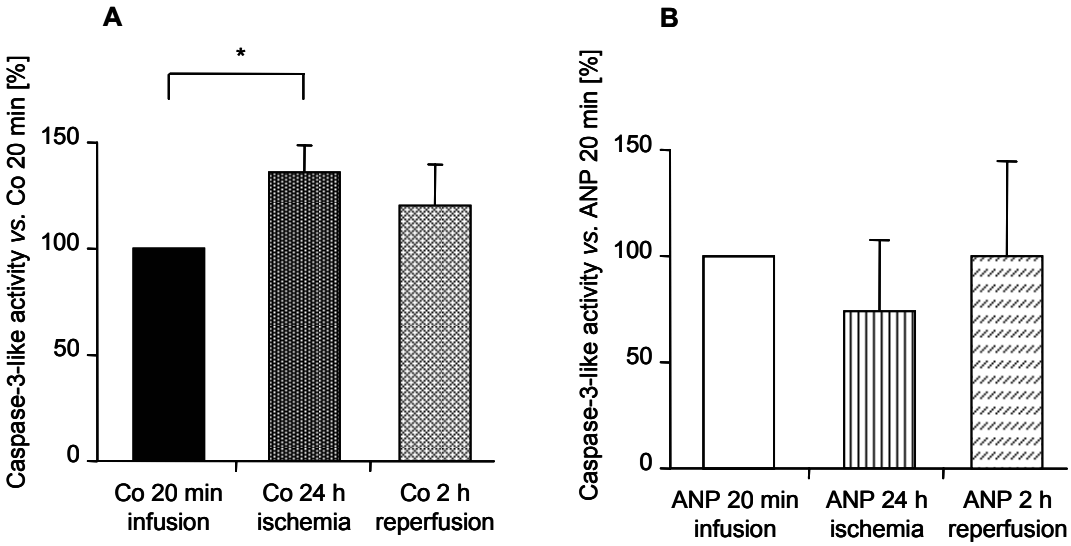

figure 15: Hepatic caspase-3-like activity increases during IR **(A)** but is unaffected in ANP-pretreated animals **(B)**. Donor livers were explanted after intravenous infusion of NaCl and kept under ischemic conditions (4 °C, UW solution) for 24 h. Livers were transplanted into recipients and reperfused for 2 h. At indicated times (after 20 min infusion, 24 h ischemia, and 2 h reperfusion), organs were snap-frozen, homogenized and caspase-3-like activity was measured as described in "Materials and Methods" with 4 rats in each group. Data are expressed as percent of caspase-3-like activity after 20 min infusion of NaCl (Co, **A**), and ANP (**B**) respectively. * $p \leq 0.05$ vs. control after 20 min infusion.

Compared to untreated controls, ANP preconditioning caused a significant attenuation of caspase-3-like activity, which was most evident in livers after 24 h of cold storage (see **figure 16**).

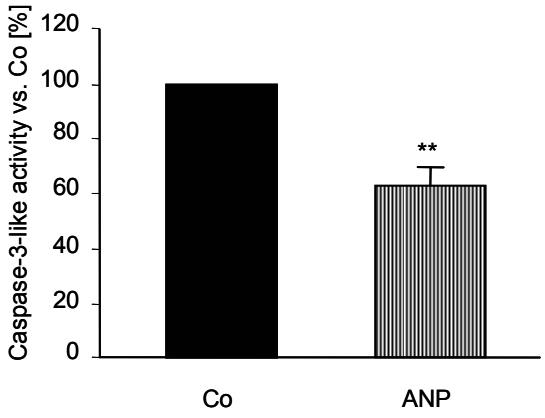

figure 16: ANP preconditioning decreases caspase-3-like activity in the liver after 24 h ischemia. Animals received an intravenous infusion of NaCl (Co) or ANP (5 µg/kg b.w.) for 20 min and livers were then kept under ischemic conditions (UW solution, 4 °C) for 24 h. Tissue was snap-frozen, homogenized, and caspase-3-like activity was determined as described (see3.2.6 for details) with 4 animals in each group. Data are expressed as percentage of caspase-3-like activity in untreated livers (100%). ** $p \leq 0.01$ vs. untreated controls.

TUNEL-staining and hematoxylin/eosin (HE) staining of liver sections confirmed the results of caspase-3-like activity measurement. After 24 h cold ischemia apoptosis was most prominent and accounted for about 0.5% of cells.

Both hepatocytes and endothelial cells showed positive TUNEL staining combined with characteristic apoptotic morphology. Preconditioning of donor livers with ANP (5 µg/kg b.w.) significantly decreased the number of TUNEL-positive cells after 24 h cold ischemia (see **figure 17**). A similar effect was observed after 2 h of reperfusion, although less pronounced (data not shown)

Thus, we clearly demonstrated that ANP reduces apoptotic cell death in ischemic liver cells.

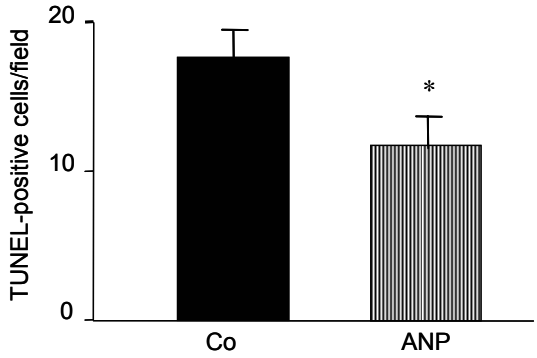

figure 17: ANP pretreatment reduces the number of apoptotic cells after ischemia in the liver. Animals received intravenous infusion of NaCl (Co) or ANP (5 µM) for 20 min. Livers were excised, kept under ischemic conditions (UW solution, 4 °C) for 24 h, and embedded in paraffin wax before tissue was cut into 6 µm slices. TUNEL-staining was performed as described (see 3.4.1) and apoptotic liver cells per field of view (1.96 mm^2) were counted with tissue samples from 4 animals of each treatment group (see 3.2.3 and 3.4.1 for details). * $p \leq 0.05$ *vs.* controls.

4.1.2.2 Effect of ANP on transaminase activity, LDH activity, and bile flow

ANP preconditioning did not affect the plasma levels of liver transaminases alanine transaminase (ALT), aspartate transaminase (AST), or lactate dehydrogenase (LDH) as parameters for necrotic liver damage. Enzyme activities were measured in the serum of rats after 2 h of liver reperfusion (**figure 18 A-C**). In accordance, morphological analysis of necrotic liver cells by HE-staining did not reveal a difference between the ANP and the control group at both, 24 h ischemia as well as 2 h reperfusion. Furthermore, no significant effect on bile flow was observed during reperfusion (**figure 18 D**).

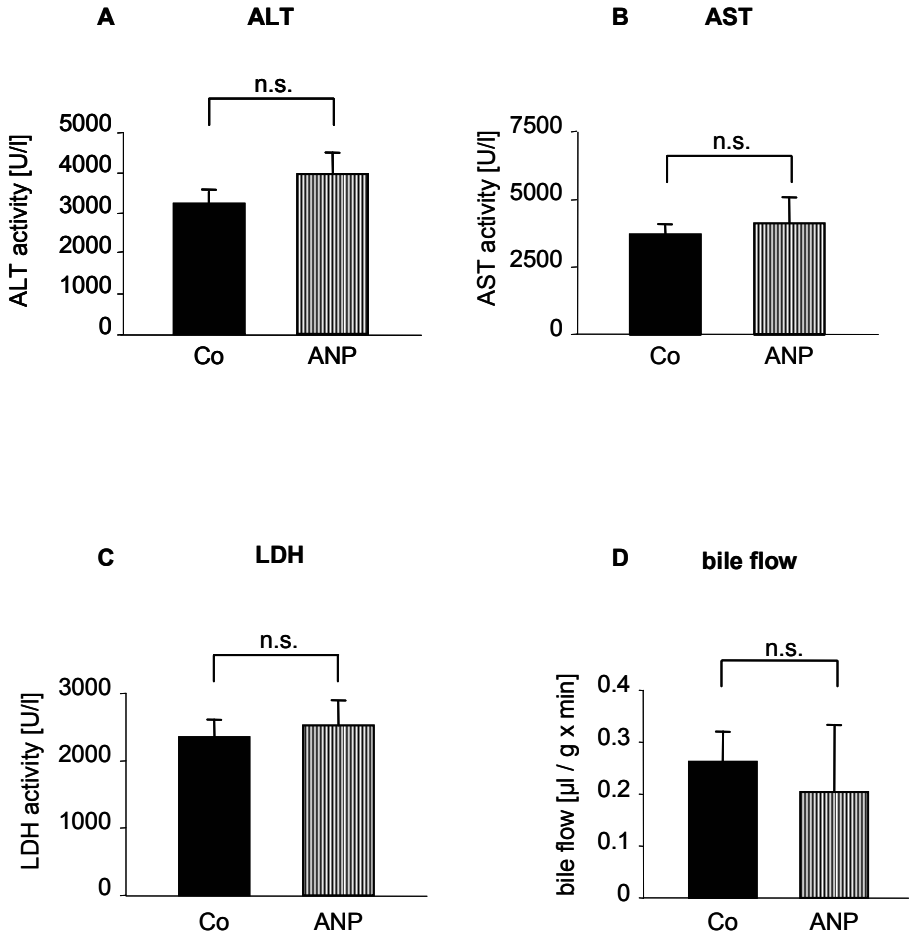

figure 18: ANP preconditioning does not influence serum transaminases and bile flow. Animals received an intravenous infusion of NaCl (Co) or ANP (5 µg/kg b.w.) for 20 min before the donor liver was explanted and kept under ischemic conditions (UW solution, 4 °C) for 24 h. **A-C:** after implantation of the organ into the recipient and reperfusion for 2 h, plasma samples (400 µl) were obtained. Activities of ALT, AST and LDH were measured as described in "Materials and Methods" and specified as units per liter serum with 7 animals in each group. **D:** bile was collected and quantified volumetrically (see 3.2.3 for details) with 7 animals in each group.

4.1.2.3 PKA is not involved in the anti-apoptotic effect

Since it has been shown that ANP mediates anti-apoptotic effects in the isolated perfused liver *via* phosphorylation of Bad by protein kinase A (PKA) (Kulhanek-Heinze,S. et al., 2004b), we investigated a possible participation of this pathway *in vivo*. Interestingly, however, ANP pretreatment of animals *in vivo* did not influence the activity of protein kinase A (PKA), as shown in **figure 19**.

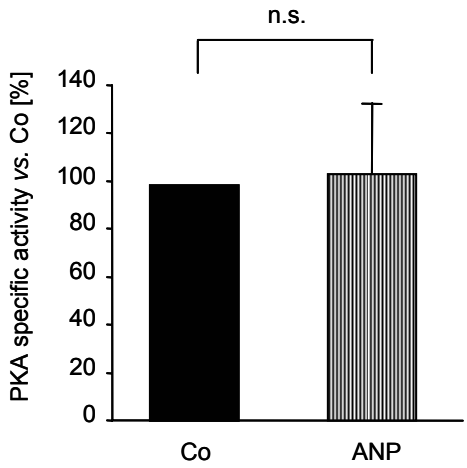

figure 19: PKA is not involved in ANP-mediated hepatic effects. Animals received an intravenous infusion of NaCl (Co) or ANP (5 µg/kg b.w.) for 20 min. Livers were excised and homogenized as described in chapter 3.2.3 and 3.2.5). PKA activity was determined in liver homogenates by *in vitro* phosphorylation. Results are expressed as % of PKA activity in NaCl-treated livers (n=4).

Also, in contrast to isolated perfused rat livers, ANP treatment *in vivo* did not alter Bad phosphorylation at the PKA specific phosphorylation site Ser 112 (see **figure 20**).

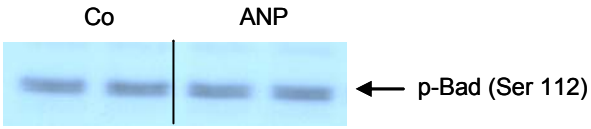

figure 20: Phosphorylation of Bad at Ser 112 is not influenced by ANP pretreatment. After intravenous infusion of animals with NaCl (Co) or ANP (5 µg/kg b.w.) for 20 min, livers were excised and homogenized. Homogenates were immunoprecipitated using anti-Bad antibody (see 3.2.3 and 3.5). Levels of phospho-Bad (Ser 112) (p-Bad) were detected by Western Blot.

4.1.2.4 Involvement of the PI3-kinase pathway

Since PKA-mediated Bad phosphorylation at Ser 112 could be excluded as the signaling pathway of anti-apoptotic actions of ANP *in vivo*, we aimed to investigate the PI3-kinase pathway. PI3-kinase has been shown to mediate cytoprotective effects by pharmacological preconditioning (Müller,C. et al., 2003).

As shown in **figure 21**, application of the PI3-kinase inhibitor Wortmannin (WM; 16 µg/kg b.w.) prior to ANP infusion completely blocked the inhibition of caspase-3-like activity by ANP seen in ischemic liver tissue. Thus, the PI3-kinase pathway is crucially involved in the anti-apoptotic effect of ANP preconditioning.

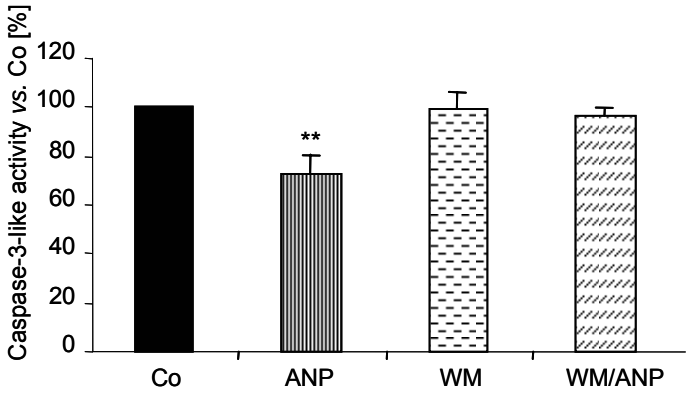

figure 21: PI3-kinase mediates anti-apoptotic effects of ANP *in vivo*. Animals received an intravenous infusion of NaCl (Co), ANP (5 µg/kg b.w.), WM (16 µg/kg b.w.), or a combination of both (WM/ANP) for 20 min. Livers were excised and snap-frozen. After 24 h ischemia livers were homogenized and caspase-3-like activity was determined as described in "Materials and Methods". Data are expressed as percent of caspase-3-like activity in untreated livers (Co, 100%) with 4 animals in each group. ** $p \leq 0.01$ vs. controls.

4.1.2.5 ANP preconditioning causes phosphorylation and translocation of Akt

As the protein kinase Akt is an important downstream target of PI3-K we next investigated a possible effect of ANP on phosphorylation of Akt.

Infusion of donor animals with ANP (5 µg/kg b.w.) for 20 min resulted in a marked increase of phosphorylated Akt (p-Akt) in the liver. This increase was abrogated in the presence of WM (**figure 22**). Akt activation by ANP seems to be a transient effect since it could not be observed after ischemia (24 h) or reperfusion (2 h) (data not shown).

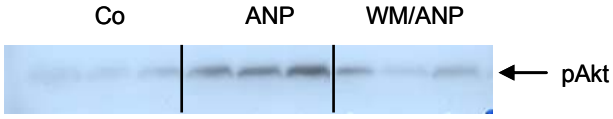

figure 22: ANP pretreatment increases Akt phosphorylation at Ser 473. After intravenous infusion of NaCl (Co), ANP (5 µg/kg b.w.), WM (16 µg/kg b.w.), or a combination of both (WM/ANP) for 20 min livers were excised and snap-frozen. Phospho-Akt (Ser 473) (p-Akt) was examined by Western Blot (see 3.2.3 and 3.5).

Total levels of Akt were analyzed by Western Blot to confirm the uniformity of protein contents in the liver homogenates (**figure 23**).

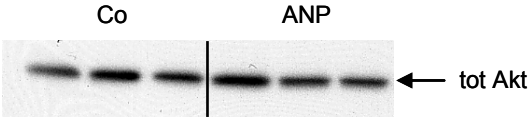

figure 23: Total protein levels of Akt after ANP preconditioning are unchanged in ischemic livers. Animals received an intravenous infusion of NaCl (Co) or ANP (5 µg/kg b.w.) for 20 min. Livers were excised and snap-frozen. Total amounts of Akt were examined by Western Blot (see 3.2.3 and 3.5).

Analysis of p-Akt in liver tissue by confocal microscopy confirmed that ANP pretreatment led to a marked increase of p-Akt as already shown by Western Blot (see **figure 22**). Moreover, this analysis revealed a strong localization of p-Akt at the plasma membrane of hepatocytes (**figure 24 A, B**). Co-treatment of

animals with WM abrogated the increase as well as the plasma membrane localization of p-Akt induced by ANP (**figure 24 C**).

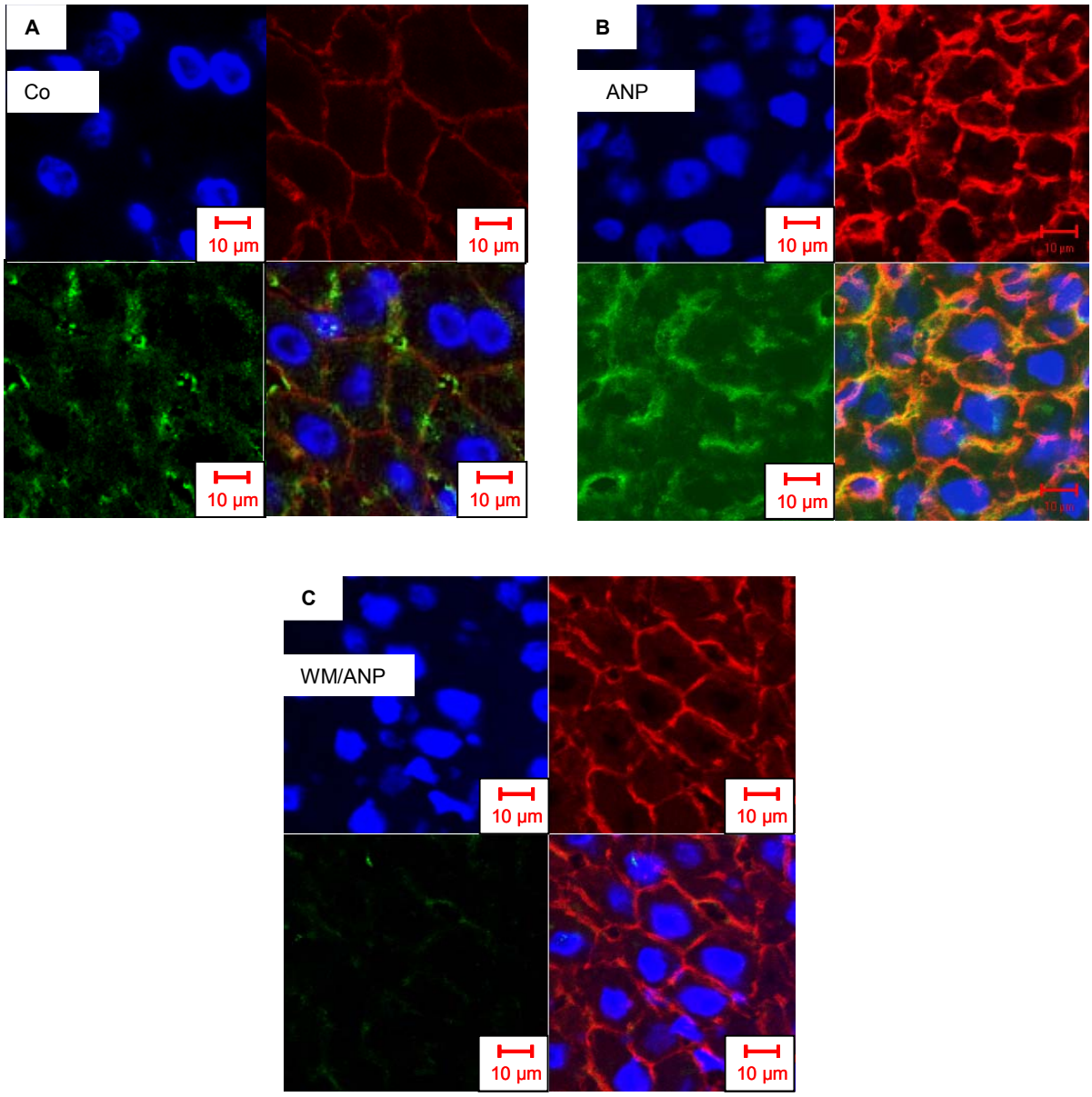

figure 24: Increase in Akt phosphorylation and changes in distribution of phospho Akt in liver tissue after ANP pretreatment. Animals were infused with NaCl (Co, **A**), ANP (5 µg/kg b.w.; **B**), or a combination of ANP and WM (16 µg/kg b.w.) (WM/ANP, **C**) for 20 min. Images were obtained as described in chapter 3.2.3 and 3.4.3 and examined by confocal microscopy (63-fold amplification). Blue: nuclei, red: F-actin, cytoskeleton; green: p-Akt.

4.1.2.6 ANP-mediated phosphorylation of Bad

To further examine the anti-apoptotic signaling by ANP in the liver induced *in vivo*, the Bad protein, an important downstream target of p-Akt, was investigated. The results indicate that Bad is phosphorylated at Ser 136 *via* p-Akt (**figure 25**). This phosphorylation is induced by ANP and completely independent of PKA-activation, since Ser 112 is the PKA specific phosphorylation site.

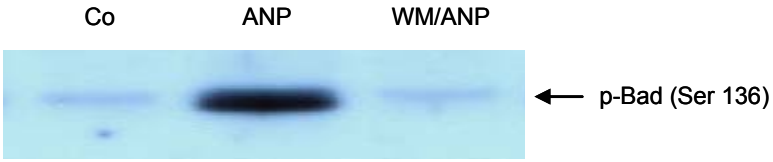

figure 25: Phosphorylation of Bad at Akt-phosphorylation site Ser 136 is enhanced in ANP-pretreated livers after ischemia. After intravenous infusion of NaCl (Co), ANP (5 µg/kg b.w.), or ANP and WM (WM/ANP) for 20 min livers were excised and snap-frozen. Homogenized livers were immunoprecipitated using anti-Bad antibody. Examination of levels of phosphorylated Bad (Ser 136) (p-Bad) was done by Western Blot (for details see 3.2.3 and 3.5).

4.2 EFFECTS OF ANP DURING LPS-INDUCED SEPTIC SHOCK

4.2.1 Effect of ANP pretreatment on survival after septic shock

In order to investigate the effects of ANP on the survival of animals suffering from LPS-induced sepsis, we first determined the lethal LPS-dose for the present experimental setting. Administration of 1 mg LPS/kg b.w. caused worsening of the animals' state of health (slight diarrhea, increase in body temperature, food refusal), which persisted during the entire experiment (food refusal until about 40 h after LPS injection) but did not affect survival (see **figure 26**). ANP treatment without LPS had also no effect on survival (see **figure 26** and **figure 27**). Septic animals which were pretreated with ANP initially showed deterioration of their state of health but completely recovered from symptoms of LPS-induced sepsis.

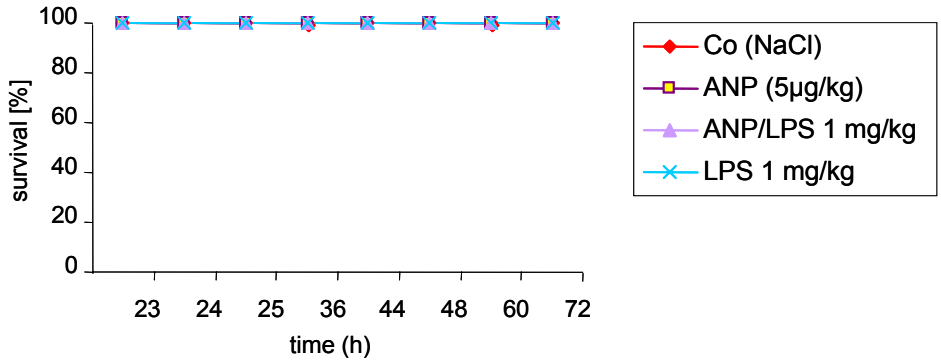

figure 26: Sublethal dose of LPS does not affect survival of animals. Animals were injected i.v. with ANP (5 µg/kg b.w.; ANP- and ANP/LPS-group) or NaCl (Co and LSP-group), followed by an i.p. injection of NaCl (Co and ANP-group) or LPS (1 mg/kg b.w.; LPS- and ANP/LPS-group) after 20 min. The animals' state of health was observed continuously for 72 h. For details see 3.3.3.1.

Administration of LPS in a dose of 2.5 mg/kg b.w. caused an immediate worsening of the animals' state of health already 1 h after i.p. injection of LPS. Mice had clotted eyelids, developed severe diarrhea and

fever, refused food and water, and became apathetic. These symptoms were observed in LPS- as well as in ANP/LPS-treated animals. All animals without ANP preconditioning died within 24.5 h due to severe symptoms of sepsis (**figure 27**). Interestingly, 80% of mice which had been pretreated with ANP recovered from septic shock (**figure 27**). After 24 h diarrhea attenuated and animals were more agile. They started drinking 27 h after LPS injection. Eyelid clotting diminished after 36 h and after 48 h mice still seemed to be debilitated but no longer refused food. Furthermore, blood heat declined and at the end of the survival experiment (72 h), ANP-pretreated mice showed an almost normal state of health without any fever or diarrhea.

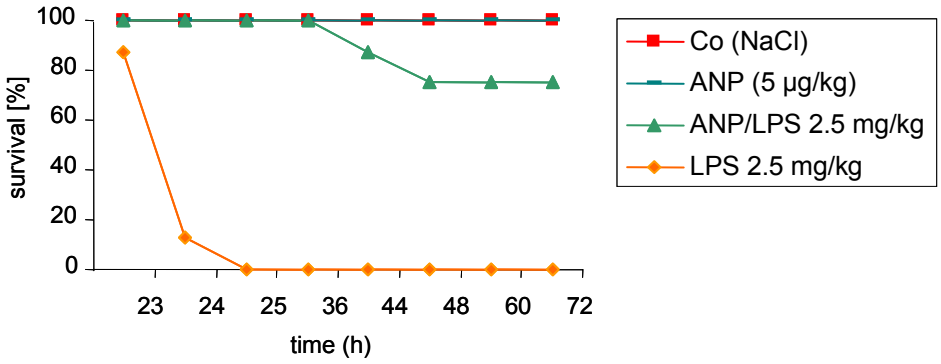

figure 27: Pretreatment with ANP reduces mortality after LPS-induced sepsis. Mice were injected i.v. with NaCl (Co and LPS-group) or ANP (5 µg/kg b.w., ANP- and ANP/LPS-group). After 20 min animals received an i.p. injection of NaCl (Co and ANP-group) or LPS (2.5. mg/kg b.w.; LPS- and ANP/LPS-group). The animals' state of health was observed continuously for 72 h. For details see 3.3.2.2 and 3.3.3.1.

4.2.2 Effects of ANP preconditioning on blood TNF-α levels after LPS-induced septic shock

In order to investigate whether survival after ANP preconditioning is associated with changes in TNF-α levels in blood as an important parameter during sepsis, we determined the TNF-α concentrations in serum 90 min after LPS injection. At this time point TNF-α levels in the blood of LPS-treated animals

peaked and were detected by ELISA.

As shown in **figure 28**, pretreatment with ANP caused a marked decrease in TNF-α serum levels. Sublethal (1 mg/kg b.w.) as well as lethal (2.5 mg/kg b.w.) doses of LPS caused this effect.

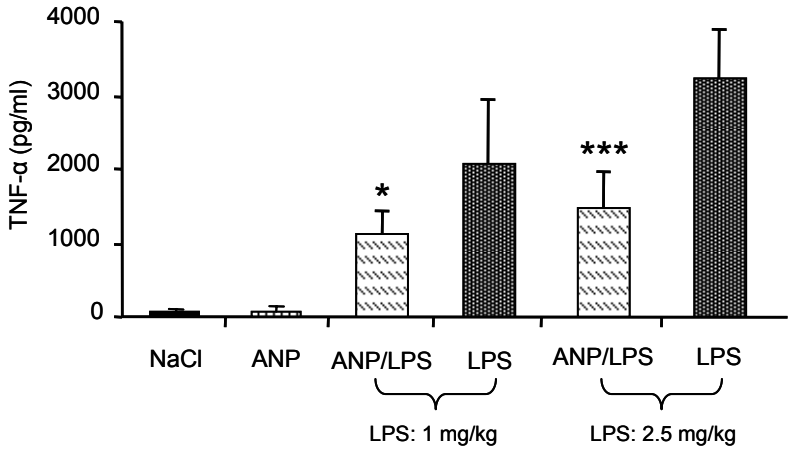

figure 28: Preconditioning with ANP reduces TNF-α levels in blood samples 90 min after LPS-induced sepsis. Mice received an i.v. injection of NaCl or ANP (5 µg/kg b.w.) at the onset of the experiment. After 20 min, LPS in a sublethal (1 mg/kg b.w.) or lethal (2.5 mg/kg b.w.) dose, or NaCl (ANP-group) was administered i.p.. 90 min later, 50 µl of tail-blood were used for determination of TNF-α concentration by ELISA (for details see 3.3). * $p \leq 0.05$ vs. LPS-group, *** $p \leq 0.001$ vs. LPS-group of the respective concentration.

4.2.3 Effects of ANP on signaling during LPS-induced sepsis in the kidney

4.2.3.1 ANP-mediated changes in NF-κB binding activity

In our experiment, interestingly, examination of the kidneys after lethal anesthesia revealed obvious differences between LPS- and ANP-treated groups. While kidneys of LPS-injected mice were smaller than kidneys from control and their surface was wizened, the appearance of kidneys from ANP-treated animals resembled control organs with regular size and a smooth surface. Therefore, possible

mechanisms responsible for the ANP-mediated effects on TNF-α level (4.2.25.2.2) and survival of animals (4.2.15.2.1) where further investigated in the kidney.

NF-κB is one of the most important mediators of the LPS signaling pathway. Thus, modifications in binding activity of NF-κB as a possible reason for changes in serum TNFα-levels were investigated.

As illustrated in **figure 29**, LPS treatment caused a significant increase in NF-κB binding activity in the kidney. This effect is characteristic for LPS-induced sepsis. The basal NF-κB binding activity in tissue of NaCl-treated animals was very low and ANP injection alone had no effect on this parameter. Preconditioning with ANP, however, provoked a significant decrease of NF-κB binding activity during endotoxemia as soon as 15 min after LPS injection (**figure 29 A**), further decreasing after 30 min (**figure 29 B**).

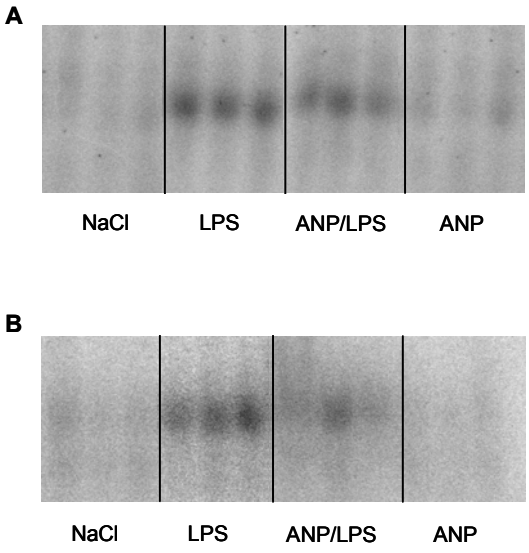

figure 29: ANP pretreatment reduces NF-κB binding activity in the kidney after LPS-induced sepsis. Animals received an i.v. injection of NaCl (Co) or ANP (5 µg/kg b.w.) 20 min prior to i.p. injection of LPS (1 mg/kg b.w.). Co: NaCl-treated; ANP: ANP i.v., after 20 min NaCl i.p.; LPS: NaCl i.v., after 20 min LPS i.p.; ANP/LPS: ANP i.v., 20 min later LPS i.p.. 15 min (**A**) or 30 min (**B**) after LPS injection kidneys were excised, snap-frozen, homogenized, and investigated by EMSA (for details see 3.3 and 3.6).

4.2.3.2 Effect of ANP on distribution of the NF-κB subunit p65 after LPS-induced sepsis

To confirm the data of NF-κB binding activity detected by EMSA, we focused on the distribution of the NF-κB subunit p65. NF-κB-translocation was investigated by staining of the p65 subunit in snap-frozen slices of kidney tissue and images were obtained by confocal microscopy.

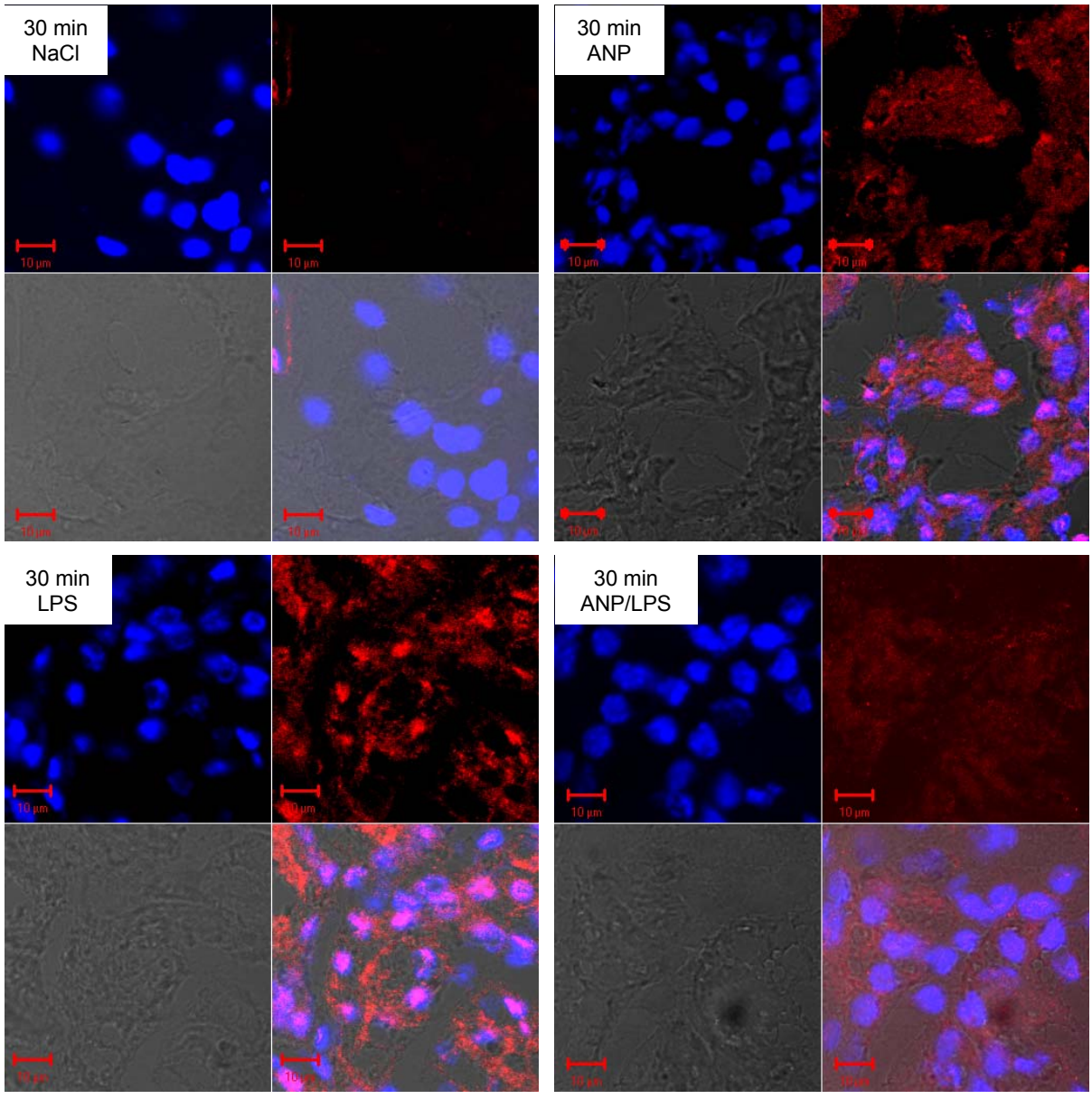

figure 30: ANP prevents translocation of the NF-κB subunit p65 into the nucleus. Animals received an i.v. injection of NaCl or ANP (5 µg/kg b.w.) 20 min before i.p. injection of NaCl or LPS (1 mg/kg b.w.). Four treatment groups were investigated: Controls (NaCl) received NaCl injections only; ANP: ANP i.v. and NaCl i.p. 20 min later; LPS: NaCl i.v. and LPS i.p. 20 min later; ANP/LPS: ANP i.v., LPS i.p. after 20 min. 30 min after LPS-injection (t = 30 min) kidneys were excised, snap-frozen, and slices were stained. Blue: nuclei, red: p65. Images were obtained as described in chapter 3.3.3.2 and 3.4.3.

As illustrated by confocal microscopy, p65 translocated into the nuclei of kidney cells after LPS treatment (**figure 30**). Pretreatment with ANP prevented this translocation shown by marginal location of p65 in the cytosol.

4.2.3.3 Protein level of total IκBα and phosphorylation of IκBα

We were now interested in the mechanisms responsible for ANP-mediated inhibition of NF-κB.

In NaCl-treated animals (Co) basal levels of total IκBα and phosphorylated IκBα could be detected (**figure 31**). ANP treatment had no effect on the amount of total IκBα or the phosphorylation of IκBα. Increased phosphorylation of IκBα was detectable 15 min after injection of LPS (**figure 31 A**). This effect became even more obvious in kidneys excised 30 min after LPS injection (**figure 31 B**). Simultaneously, we observed decreased levels of total IκBα in kidneys of LPS-treated animals, pointing to an increased degradation of the NF-κB inhibitor. In contrast, ANP preconditioning caused only a slight decrease in phosphorylation of IκBα. This resulted in minor degradation of IκBα and thus a higher content of total IκBα in the samples (**figure 31**).

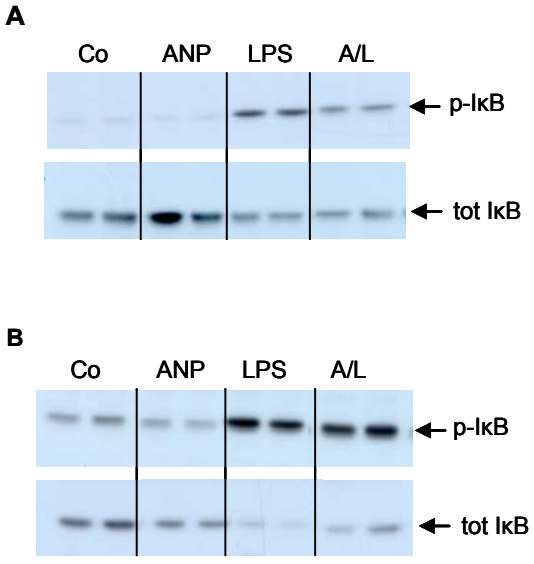

figure 31: After LPS-induced sepsis ANP pretreatment causes decreased phosphorylation of IκBα and increased content of total IκBα compared to samples of the LPS-group. 20 min after i.v. injection of NaCl or ANP (5 µg/kg b.w.) animals were injected i.p. with NaCl or LPS. Co: NaCl treatment only; ANP: ANP i.v., 20 min later injection of NaCl; LPS: NaCl i.v. before LPS i.p. after 20 min; ANP/LPS: ANP i.v., 20 min later LPS i.p.. 15 min **(A)**, and 30 min after LPS injection respectively **(B)**, kidneys were excised, snap-frozen and analyzed by Western Blot (3.3.3.2 and 3.5.)

5. Discussion

5.1 EFFECTS OF ANP DURING ISCHEMIA AND REPERFUSION IN THE LIVER

5.1.1 *Ex vivo* pretreatment with ANP causes p38 MAPK-dependent changes in hepatocytes cytoskeleton

In previous studies we demonstrated that ANP and IR influence the cytoskeleton in liver tissue (diploma thesis). Although hepatic content of total actin is affected neither by IR alone nor by pretreatment of livers with ANP, we observed increased levels of filamentous actin (F-actin) in ANP-pretreated livers. This augmentation of F-actin content was observed in fractions containing plasma membranes of whole livers, but the involved cells were not identified. Since F-actin is know to play a crucial role in stabilization of the cytoskeleton and consequently in preservation of an intact cell morphology (Banan,A. et al., 2000), these ANP-mediated changes were suspected to be an essential part of the peptide's cytoprotective action during IR (Gerbes,A.L. et al., 1998b).

Our data also showed that ANP preconditioning *ex vivo* provokes changes in protein levels and in localization of the small heat shock protein Hsp27. While ischemic livers showed a shift of Hsp27 from the cytosolic to the cytoskeletal fraction, ANP pretreatment completely abrogated this translocation of the heat shock protein. These findings correlated with a translocation of Hsp27 to the cytoskeleton which was observed after ischemia and reoxygenation in a myoblast cell line (Sakamoto,K. et al., 1998), in the isolated rat heart (Sakamoto,K. et al., 2000a), and *in vivo* in the rabbit heart (Sakamoto,K. et al., 2000d, Sanada,S. et al., 2001c). Moreover, Huot et al. showed that Hsp27 has protective properties in CCL39 cells with respect to cytoskeletal damage which can be caused by oxidative stress (Huot,J. et al., 1996).

Subsequently, the results of the diploma thesis led to the question about possible signaling pathways participating in alteration of actin polymerization, translocation of Hsp27, and the potential of activation/phosphorylation of the heat shock protein. In addition, it had to be analyzed which cell type in the liver shows cytoskeletal changes mediated by ANP and whether there are any functional consequences during IR.

5.1.1.1 Role of p38 MAPK in ANP-mediated cytoskeletal changes in the liver after ischemia

ANP has previously been shown to lead to a marked activation of the mitogen-activated protein kinase (MAPK) p38 (Kiemer,A.K. et al., 2002h). However, to date there are no data about functional consequences of this activation in the liver. Interestingly, this protein kinase has been shown to cause cytoskeletal changes in various *in vitro* systems, for example in cultured Chinese hamster CCL39 cells, HeLa cells, Chinese hamster fibroblasts, and pancreatic acini (Guay,J. et al., 1997, Landry,J. et al., 1995f, Schafer,C. et al., 1998). Nevertheless, similar pathways in the liver are as yet unknown. We were therefore interested whether p38 MAPK is involved in the cytoskeletal changes we found in ANP-treated livers after ischemia.

In order to test this hypothesis, we applied the p38 MAPK inhibitor SB203580 at a concentration of only 2 μM to assure its specificity (McGovern,S.L. et al., 2003). Importantly, the inhibitor completely abolished the ANP-induced effect of increased F-actin content in pretreated livers. Thus, we show for the first time that ANP-mediatedp38 MAPK activation (Kiemer,A.K. et al., 2002g) leads to hepatic cytoskeletal changes by an increase of the hepatic F-actin content in rat livers.

5.1.1.2 Phosphorylation status of Hsp27

In previous studies we showed that *ex vivo* preconditioning with ANP causes changes in protein levels and localization of the small heat shock protein Hsp27 (diploma thesis). It is known that these changes, as well as the function of Hsp27, largely depend on the phosphorylation status of the heat shock protein (Benndorf,R. et al., 1994a, Lutsch,G. et al., 1997). Thus, in subsequent investigations we focussed on this parameter.

In fact, our results clearly point to an influence of ANP on the phosphorylation status of Hsp27 after the 20 min preconditioning period as well as after 24 h ischemia. The correlation between ANP preconditioning and phosphorylation of Hsp27 resembles data presented by Sanada *et al*., showing increased Hsp27 phosphorylation after ischemic preconditioning in the heart *in vivo* (Sanada,S. et al., 2001b).

In addition, our data show that ischemic conditions cause phosphorylation of Hsp27 in isolated perfused rat livers. This is in line with the results of Huot *et al.* demonstrating an increased content of phosphorylated Hsp27 in Chinese hamster CCL39 cells and human umbilical vein endothelial cells (HUVECS) respectively, after exposure to cellular stressors like TNF-α or H_2O_2 (Huot,J. et al., 1995, Huot,J. et al., 1997).

Little is known about the downstream targets of hepatic p38 MAPK activation by ANP. However this protein kinase has been demonstrated to play an important role in the liver for example in regulation of translational processes upon cellular stress, influencing production of TNF-α and IL-1 after burn-induced liver injury, and in vivo p38 has been demonstrated to activate the transcription factor AP-1 and MAPKAPK2 after heat shock (Chen,X.L. et al., 2005, Maroni,P. et al., 2000, Wang,X. et al., 1998). Previous experiments have shown that phosphorylation of Hsp27 is exhibited by the p38 MAPK / MAPKAPK2 pathway in other systems (Freshney,N.W. et al., 1994a, Landry,J. et al., 1995g, Murashov,A.K. et al., 2001a, Nakano,A. et al., 2000b, Sanada,S. et al., 2001a). For this reason, we investigated the importance of p38 MAPK in the phosphorylation of Hsp27 in ANP-pretreated livers after ischemia.

Livers were perfused with SB203580 in combination with ANP. In fact, an inhibition of p38 MAPK decreased the phosphorylation status of Hsp27 to basal levels. Thus, our results clearly prove the dependency of Hsp27 phosphorylation on previous activation of p38 MAPK in the liver.

5.1.1.3 Functional consequences of changes in hepatocytes cytoskeleton

The results of the final year project (diploma thesis) pointed out ANP-mediated changes in cytoskeletal structures of preconditioned liver cells during IR. In the present work, we clearly demonstrate that ANP pretreatment causes an increase in F-actin content, which occurs particularly at the plasma membranes of hepatocytes after ischemia.

In hepatocytes, a decrease in hepatic F-actin content determines inhibition of store-operated calcium-channels (SOCs), disruption of the organization of the endoplasmic reticulum, and functional disturbances of tight junctions (Marceau,N. et al., 2001, Song,J.Y. et al., 1996b, Wang,Y.J. et al., 2002b). In addition,

increased hepatic F-actin levels are thought to stabilize the cytoskeleton and to improve contractility which results in improved bile flow after ischemia (Rungger-Brandle,E. *et al.*, 1983).

Recently, Gomes *et al.* proved that ischemic preconditioning (IPC) preserves rat kidneys from IRI. The improved function and morphology of kidney tissue was accompanied by an upregulation of genes encoding for cytoskeletal proteins. The demonstrated increase in mRNA levels of these proteins, which have an F-actin-stabilizing function and are therefore crucial for the maintenance of cellular structures, is suggested to improve tolerance of preconditioned tissue to ischemia (Gomes,M.D. *et al.*, 2003). The augmented transcription of cytoskeletal proteins is already observed after 30 min of preconditioning. Our findings of an increased F-actin content in ANP-preconditioned livers are therefore suggested to be a consequence of enhanced synthesis of this cytoskeletal protein. Thus, the influence of protective preconditioning (IPC or ANP pretreatment) on F-actin content of the affected tissue is confirmed. ANP preconditioning as well as IPC defends the liver from IRI (for review see (Carini,R. et al., 2003c)). Furthermore, F-actin plays a crucial role in regulation of cell morphology, intracellular transport processes, exo- and endocytosis, and canalicular motility responsible for bile flow (Denk,H. et al., 1986, Fisher,M.M. et al., 1979, Marceau,N. et al., 2001). In addition cytoskeletal structures are important to ensure stability and mobility of liver cells (Arias I.M., 2001a).

We therefore hypothesized that an augmentation of hepatic F-actin after ischemia might be a feature of the cytoprotective activities of ANP. Surprisingly, we found no improvement of bile flow although F-actin content increases in the pretreated organs. In consequence, in the isolated perfused liver the ANP-mediated effect on the cytoskeletal structure of hepatocytes might just not be strong enough to facilitate a correlation between ANP pretreatment and protection of the liver against IRI with respect to functional parameters. Thus, further targets of ANP-induced signaling which might be responsible for cytoprotective effects of the peptide have to be investigated in the future.

5.1.2 ANP treatment *in vivo* has anti-apoptotic effects mediated *via* the PI3-kinase pathway

5.1.2.1 ANP reduces apoptotic cell death in liver tissue

The type of cell death occurring during hepatic IR as well as the importance of apoptosis versus necrosis remains a controversial issue. In this context, Gu *et al.* and Rosser *et al.* proposed that necrotic cell death is the main cause of tissue damage during IR (Gu,X.P. et al., 2004, Rosser,B.G. et al., 1995b). Gujral *et al.* share this opinion in a paper in which they assess this type of cell death in liver tissue during IR according to morphological criteria, TUNEL staining of tissue, and caspase-activity in liver samples as the only relevant (Gujral,J.S. et al., 2001a). On the other hand, there are numerous studies demonstrating that liver cells die of apoptotic cell death. It has been shown that 50-70% of endothelial cells and 40-60% of hepatocytes undergo apoptosis during reperfusion (Gao,W. et al., 1998b, Kohli,V. et al., 1999a). Furthermore, it has previously been demonstrated that *in vivo* application of caspase inhibitors leads to a complete abrogation of cell death in hepatocytes and protects against IRI, thus referring to apoptosis as the major type of cell death during IR (Cursio,R. et al., 1999b). Application of a specific inhibitor of caspase 3 and 7 even causes improved survival after IR in rat liver transplantation supporting a critical role of apoptotic cell death for loss of function of this organ (Mueller,T.H. et al., 2004b). Since Gerwig *et al.* proved that ANP pretreatment prevents apoptosis in the model of the isolated perfused liver with major effects after 24 h cold ischemia (Gerwig,T. et al., 2003b), we were now interested whether preconditioning *in vivo* protects the liver from IRI and which signaling pathways are involved in the effect of the peptide hormone with regard to apoptotic cell death during IR.

Our results clearly demonstrate that ANP-treated livers display a marked decrease of caspase-3-like activity by around 30% compared to control. Additionally, the number of TUNEL-positive cells in ANP-preconditioned livers is significantly lower than in controls. This reduction of apoptotic cell death is an important parameter for cytoprotective effects *in vivo* following IR, as demonstrated in numerous studies. Vilatoba *et al.* for example presented a correlation of decreased apoptotic cell death and improvement of

liver regeneration and survival after warm ischemia in 17-betaestradiol-treated mice (Vilatoba,M. et al., 2005). Ischemic preconditioning, which has been proven to reduce IRI in many cases, also leads to an inhibition of apoptosis of liver cells demonstrating the importance of this type of cell death during IR (Glanemann,M. et al., 2004). Moreover, Contreras *et al.* showed that the application of caspase-3 small interference RNA, which specifically suppresses the expression of the gene encoding for caspase-3, protects from IRI by improving functional parameters and morphology of liver tissue *in vivo* (Contreras,J.L. et al., 2004b). As our results revealed that ANP *in vivo* causes a decline in apoptotic cell death in liver tissue after ischemia, these data point to a beneficial effect of the peptide in liver transplantation.

5.1.2.2 Anti-apoptotic effects of ANP preconditioning in vivo are not mediated via PKA

Based on recent findings of Kulhanek *et al.* indicating activation of PKA as a key event in prevention of hepatic IRI after cold ischemia *ex vivo* (Kulhanek-Heinze,S. et al., 2004a), we investigated the role of PKA activity in our *in vivo* model of liver IR. In contrast to Kulhanek *et al.*, who showed an inhibition of anti-apoptotic effects of ANP *via* inhibition of PKA, we could clearly demonstrate that this kinase is not involved in ANP-mediated anti-apoptotic effects *in vivo:* there was neither a detectable change in PKA activity nor in the phosphorylation status of the downstream target Bad at its PKA-specific phosphorylation site Ser 112 (Harada,H. et al., 1999). Since Kulhanek *et al.* performed their experiments *ex vivo* in the model of isolated perfused rat liver the controversial observations are explainable by the different experimental settings used. Therefore, we concentrated on other signaling pathways possibly involved in the anti-apoptotic effects of ANP.

5.1.2.3 ANP-mediated in vivo effects involve the PI3-kinase pathway: Phosphorylation and translocation of Akt

Cullen *et al.* recently demonstrated anti-apoptotic effects in hepatocytes to be mediated *via* PI3-kinase activation and completely independent of PKA (Cullen,K.A. et al., 2004). Due to the importance of this kinase as a mediator of cellular survival (Datta,S.R. et al., 1999b, Osaki,M. et al., 2004b), we subsequently focused on the PI3-kinase pathway.

By using the PI3-kinase inhibitor Wortmannin (WM) which completely abrogated inhibition of caspase-3-like activity after ANP pretreatment, we demonstrated that anti-apoptotic effects of ANP depend on a preceding activation of PI3-kinase. This observation is in line with results obtained by Tong *et al.* and Mocanu *et al.* showing that in the heart repression of the PI3-kinase pathway by administration of WM abrogated protective effects of ischemic preconditioning (IP) (Mocanu,M.M. et al., 2002, Tong,H. et al., 2000). Also in preconditioned hepatocytes PI3-kinase inhibition abolishes the tolerance to hypoxic damage, therefore confirming the assumption that protective effects of IP are mediated *via* the PI3-kinase pathway (Carini,R. et al., 2004).

To elucidate ANP-mediated signaling mechanisms involving the PI3-kinase pathway we next focussed on an important downstream target of the PI3-kinase: the survival kinase Akt. This kinase plays a pivotal role in anti-apoptotic signaling (Armstrong,S.C., 2004, Downward,J., 2004a). As previously demonstrated, Akt is rapidly activated after partial hepatectomy and is important for survival of hepatocytes, regeneration of the liver, and also protection of the heart against IRI (Hausenloy,D.J. et al., 2005a, Hong,F. et al., 2000). It is known that activity of Akt is regulated *via* binding of phosphatidylinositol 3,4,5 triphosphate (PIP_3) and $PI(3,4)P_2$ located at the plasma membrane of the affected cells. This causes the translocation of Akt from the cytosol to the plasma membrane followed by its phosphorylation (Datta,S.R. et al., 1999a). Regarding these facts we investigated whether ANP pretreatment influences the distribution and phosphorylation status of Akt in hepatic cells.

Our findings which showed a significant augmentation of phospho-Akt levels in ANP-pretreated livers support results of Kook *et al.* (Kook,H. et al., 2003) who found an increased phosphorylation of Akt in endothelial cells after ANP preconditioning. According to recent findings, an activation of Akt in the liver during warm IR *in vivo* is also protective (Harada,N. et al., 2004c). This strengthens our hypothesis that ANP exerts protective, anti-apoptotic effects *via* an activation of Akt.

With respect to the localization of Akt we observed a translocation of the kinase from the cytosol to the plasma membrane in hepatocytes of ANP-pretreated livers. This translocation has been suggested to be a consequence of binding of the PIP_2 and PIP_3 to Akt and is essential for activation of Akt (Datta,S.R. et al., 1999g). This has also been demonstrated by studies investigating the protective effects of Akt *in vitro* in cardiomyocytes as well as *in vivo* in heart and liver using an adenoviral construct encoding for a constitutively-active, membrane-targeted form of Akt (Harada,N. et al., 2004b, Hausenloy,D.J. et al., 2005b, Matsui,T. et al., 1999, Miao,W. et al., 2000).

Based on our findings demonstrating ANP-mediated anti-apoptotic effects on ischemic liver cells *in vivo via* Akt, we subsequently focused on possible downstream targets of the survival kinase involved in the anti-apoptotic signaling.

5.1.2.4 Bad is phosphorylated by Akt after ANP pretreatment in vivo

In its unphosphorylated form, the apoptosis-regulating factor Bad acts as an inhibitor of anti-apoptotic proteins like Bcl-2 or Bcl-X_L and thus promotes apoptotic processes (Chao,D.T. et al., 1998, Wang,H.G. et al., 1999). After phosphorylation at Serine (Ser) 112 by PKA or at Ser 136 by Akt, Bad detaches from Bcl-2 or Bcl-X_L thereby facilitating anti-apoptotic effects (Datta,S.R. et al., 1999i). Phosphorylation of Bad *via* Akt at Ser 136 is one essential event responsible for the anti-apoptotic effects of the survival kinase (Datta,S.R. et al., 1999h). Thus, inhibition of PI3-kinase and subsequently decreased phosphorylation of its downstream target Akt reverses the protective effects of Akt-activation. In this context, Harada *et al.* showed in a different model of IR *in vivo* that Bad phosphorylation plays a crucial role in protection of liver cells against apoptotic cell death (Harada,N. et al., 2004a). Our data demonstrate that application of WM, thus inhibiting the PI-3kinase pathway, not only prohibits phosphorylation and subsequently activation of Akt but also prevents Bad phosphorylation. With regard to the phosphorylation site of Bad being responsible for the anti-apoptotic effects of Akt we demonstrated that Ser 136 is phosphorylated while phosphorylation at Ser 112 is completely unaffected after ANP preconditioning. It has been shown that mutation of Ser 136 to alanine causes abrogation of survival-promoting properties of Akt. On the other hand, mutation of Bad at Ser 112 has no influence on Akt-mediated anti-apoptotic signaling (Datta,S.R. et

al., 1999j).

Based on this knowledge we clearly demonstrated that ANP mediates its anti-apoptotic effects in the liver after IR *in vivo via* the PI3-kinase / Akt pathway and subsequent phosphorylation of Bad at Ser 136.

5.1.2.5 No effect on transaminase activity and bile flow after ANP pretreatment in vivo

Due to our findings that ANP pretreatment *in vivo* reduces apoptosis in liver tissue after IR we finally aimed to elucidate functional consequences of this effect in the liver. The data of Gerwig *et al.* (Gerwig,T. et al., 2003a) clearly demonstrate a decrease in necrotic cell death in combination with anti-apoptotic effects in ANP-preconditioned livers. In contrast, we were not able to show anti-necrotic effects of the peptide *in vivo*. Interestingly, Cursio *et al.* found out that an application of caspase-inhibitors blocks apoptosis in liver tissue thereby assuring survival of 95 % of the animals after lethal warm liver ischemia, and assigning the major role in cell death during IR to apoptosis (Cursio,R. et al., 1999a). However, they furthermore showed that despite a complete inhibition of caspases, the activity of transaminases, though drastically reduced, remains relatively high. Apoptotic cell death is therefore assumed to be accompanied by necrosis of liver cells during IR. Regarding the nonexisting improvement of bile flow after transplantation, the results of Mueller *et al.* are interesting to discuss (Mueller,T.H. et al., 2004a). They demonstrated improved survival of rats after liver transplantation provoked by application of a caspase-3-inhibitor but could not observe any melioration regarding bile flow after transplantation. In our setting, ANP preconditioning *in vivo* only reduced caspase-3-like activity after ischemia to about 60%. This inhibitory effect might not be strong enough to have an influence on the amount of necrotic cells pointing to a possible explanation for the inability of ANP to influence transaminase activity.

With regard to parameters of liver function, our results furthermore indicate that apoptotic cell death in liver cells is not the only parameter leading to tissue damage and dysfunction. Though caspase-3-like activity increases after ischemia in untreated cells and ANP preconditioning prevents this augmentation, the portion of liver cells dying from apoptosis may be too small to be solely responsible for tissue damage. Therefore, inhibition of apoptosis during IR does not necessarily cause general protection of the organ. This opinion is also represented by Gujral *et al.*, reasoning that if apoptosis was the primary

mechanism of cell death leading to IRI, inhibition of apoptotic processes, like caspase activity, should rapidly ameliorate organ function (Gujral,J.S. et al., 2001d).

Another explanation for the lack of beneficial effects by ANP on functional parameters might be the experimental setting itself. Although Cottart *et al.* investigated influence of the peptide in the liver *in vivo* and observed a decrease in AST (-30%) and LDH (-40%) plasma activity after 1 h reperfusion (Cottart,C.H. et al., 2003d), it has to be mentioned that a completely different experimental setting was used. Warm partial ischemia (37 °C) was performed for 45 min followed by liver perfusion for up to 6 h. Furthermore, the ANP dose used was twice as high as in our experiments (10 µg/kg b.w.) and livers were not transplanted, but reperfused after ischemia. Thus, in our model of ANP preconditioning before cold ischemia *in vivo*, the protective impact of the peptide may just not be strong enough to lead to a significant beneficial outcome after liver transplantation.

5.2 EFFECTS OF ANP DURING LPS-INDUCED SEPTIC SHOCK

5.2.1 ANP preconditioning maintains survival after LPS-induced septic shock

In previous studies of our group ANP has been shown to possess anti-inflammatory and cytoprotective properties *in vitro* after stimulation of HUVECs with TNF-α (Kiemer,A.K. et al., 2002u, Kiemer,A.K. et al., 2002q, Weber,N.C. et al., 2003a). Moreover, we demonstrated that ANP exhibits anti-inflammatory effects in bone marrow derived macrophages after stimulation with LPS (Kiemer,A.K. et al., 2000c, Kiemer,A.K. et al., 2002n). In LPS-mediated activation of KC the peptide increased phagocytosis activity of KC and reduced TNF-α secretion (Kiemer,A.K. et al., 2002c). Besides these *in vitro* observations, inhibition of the ANP receptor binding involved inflammation in *vivo* in rats with serotonin ($5\text{-}HT_{2A)}$ receptor- mediated acute hemodynamic changes (Tozawa,Y. et al., 2002a). In LPS-treated mice, preconditioning with ANP markedly reduced thromboxane B_2 level in the blood, also pointing to anti-inflammatory properties of ANP (Kiemer,A.K. et al., 2002o).

The findings of Qu et al. point to a possible participation of ANP in regulation of plasma volume during endotoxemia (Qu,X.W. et al., 1998b). Additionally, increased plasma ANP levels have been observed in patients with septic shock (Lubbesmeyer,H.J. et al., 1988, Mitaka,C. et al., 1993), and Morgenthaler et al. even discuss Pro-ANP as a prognostic marker in sepsis (Morgenthaler,N.G. et al., 2005c). During endotoxic shock augmentation of ANP concentrations in the plasma persists until 24 h after LPS injection (Aiura,K. et al., 1995a). These findings allude to a role of ANP as a protective endogenous regulator of defensive mechanisms during inflammatory processes in vivo. To date, however, there are no data explaining the signaling mechanism responsible for ANP-mediated effects during endotoxemia. Therefore we were now interested whether ANP exhibits anti-inflammatory actions in vivo in a murine model of LPS-induced endotoxemia thereby influencing survival of animals.

Lethal LPS doses in murine models of endotoxemia highly vary and depend on origin, quality, and manufacturer of LPS samples used for experiments (Gorgen,I. et al., 1992c, Kuhnle,S. et al., 1999,

Tiegs,G. et al., 1994). On this account, we first determined the lethal LPS dose for our experimental setting of LPS-induced sepsis in mice. Using LPS from *Salmonella abortus equi*, we figured out that i.p. injection of 3 mg/kg b.w. LPS is lethal to mice, whereby 100 % of the animals die within 25 h. Interestingly, ANP pretreatment protects mice against LPS-induced sepsis in vivo and guarantees 80 % survival of the animals even 72 h after LPS administration.

Based on these results we subsequently focused on the mechanism involved in ANP-mediated survival after LPS-induced sepsis.

5.2.2 ANP causes reduction of serum TNF-α concentration after LPS-induced sepsis

TNF-α is one of the central pro-inflammatory cytokines and a prominent marker for inflammatory processes during sepsis (Gorgen,I. et al., 1992b, Guha,M. et al., 2002, Munoz,C. et al., 1991).

The serum levels of this cytokine are markedly increased during the inflammatory response to LPS in a murine model of septic shock (Barsig,J. et al., 1995). As Gorgen *et al*. point out coherence between suppression of TNF-α production and survival of mice after LPS-induced septic shock (Gorgen,I. et al., 1992a), we were interested in the effects of ANP on serum TNF-α levels during endotoxemia.

Interestingly, ANP preconditioning reduces TNF-α concentration up to 50 % compared to LPS-treated animals. *In vitro* ANP has previously been demonstrated to possess anti-inflammatory activities. Prior studies showed that the production of TNF-α is inhibited by ANP pretreatment in murine macrophages stimulated with LPS, and Interferon-gamma respectively (Kiemer,A.K. et al., 2000d, Tsukagoshi,H. et al., 2001c). Furthermore, ANP treatment abrogates LPS-induced increase of TNF-α in human blood and reduces TNF-α secretion and phagocytotic activity of rat Kupffer cells (KC) (Kiemer,A.K. et al., 2000e, Kiemer,A.K. et al., 2002d).

However, the role of the TNF-α level in serum as a prognostic marker during sepsis for survival of patients and the clinical relevance of an anti-TNF-α therapy are discussed controversially. On the one hand, Atici *et al.* reported that in infants suffering from sepsis, TNF-α levels are significantly elevated, but

are not different in surviving and non-surviving neonates (Atici,A. et al., 1997). Thus, the authors conclude that there is no correlation between TNF-α serum concentration and the outcome of neonatal sepsis. On the other hand, Collighan *et al.* and Damas *et al*. showed that a decrease in serum TNF-α level is directly linked to survival of septic patients and is therefore suitable for an estimation of sepsis severity score (Collighan,N. et al., 2004, Damas,P. et al., 1989). With respect to efficacy of anti-TNF-α compounds during sepsis some studies revealed only small survival benefit as described by Reinhart *et al.* (Reinhart,K. et al., 2001). However, it has recently been demonstrated that Afelimomab, a monoclonal anti-TNF-α antibody fragment, reduces mortality and attenuates the severity of organ dysfunction in patients with severe sepsis (Panacek,E.A. et al., 2004). Moreover, Matsumoto *et al.* demonstrated in a murine model of gut-derived sepsis that treatment with an anti-TNF-α monoclonal antibody significantly increases survival of animals, whereas application of recombinant human TNF-α increases the mortality rate in comparison to saline-treated mice (Matsumoto,T. et al., 1997). In fact, the results of the present study suggest for our experimental setting an inverse correlation between the serum TNF-α levels and survival of animals. Pretreatment and thus inhibition of production of TNF-α might prevent increase in serum levels of this cytokine and could be a key event in facilitating survival in the present study. Therefore, signaling pathways leading to TNF-α release are an important target for ANP mediated protective effects in LPS-induced sepsis and were therefore further investigated.

5.2.3 Intervention of ANP into LPS-signaling in the kidney

ANP has been demonstrated to play a protective role during inflammatory processes and in the kidney regarding acute renal failure (Lieberthal,W. et al., 1990, Raine,A.E. et al., 1989). Due to leukocytes infiltration, inflammatory processes such as activation of NF-κB and subsequent increased expression of inflammatory genes in mesangial cells, renal tubular epithelial cells, and urothelial cells this organ is seriously affected during sepsis (Gjertsson,I. et al., 2001b, Guijarro,C. et al., 2001b). Furthermore, ANP levels have been demonstrated to be increased in kidneys of sheep suffering from endotoxemia, thereby influencing renal functions (Hinder,F. et al., 1996).

Our experiments, interestingly, revealed that kidneys from LPS-treated animals were obviously damaged,

those from ANP-injected mice were hardly affected in their structural appearance pointing to ANP-induced effects in this organ.

Based on the observation of decreased serum TNF-α levels in ANP-treated septic animals and the knowledge that this cytokine is regulated *via* NF-κB (Barnes,P.J. et al., 1997), we next investigated the role of NF-κB in ANP-mediated effects during endotoxemia. NF-κB is an important component regulating the inflammatory and immune response by inducing the expression and release of cytokines and other pro-inflammatory mediators (Blackwell,T.S. et al., 1997b, Hayden,M.S. et al., 2004). Abraham *et al.* furthermore demonstrated that activation of NF-κB dependant pathways is crucial for the development of septic shock and thus, regulation of this parameter is an attractive therapeutic option (Abraham,E., 2003).

Activation of NF-κB plays a crucial role in inflammatory processes causing severe damage of the kidneys as shown in models of systemic inflammation, nephritis, tubulointerstitial disorders, and proteinuria (Guijarro,C. et al., 2001c). Pocock *et al.* observed in kidneys of LPS-tolerant rats a significant downregulation of NF-κB activity (Pocock,J. et al., 2003). Although Gjertsson *et al.* show that administration of antisense oligonucleotides to the p65-subunit of NF-κB has no effect on the clinical outcome of septic arthritis or sepsis (Gjertsson,I. et al., 2001a), there are definitely more data indicating the inhibition of NF-κB activity as a promising target for anti-inflammatory therapies of renal diseases. Thus, Tamada *et al.* demonstrate that inhibition of NF-κB activation prevents renal fibrosis and interstitial inflammation in chronic tacrolimus (FK506) nephropathy (Tamada,S. et al., 2003). Additionally, prevention of NF-κB activation by an application of anti-inflammatory substances like partenolide and gliatoxin improves the outcome of experimental glomerulonephritis in rats (Lopez-Franco,O. et al., 2002a). The kidneys of either partenolide- or gliatoxin-treated animals showed an inhibition of NF-κB activity combined with protection against renal lesions, proteinuria and monocyte infiltration. Further *in vivo* studies also suggest the inhibition of NF-κB activity to be responsible for anti-inflammatory and thus renal protective effects of the glucocorticoid prednisolon making this transcription factor an important target for continuing investigation (Komiya,T. et al., 2004, Sakurai,H. et al., 1997).

In vitro ANP has been shown to possess anti-inflammatory properties causing a reduction of NF-κB binding activity in LPS-stimulated, and Interferon-gamma-activated murine macrophages respectively (Kiemer,A.K. et al., 1998b, Tsukagoshi,H. et al., 2001a). In human cells (HUVECs) ANP-treatment prevented TNF-α-induced NF-κB activity (Kiemer,A.K. et al., 2002t). Moreover, it has been demonstrated that ANP prevented the increase of NF-κB activity occurring during reperfusion in ischemic rat livers and

Irwin *et al.* previously demonstrated that treatment with ANP prevents hypoxia-induced activation of NF-κB in bovine pulmonary endothelial cells (Irwin,D.C. et al., 2005a, Kiemer,A.K. et al., 2000j). Vellaichamy *et al.* additionally observed an activation of NF-κB *in vivo* due to absence of ANP-mediated anti-inflammatory actions in hearts of mice lacking the gene encoding for the ANP receptor (NPR-A) (Vellaichamy,E. et al., 2005).

In the present study investigation of NF-κB binding activity in kidneys of septic mice clearly reveals that the NF-κB binding activity increases in the kidney during endotoxaemia. Interestingly, pretreatment with ANP completely compensates this inflammatory action, causing a decrease in NF-κB binding activity during LPS-induced sepsis. Therefore, we presumed that ANP mediates its anti-inflammatory effects *via* the NF-κB pathway and subsequently concentrated on studying further processes involved in NF-κB activation.

Dimerization of the p50 and p65 subunit is essential for activation of NF-κB. The p50 subunit lacks the transactivating domain which enables NF-κB to activate gene transcription (Adrie,C. et al., 2000, Blackwell,T.S. et al., 1997a). Consequently, homodimers of p50 cause NF-κB dysfunction *in vitro* (Viatour,P. et al., 2005, Ziegler-Heitbrock,H.W. et al., 1994). The p65 subunit, however, which contains a transactivation domain at the C-terminal end, is crucial for transcriptional actions of NF-κB. Under normal conditions, NF-κB is present in the cytoplasm in complex with its inhibitor IκBα (Annane,D. et al., 2005a). After stimulation, for example by binding of LPS to its receptor, the transcription factor translocates into the nucleus in order to bind to specific DNA recognition sites (Annane,D. et al., 2005d).

In vitro ANP has been demonstrated to decrease TNF-α-induced translocation of p65 into the nucleus in HUVECs (Kiemer,A.K. et al., 2002s). Furthermore, pretreatment of isolated rat livers with ANP before IR caused markedly reduced protein levels of p50 and p65 in nuclear extracts of liver tissue after reperfusion (Kiemer,A.K. et al., 2000i). Such capture of NF-κB in the cytosol has been described to correlate with protective anti-inflammatory effects of peroxisome proliferator-activated receptor-gamma (PPR-gamma) activators in autoimmune myocarditis as well as with cardioprotective properties of [N-(3,5-bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide] (IMD-0354), an inhibitor of NF-κB activation (Onai,Y. et al., 2004a, Yuan,Z. et al., 2005).

In our model of endotoxemia translocation of p65 into nuclei was observed in kidney cells of septic mice. Interestingly, ANP caused an inhibition of LPS-induced translocation, thus retaining the NF-κB complex in the cytosol. Our data therefore demonstrate anti-inflammatory effects of ANP during endotoxemia by

means of reduced translocation of the p65 subunit into the nucleus causing decreased NF-κB binding activity *in vivo* and we were now interested in mechanisms involved in this inhibition of NF-κB activity.

5.2.3.1 Decreased phosphorylation of IκBα by ANP

IκBα is the best characterized member of the IκB family. As demonstrated in studies with IκBα knockout mice, this protein plays a pivotal role in limiting NF-κB activation (Beg,A.A. et al., 1995a, Klement,J.F. et al., 1996b). Regulation of NF-κB transcriptional activity is facilitated by reversible binding of IκBα to the NF-κB complex, but the suppositions for NF-κB separation, translocation, and subsequent activation have been discussed intensively. On the one hand, Henkel *et al.* represent the opinion that the activation of NF-κB depends exclusively on a previous degradation of IκBα, whereas phosphorylation of the inhibitor is not mandatory (Henkel,T. et al., 1993). On the other hand, Beg *et al*. and Gosh *et al*. clearly demonstrate the dependence of NF-κB activation on a previous phosphorylation of its inhibitor (Beg,A.A. et al., 1993, Ghosh,S. et al., 1990). They show that IκBα has to be phosphorylated before it becomes separated from the NF-κB complex and immediately degraded, whereas free NF-κB translocates into the nucleus, subsequently activating further signaling cascades (for review see (Karin,M. et al., 2000). The crucial role of IκBα phosphorylation has also been described for LPS-induced NF-κB activation *in vitro* and *in vivo* (Chen,L.F. et al., 2004c, Chen,L.F. et al., 2004a, Guha,M. et al., 2001).

In TNF-α-stimulated HUVECs treatment with ANP induced the expression of IκBα (Kiemer,A.K. et al., 2002r). In contrast, in a model of IR in the isolated rat liver, Kiemer *et al.* did not see any influence of ANP on IκBα levels although NF-κB activity in ANP-treated livers was decreased, therefore suggesting NF-κB activation to be independent of IκB-α in this experimental setting (Kiemer,A.K. et al., 2000h). Onai *et al.* suggested the inhibition of IκBα phosphorylation by IMD-0354 (N-(3,5-Bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide) to be causally involved in the protection of rat hearts from myocardial IRI (Onai,Y. et al., 2004b). Furthermore, the results of Lopez *et al.* point to anti-inflammatory and therefore protective properties of an inhibition of phosphorylation and degradation of IκBα with respect to glomerulonephritis (Lopez-Franco,O. et al., 2002b). In LPS-treated cultured mesangial cells and monocytes the anti-inflammatory substances parthenolide and gliotoxin inhibit the phosphorylation and

degradation of IκBα. Additionally, in an *in vivo* model of experimental glomerulonephritis in rats these substances prevent renal lesions, proteinuria, and monocyte infiltration suggesting a crucial role for IκBα during inflammatory processes in the kidney (Lopez-Franco,O. et al., 2002c). Moreover, Tashiro *et al.* showed that an inhibition of NF-κB by stabilization of IκBα leads to attenuation of renal fibrosis in rats (Tashiro,K. et al., 2003). Therefore, we aimed to investigate the role of IκBα in our model of endotoxemia and the influence of ANP on this NF-κB inhibitor in the kidney.

Our data show a strong phosphorylation of IκBα in kidneys of LPS-treated animals paralleled by decreased levels of IκBα pointing at a fast degradation of the inhibitor after phosphorylation. Interestingly, ANP causes a reduction in IκBα phosphorylation and therefore reduced degradation of the inhibitor in the kidney. Therefore we suggest that ANP prevents translocation and activation of NF-κB by maintaining IκBα in its non-phosphorylated state thus preventing inflammatory processes during sepsis in mice.

With respect to second messengers involved in ANP-mediated effects the cyclic nucleotide cGMP (3´,5´-cyclic guanosine monophosphate) has been shown to play a crucial role. Protective and anti-inflammatory properties of ANP such as prevention of IRI in isolated perfused rat livers (Gerbes,A.L. et al., 1998a, Kiemer,A.K. et al., 2000g) , as well as the inhibition of macrophages activation by ANP (Kiemer,A.K. et al., 1997a, Kiemer,A.K. et al., 2000f) were shown to be mediated *via* cGMP. In renal diseases the angiotensin II type 2 receptor (AT_2-R) was demonstrated to exhibit protective effects *via* increased production of cGMP in the kidney (Matsubara,H., 1998). Moreover, an augmented renal cGMP production has been shown to contribute to protection of the kidney in chronic nephritis in rats (Uhlenius,N. et al., 2002). The beneficial properties of ANP with regard to nephrotoxicity of immunosuppressive agents and antibiotics were also attributed to cGMP as the pivotal second messenger: Polte *et al.* observed in cultured proximal tubular cells (LLC-PK1 cells) that the cGMP analogue 8-bromo-cGMP mimics the cytoprotective effects of ANP and prevents cyclosporine A-dependent nephrotoxicity (Polte,T. et al., 2002). Even more importantly, the results of Murakami *et al.* evidence that application of an adenovirus carrying the human ANP gene protects rats from gentamycine-induced renal failure, with cGMP levels being significantly increased in animals which received the ANP gene (Murakami,H. et al., 1999). As these findings suggest an involvement of cGMP in protective actions of ANP, we hypothesize that this second messenger might play a role in ANP-mediated ant-inflammatory properties *in vivo*.

Regarding therapeutic potentials of ANP, pretreatment with this peptide might be a prophylactic strategy in treatment of patients at risk preventing the onset of inflammatory processes which cause the

progression of endotoxemia. However, intense research on further properties of ANP during sepsis in other organs and the elucidation of the involved signaling mechanism is required before final conclusions can be made about the therapeutic potential of ANP in sepsis.

6. Summary

The Atrial Natriuretic Peptide (ANP) has recently been shown to possess cytoprotective potential in a variety of experimental settings. Most of the underlying signaling pathways however, remain to be clarified.

In the model of IRI in the isolated perfused rat liver we could show that ANP preconditioning affects hepatic cytoskeletal structures. In detail, *via* activation of p38 MAPK and subsequently phosphorylation of Hsp27 ANP treatment of livers leads to enhanced cytoskeletal F-actin content.

In the *in vivo* setting of liver-transplantation anti-apoptotic effects of ANP could characterized. ANP preconditioning leads to activation of the PI3-K/Akt pathway and inactivation of the pro-apoptotic protein Bad.

In a murine model of sepsis ANP pretreatment was shown to increase survival of the animals, which was paralleled by reduced serum TNF-α level.

Focussing on the kidney, ANP treatment results in inhibition of IκBα degradation.

In summary, this work contributes to a better understanding of the mechanism of action underlying the anti-inflammatory and cytoprotective effects of the Atrial Natriuretic Peptide.

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8. Appendix

8.1 ABBREVIATIONS

A	Ampère
ADF	Actin depolymerizing factor
ALT	alanine transferase
ANP	Atrial Natriuretic Peptide
AP-1	Activator protein-1
AST	aspartate transferase
ATP	Adenosine-5`-triphosphate
BNP	Brain Natriuretic Peptide
Bp	Basepair
8-Br-cGMP	8-Bromo-3`,5`-cyclic monophosphate
BSA	Bovine serum albumine
°C	Degree Celsius
cAMP	Cyclic Adenosine-5`-monophosphate
cGMP	Cyclic Guanosine-5`-monophosphate
Ci	Curie (1 Ci=3.7x10^7 Bequerel)
CNP	C-type Natriuretic Peptide
Co	Control
cpm	Counts per minute
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(aminoethylether)-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
Erk	Extracellular-regulated kinase

EtOH	Ethanol
G	Gram
GDP	Guanosine-5`-diphosphate
GTP	Guanosine-5`-triphosphate
H	Hour
HEPES	N-(2-hydroxyethyl)piperazine
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IκBα	Inhibitor of κB α
IR	Ischemia/Reperfusion
IRI	Ischemia reperfusion injury
JNK	c-Jun N-terminal kinase
KC	Kupffer cell
kDa	Kilo Dalton
l	Liter
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
m	Milli (10^{-3})
M	Molar
µ	Micro (10^{-6})
MAPK	Mitogen-activated kinase
MCP-1	Monocyte chemoattractant protein-1
MeOH	Methanol
min	Minute
MKP	Mitogen-activated kinase phosphatase
MPT	Mitochondrial permeability transition
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
NF-κB	Nuclear factor-κB
NO	Nitric oxide

NP	Natriuretic Peptide
NPR	Natriuretic Peptide receptor
PAA	Polyacrylamide
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate buffered saline
PI3-K	Phosphatidylinositol-3 kinase
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of mean
TBS-T	Phosphate buffered saline solution with Tween
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor alpha
Tris	Tris-hydromethyl-aminomethan
U	Unit
V	Volt
W	Watt

8.2 ALPHABETICAL LIST OF COMPANIES

Alexis Biochemicals	Grünberg, Germany
AGFA	Cologne, Germany
Amersham	Braunschweig, Germany
Bachem	Heidelberg, Germany
BD Biosciences	Heidelberg, Germany
Beckman Instruments	Munich, Germany
Biomol	Hamburg, Germany
Bio-Rad Laboratories	Munich, Germany
Biosource	Nivella, Belgium
Biozol	Eching, Germany
Boehringer	Mannheim, Germany
Braun	Melsungen, Germany
Calbiochem	Schwalbach, Germany
Canberra-Packard	Dreieich, Germany
Cell signaling/NEB	Frankfurt/Main, Germany
Charles River GmbH	Sulzfeld, Germany
Dianova	Hamburg, Germany
Eppendorf	Maintal, Germany
Fuji	Düsseldorf, Germany
Greiner	Frickenhausen, Germany
Heraeus	Hanau, Germany
Kodak	Rochester, USA
Leinco/Biotrend	Cologne, Germany
Merck-Eurolab	Munich, Germany
Millipore	Eschborn, Germany
Molecular Probes/Invitrogen	Karlsruhe, Germany
NEN	Cologne, Germany

Olympus Optical	Hamburg, Germany
Perkin-Elmer	Rodgau-Jügesheim, Germany
Peske	Aindling-Pichl, Germany
Pharmacia Biotech	Heidelberg, Germany
Pierce	Rockford, USA
Roche Diagnostics	Mannheim, Germany
Roth	Karlsruhe, Germany
Santa Cruz	Heidelberg, Germany
Siemens	Erlangen, Germany
Sigma-Aldrich	Taufkirchen, Germany
Ssniff	Soest, Germany
Stressgen	San Diego, USA
Upstate/Biomol	Hamburg, Germany
Zeiss	Oberkochen, Germany

8.3 PUBLICATIONS

8.3.1 Original publications

Keller M, Gerbes Al, Gerwig T, Grützner U, Vollmar AM, Kiemer AK. The hepatocyte cytoskeleton during ischemia and reperfusion – influence of ANP-mediated p38 MAPK activation.
World J. Gastroenterol.. In press.

Grützner U[#], Keller M[#], Bach M[#], Kiemer AK, Meissner H, Bilzer M, Zahler S, Gerbes AL, Vollmar AM. PI 3-Kinase pathway is responsible for anti-apoptotic effects of Atrial Natriuretic Peptide (ANP) in rat liver transplantation.
Liver Transplantation. Submitted.

8.3.2 Oral presentations

Keller M, Gerwig T, Gerbes AL, Vollmar AM, Kiemer AK. Steigerung der Aktinpolymerisation durch Präkonditionierung mit Atrialem Natriuretischem Peptid – Einfluß von p38 MAPK und Hsp27.

14. Workshop für Hepatologie und Lebertransplantation, 2003, Wilsede, Germany.

German J Gastroenterol., 2003, 6: 618

8.3.3 Poster presentations

Keller M, Grützner U, Zahler S, Kiemer AK, Gerbes AL, Vollmar AM. Pretreatment with the Atrial Natriuretic Peptide leads to inhibition of Caspase-3 *in vivo* – involvement of PI 3-kinase-pathway and Bad.

21st Annual Meeting of the German Working Team on Liver Studies, 2005, Ulm, Germany.

German J Gastroenterol, 2005, 1:91

Keller M, Grützner U, Kiemer AK, Meissner H, Bilzer M, Zahler S, Gerbes AL, Vollmar AM. Vermittlung der antiapoptotischen Effekte des Atrialen Natriuretischen Peptids in der Rattenlebertransplantation: Beteiligte Signaltransduktionswege.

46th Spring Meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, 2005, Mainz, Germany.

Naunyn Schmiedeberg`s Arch Pharmacol. 2005, 370 Suppl.1:R 373

Keller M, Kiemer AK, Vollmar AM, Wendel A. Pretreatment with Atrial Natriuretic Peptide pervents formation of tumor necrosis factor alpha (TNF-α) in LPS-induced sepsis *in vivo*.

45th Spring Meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, 2004, Mainz, Germany.

Naunyn Schmiedeberg`s Arch Pharmacol. 2004, 369 Suppl.1:R 308

Koch E, Ladetzki-Baehs K, Keller M, Kiemer AK, Wendel A, Vollmar AM. Preconditioning with Atrial Natriuretic Peptide modulates LPS-induced NF-κB activation *in vivo.*

45th Spring Meeting of the Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie, 2004, Mainz, Germany.

Naunyn Schmiedeberg`s Arch Pharmacol. 2004, 369 Suppl.1:R 309

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