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Prevention of Ischemia-Reperfusion Injury in the Rat Liver by Atrial Natriuretic Peptide: Insights into Mechanisms and Mode of Cell Death

von
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Erklärung

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Ehrenwörtliche Versicherung

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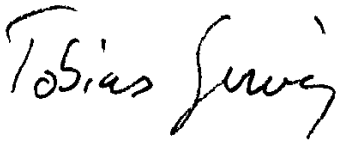

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To my family

“The trouble with experience is

that by the time you have it

you are too old to take advantage of it”

Jimmy Connors

Contents

A. Introduction

1. Background and overview

The temporary disconnection of blood flow is a necessity in a number of surgical procedures on the liver. This event, also referred to as ischemic period, can last for a short time as in liver resection at maintained organ temperature or can last up to several hours in the cold as it is the case in liver transplantation. Both insults cause detrimental cellular mechanisms that are even augmented by the subsequent restoration of blood flow after surgery or insertion of an organ, culminating in the manifestation of ischemia reperfusion injury (IRI).

In the field of hepatic transplantation, the preservation injury, a severe form of IRI, contributes to serious complications. Primary non-function, dysfunction, and non-anastomotic biliary strictures frequently question the outcome of liver transplantation and are major causes of retransplantation and mortality. Because of the increasing numbers of orthotopic liver transplantations and the concomitant lack of suitable donor organs, there is a great demand for better protection against IRI to decrease the rate of preservation–related complications. Additionally, successful liver transplantation reduces the need of retransplantation and therefore increases the number of urgently required organs available for liver transplantation.

Several approaches have been made in the recent time in order to protect from hepatic IRI. Among them, preconditioning with the Atrial Natriuretic Peptide (ANP) (Gerbes *et al.*, 1998) emerged as a potential candidate making livers more resistant against IRI. It was demonstrated in the model of the isolated perfused rat liver, that ANP is able to reduce cell damage and improve liver function. Cytoprotection is thereby mediated via the guanylyl cyclase-coupled A receptor. First mechanistical investigations revealed that activation of transcription factors and expression of cytokines, known to participate in cell damage, are mitigated, and cytoprotective pathways are enabled.

Aim of the present work was the detailed characterization of cell death and ANP protection in IRI of the isolated perfused rat liver. The following questions should be answered:

1. Is apoptotic cell death involved in IRI of the isolated perfused rat liver? Which liver cell types are affected and does ANP influence this kind of cell death?
2. Elucidation of signal transduction processes: which cGMP-dependent proteins are involved in ANP mediated protection?
3. Does ANP induce the expression of the heme oxygenase-1 (HO-1), a cytoprotective heat shock protein? Which liver cell types are affected and is there a causal link between HO-1 induction and ANP cytoprotection?

2. Ischemia-reperfusion injury of the liver

2.1 Significance

Since Thomas Starzl in 1963 performed the first human liver transplantation (Starzl, 1978), this technique has evolved to an established therapy for end stage liver disease or acute liver non-function. Due to the increasing experience and optimization of surgical technique and immunosuppression, survival rates were improved significantly and are today about 85%-90% after one year (Keeffe, 2001). Superior organ preservation also accounts for this development. The commonly used University of Wisconsin (UW) solution (Southard *et al.*, 1995) facilitates preservation times for up to 20 h and therefore replaced the former, less effective Euro-Collins solution (Crenesse *et al.*, 1994).

Despite improved preservation techniques, the success of liver transplantations is still influenced by IRI (Bilzer *et al.*, 2000). Long preservation times are in direct proportion to the incidence of retransplantation, indicating that preservation injury is a major cause for graft loss. In 5 to 15% of transplanted livers a primary non-function occurs that can be defined by transaminase increase, loss of bile production and disturbances in clotting. This event highly correlates with preservation time and strongly increases after more than 12 h of preservation (Furukawa *et al.*, 1991). In this case, retransplantation is inescapable.

30% of graft recipients suffer from primary dysfunction, another grave complication. It is characterized by a post-surgical increase of liver enzymes, accompanied by a retarded regeneration of hepatic synthesis (Lemasters *et al.*, 1995) resulting in a three times higher risk of graft loss.

Non-anastomotic biliary strictures also show a correlation with the preservation time (Sanchez-Urdazpal *et al.*, 1993). These ischemic bile duct strictures emerge during the first three months after transplantation and are difficult to cure. In hard cases, retransplantation is the only way for therapy.

In summary, improved protection against IRI could decrease the rate of preservation related complications and, moreover, should increase the number of organs available for liver transplantation. Elucidation of the involved pathomechanisms and developing protective strategies are therefore important objectives.

2.2 Pathomechanisms in cold ischemia and reperfusion

In recent years, the knowledge of mechanisms underlying IRI markedly improved. There is general agreement that IRI is caused by a combination of cellular changes in the ischemic period and the consecutive reperfusion period, where blood flow and oxygen supply are restored.

2.2.1 Ischemia

The main feature of the ischemic phase is the loss of mitochondrial respiration. Reduced energy status leads to a breakdown of energy-dependent metabolic pathways and transport processes, finally resulting in perturbation of ion homeostasis and activation of proteases (Clavien *et al.*, 1992; Rosser *et al.*, 1995). These include aspartate proteases, matrix metalloproteases, and Ca^{2+}-requiring calpains (Takei *et al.*, 1991; Upadhya *et al.*, 1997; Calmus *et al.*, 1995), which proteolytically cleave and disrupt membrane and cytoskeletal proteins. Additionally, endonucleases cause nuclear chromatin damage, and Ca^{2+}-dependent phospholipases alter membrane fluidity and function (Trump *et al.*, 1992). The activation of ATPases by increased cytosolic calcium hastens ATP depletion, even worsening the low cellular energy state during ischemia (Rosser *et al.*, 1995). In organ transplantation, hypothermic storage itself leads to negative effects like cell swelling and calcium alterations despite the beneficial properties of low temperatures to prolong the possible storage time by reducing the metabolic rate (Hansen *et al.*, 1994; Marsh *et al.*, 1989). Sinusoidal endothelial cells (SEC) seem to be more susceptible to cold ischemia than hepatocytes (Otto *et al.*, 1984) and contribute to reduced organ viability by detaching from their cellular matrix at the beginning of the reperfusion process (McKeown *et al.*, 1988).

2.2.2 Reperfusion

When blood supply is restored in the reperfusion period, a complex network of hepatic and extrahepatic mechanisms leads to an aggravation of liver damage. Kupffer cells, the liver macrophages, are rapidly activated and are commonly seen as central mediators of hepatic pathomechanisms (Bilzer *et al.*, 2000; Mochida *et al.*, 1994). By releasing reactive oxygen species (ROS), a variety of related signal transduction processes gets started (Wanner *et al.*, 1996). The proposed direct toxicity of ROS in lipid peroxidation appears unlikely to be the main mechanism of injury. In contrast, secretion of platelet activating

factor (PAF) (Zhou *et al.*, 1992), interleukins, and TNF-α (Le Moine *et al.*, 1997) by endothelial cells and monocytes, and activation of the redox sensitive transcription factors (TF) AP-1 and NF-κB (Kiemer *et al.*, 2000b) initiate an inflammatory response with upregulation of adhesion molecules (Jaeschke *et al.*, 1996), neutrophil sequestration and priming of neutrophils and macrophages for increased toxicity. The development of delayed perfusion failure in the hepatic microcirculation further contributes to IRI. This is caused by an increased formation of vasoconstrictors, such as endothelin-1 (ET-1) and ROS and a reduction of vasodilators such as nitric oxide (NO) leading to an imbalance in liver perfusion.

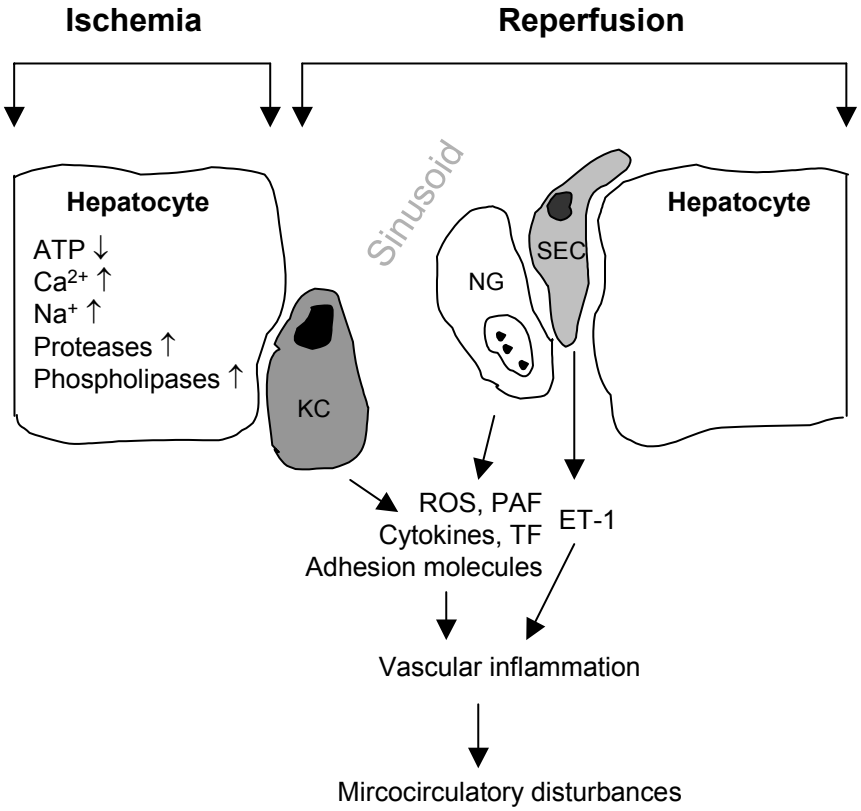

figure 1: Summary of the pathomechanisms of ischemia reperfusion injury. KC: Kupffer cell, NG: neutrophil granulocyte, SEC: sinusendothelial cell, ROS: reactive oxygen species, PAF: platelet activating factor, ET-1: endothelin-1, TF: transcription factors.

3. Mode of cell death

When blood flow is re-established after the ischemic period, damaged cells are still able to survive if they are reversibly injured. However, depending on the intensity and duration of the ischemic insult, variable numbers of cells are already irreversibly injured or may be severely damaged in the reperfusion period exceeding the point of no return. In this case, cell death can happen in two principal patterns, namely necrosis and apoptosis (reviewed in (Majno *et al.*, 1995; Raffray *et al.*, 1997; Leist *et al.*, 2001)). The switch to the respective kind of cell death is discussed to depend on the severity of death stimulus: for example, moderate hypoxia may induce apoptosis and severe hypoxia may induce necrosis (Kaplowitz, 2000). Furthermore, it was proposed that there are various intermediates between apoptotic and necrotic cell death (Raffray *et al.*, 1997; Nicotera *et al.*, 1999).

In the reperfused liver, there is evidence that reactive oxygen species and proteases cause hepatocellular necrosis (Mavier *et al.*, 1988; Nieminen *et al.*, 1995; Nieminen *et al.*, 1997). Apoptotic cell death was reported to account to a large degree to IRI (Gao *et al.*, 1998; Cursio *et al.*, 1999; Kohli *et al.*, 1999). The significance of this kind of cell death for warm (Gujral *et al.*, 2001) or cold (Redaelli *et al.*, 2002) hepatic IRI, however, has been questioned.

3.1 Necrosis

Necrosis refers to the progressive degradation of cell structure that occurs after death caused by severe, injurious changes in environmental conditions (Walker *et al.*, 1988).

Necrosis describes a passive, barely regulated form of cell demise that affects mainly cell groups. The morphological changes are characterized by increased eosinophilia and cell swelling. Necrotic cells show discontinuities in plasma and organelle membranes, marked dilation of mitochondria and nuclear changes. This non-specific DNA breakdown of DNA is called karyolysis, when the basophilia of the chromatin fades by increased DNase activity. In contrast pyknosis, as seen in apoptosis, is characterized by nuclear shrinkage and increased basophilia. Finally, necrotic cell lysis results in a significant inflammatory response by release of cytoplasmatic components attracting phagocyting macrophages (Walker *et al.*, 1988; Majno *et al.*, 1995; Raffray *et al.*, 1997).

3.2 Apoptosis

Apoptosis was initially recognized in 1972 by its distinctive morphology and named after the greek designation for “falling off” (Kerr *et al.*, 1972), referring to autumn foliage. It is a form of cell death designated to eliminate unwanted host cells through activation of a coordinated, internally programmed series of events. It occurs for example as a physiological process during development, and as a homeostatic mechanism in order to remove cells damaged by disease or noxious agents. On the other hand, dysregulation of apoptosis can also be involved in exaggerated or suppressed cell demise in a number of diseases like cancer, AIDS, and autoimmune or neurodegenerative disorders (Thompson, 1995).

Chromatin condensation represents the most characteristic feature of apoptosis. Other morphological alterations, often limited to single or only few cells, comprise shrinkage into tightly packed and smaller cells. The apoptotic cell first shows extensive surface blebbing, then undergoes fragmentation into a number of membrane bound apoptotic bodies composed of cytoplasm and tightly packed organelles, with or without a nuclear fragment. Finally, apoptotic bodies or cells are phagocyted by parenchymal cells or macrophages and are subsequently degraded within lysosomes.

In contrast to necrosis, plasma membranes are thought to remain intact during apoptosis, preventing release of potentially inflammatory content (Walker *et al.*, 1988). However, recent findings suggest that apoptotic cell death can be equally as effective as necrosis in inducing hepatic inflammation (Faouzi *et al.*, 2001; Jaeschke, 2002).

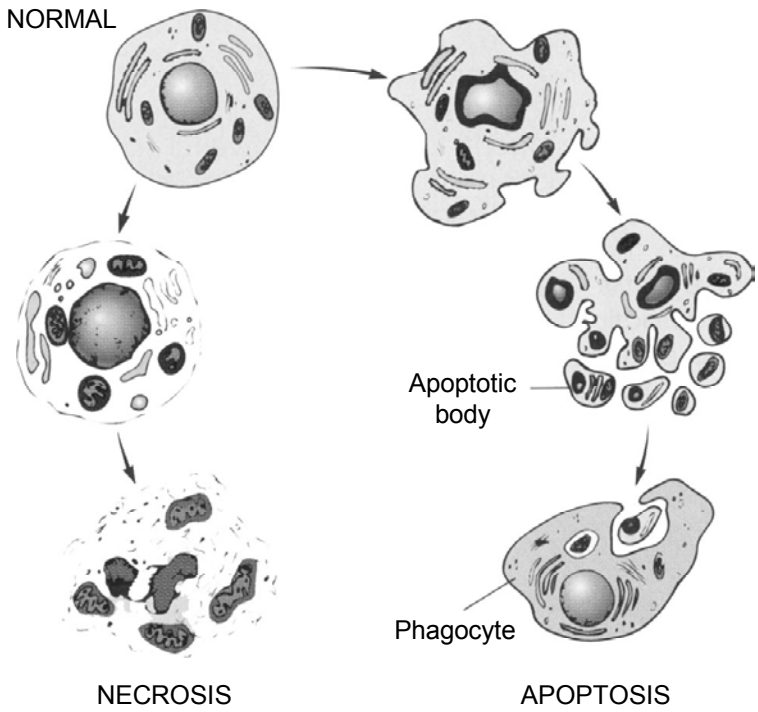

figure 2: The ultrastructural changes seen in coagulation necrosis and apoptosis. In apoptosis, the initial changes consist of nuclear chromatin condensation and fragmentation, followed by cytoplasmic budding and phagocytosis of the extruded apoptotic bodies. Signs of necrosis include chromatin clumping, organellar swelling, and membrane damage (adapted from (Walker et al., 1988).

3.2.1 Mechanisms

Apoptosis represents a highly coordinated way of cell death. This is reflected by the complexity of apoptotic signal transduction. Apoptotic stimuli generate signals that are either transmitted across the plasma membrane to intracellular regulatory molecules or address directly to targets present within the cell.

3.2.1.1 Extracellular signaling

Growth factors, certain hormones or cytokines are required as normal survival stimuli. The absence of such factors activates preexisting death programs und thus triggers cell death (Boise *et al.*, 1995; Collins *et al.*, 1994).

Distinct surface receptors were discovered transducing death signals into the cell. Among them, the death receptors TNF-R1 and CD95 (Apo-1/Fas) and their ligands TNF and CD95L, respectively, are the most prominent for inducing apoptosis in the liver (Faubion *et al.*, 1999; Ashkenazi *et al.*, 1998).

In the liver, the physiological role of the death receptor-mediated induction of apoptosis represents the maintenance of hepatic tissue homeostasis and of liver function. Therefore, senescent (Benedetti *et al.*, 1988) or toxin damaged (Leist *et al.*, 1997) hepatocytes are rapidly eliminated by apoptosis. Pathologically increased or diminished apoptosis rates are basis for several hepatic disorders. Excessive apoptosis is found, for example, in viral or autoimmune hepatitis, alcoholic hepatitis, Wilson's disease, primary biliary cirrhosis, transplant rejection, and toxic liver injury, whereas pathologically decreased apoptosis rates account for hepatic carcinogenesis (Patel *et al.*, 1999).

After receptor ligand interaction, the death signal is further transmitted *via* death domain containing adapter proteins to activation of caspase-8, a member of the caspase-family of proteases. In the case of TNF, signaling is extremely complex leading to both cell death and cell survival signals. (Faubion *et al.*, 1999).

3.2.1.2 Intracellular signaling

Intracellular signaling may also cause apoptosis. If irreversible DNA damage occurs, the tumor suppressor protein p53 accumulates and induces apoptosis if repair is impossible (Lane, 1992; Kastan *et al.*, 1991).

The mitochondrial pathway is used extensively in response to extracellular triggers, such as physicochemical agents (heat, radiation, xenobiotics, free radicals, hypoxia and viral infections) (Green *et al.*, 1998). The results are mitochondrial permeability transitions and formation of pores with subsequent reduction of mitochondrial membrane potential and mitochondrial swelling. Apoptosis is then provoked by the release of AIF and Smac/Diablo and of cytochrome-c into the cytoplasm, which in turn forms in combination with Apaf-1 and caspase-9 the apoptosome complex. These proapoptotic events can be counteracted or enforced by proteins of the Bcl-2 family (Reed, 1997).

3.2.1.3 Execution

The death signal from extracellular and intracellular origin are transmitted *via* a group of initiator caspases to effector caspases, which are the final executioners of apoptosis (Salvesen, 1999; Thornberry *et al.*, 1998). Caspases are a family of cysteine proteases that specifically cleave substrates after aspartic acid residues. They are present in all mammalian cells as latent inactive proenzymes (zymogens), and can be proteolytically processed to their active forms.

The 14 caspases known to date have been subdivided into three groups. Apoptosis related caspases are classified into initiator (Caspase-6,8,9,20) and executioner caspases

(Caspase-2,3,7), whereas a third group is implicated in the maturation of the cytokines IL-1β and IL-18 and does not have a significant role in apoptosis.

Initiator caspases can cleave executioner caspases, thereby triggering a caspase cascade, including a number of caspase-caspase interactions. The executioner caspases (i.e. caspase-2,3,7) cleave a variety of death substrates with their subsequent loss of function finally resulting in the morphological and biochemical characteristics of an apoptotic cell. For example, structural proteins such as lamins or fodrin, are inactivated. Limited proteolysis can also result in a gain of biological activity as demonstrated by fragmentation of nuclear DNA by activation of a DNase (Hengartner, 2000).

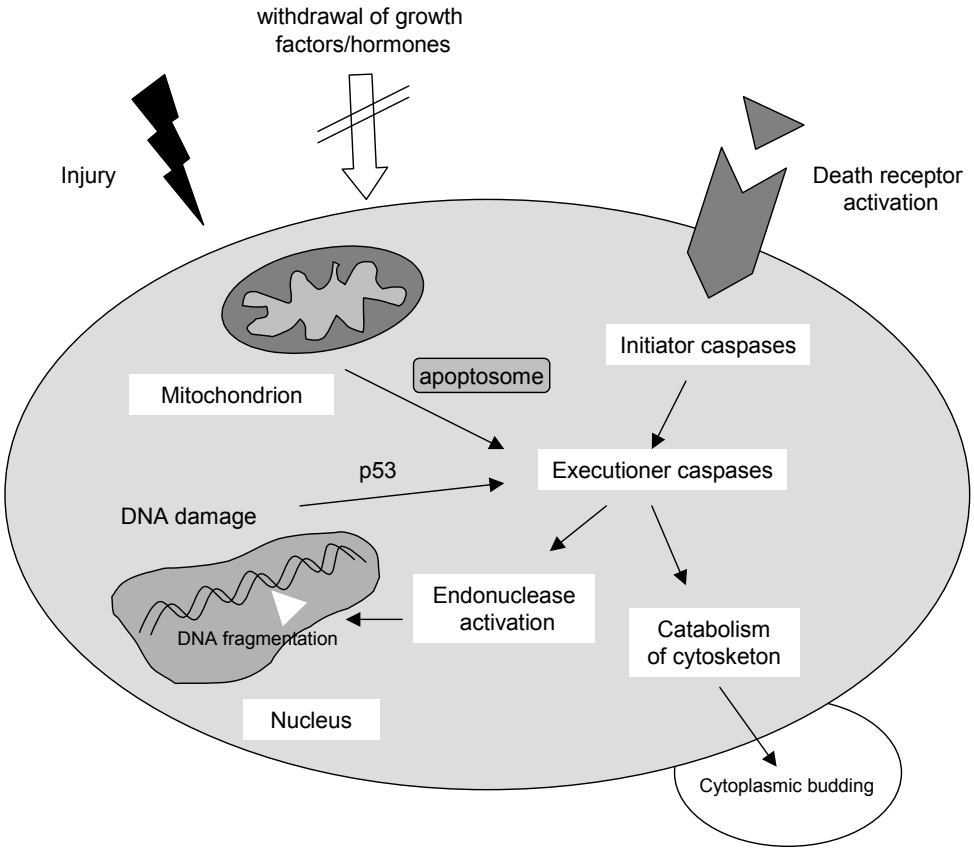

figure 3: Schematic representation of principle apoptotic events (for details see text).

4. Therapeutic strategies

At presence, only about two thirds of organs offered for transplantation are accepted and marginal organs, such as fatty livers or livers from donors with prolonged intensive care or prolonged ischemia time, are rejected (Lemasters *et al.*, 1995). These organs are more vulnerable to IRI, and graft as well as patient survival is diminished after use of such organs (Lemasters *et al.*, 1995; Trevisani *et al.*, 1996). Therefore, better protection against IRI and strategies to counteract the pathomechanisms are of great interest.

4.1 Established therapies

4.1.1 Storage and rinse solutions

Presently, liver preservation is performed in the University of Wisconsin (UW)-solution. This preservation solution has superior properties against the older Euro-Collins solution and is also applied for conservation of other organs, such as heart, kidney, and lung (Southard *et al.*, 1995). Among its complex composition, the ingredients lactobionate and glutathione were identified as protective agents during liver preservation. Lactobionate has strong osmotic properties and was shown to reduce hypothermic cell swelling (Southard *et al.*, 1995), whereas glutathione seems to be beneficial because of its antioxidant properties.

To prevent reperfusion injury, flushing of the graft with Carolina rinse solution containing antioxidants before transplantation proved to be superior to Ringer's lactate and albumin solution. Kupffer cell activation and neutrophil adherence were inhibited, and hepatic microcirculation and survival were finally improved (Post *et al.*, 1993; Gao *et al.*, 1991).

4.1.2 Prostaglandins

Prostaglandin E_1 (PGE_1) (Greig *et al.*, 1989) has been used clinically, for many years, to reduce primary graft non-function. It has been proposed that PGE_1 infusion during reperfusion of livers may improve hepatic blood flow *via* microvasodilatory properties (Richardson *et al.*, 1981), protection of the sinusoidal endothelial cells, or decreased platelet adhesion (Clavien *et al.*, 1992). Numerous studies have been performed in animal models, demonstrating beneficial effects in transplant or ischemic injury setting (Olthoff *et al.*, 1991). However, the clinical benefits of PGE_1 infusion following liver transplantation were not of advantage with respect on patient and graft survival and incidence of organ dysfunction (Olthoff, 2001). Despite the lack of clinical evidence, many programs continue

to use PGE_1 in recipients in whom there is a concern for poor graft function.

4.2 New therapeutic advances

4.2.1 Ischemic preconditioning

Ischemic preconditioning refers to a phenomenon in which tissues are rendered resistant to the deleterious effects of IRI by previous exposure to brief periods of vascular occlusion. The protective effects were first described in the myocardium (Murry *et al.*, 1986) and have also been demonstrated in the liver. In rats, ischemic preconditioning prior to harvesting of the liver for transplantation resulted in improved survival and decreased serum transaminases and TNF-α (Yin *et al.*, 1998). The protective effect is thought to result, at least in part, from the release of adenosine by the ischemic tissue (Peralta *et al.*, 1999). Moreover, attenuation of postischemic generation of reactive oxygen species (ROS) seems to mediate protection (Peralta *et al.*, 2002). First successful results in human liver resection are pointing to a potentially clinically relevant procedure (Clavien *et al.*, 2000).

4.2.2 Antioxidative strategies

Generation of ROS is a central event in the reperfusion period (Jaeschke, 1996). Kupffer cell and neutrophil activation and activation of xanthine oxidase are the major sources of these detrimental mediators. Extracellular fluids, such as blood plasma, contain little antioxidant capacity as compared to hepatocytes (Halliwell *et al.*, 1990). Therefore, administration of antioxidants during the early phase of reperfusion was suggested to have beneficial effects. In fact, various antioxidants demonstrated their protective potential: glutathione (GSH) (Bilzer *et al.*, 1999b), superoxide dismutase (Mizoe *et al.*, 1997), allopurinol (Kusumoto *et al.*, 1995), N-acetylcysteine (Koeppel *et al.*, 1996), and α-tocopherol (Marubayashi *et al.*, 1986) have all been shown to attenuate hepatic IRI.

4.2.3 Hormonal preconditioning with the atrial natriuretic peptide

The basis of this thesis is the protection of rat livers against IRI by the atrial natriuretic peptide (ANP) which was demonstrated in warm and cold ischemic and reperfused rat livers (Bilzer *et al.*, 1994; Gerbes *et al.*, 1998). Pretreatment of livers with ANP revealed decreased cell damage as assessed by less release of lactate dehydrogenase. Liver function was improved as could be seen by increased bile production. All ANP effects were mimicked by 8-Br-cGMP, an analog of ANP's second messenger cGMP, which

indicates guanylyl cyclase-A receptor/cGMP mediated signaling of cytoprotection. Mechanistic investigations revealed that ANP could mediate 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 *et al.*, 2000b). In addition, an influence of ANP on Kupffer cell (KC) mediated injury could be demonstrated by (Bilzer *et al.*, 1999a): ANP was able to protect liver cells against oxidative stress of activated Kupffer cells without influencing superoxide formation of KC.

In addition, ANP has recently been shown to activate the heat shock transcription factor (HSF) and the heat shock protein 70 (HSP70) (Kiemer *et al.*, 2002b), both mediators of the heat shock response (Jäättelä, 1999). HSP70 is not only discussed to confer thermotolerance, but also to protect against ROS toxicity and IRI by renaturation or refolding of partially denatured or unfolded proteins (Becker *et al.*, 1994) and to prevent apoptotic cell death (Beere, 2001; Ikeyama *et al.*, 2001). Induction of HSP70 in the liver was suggested to contribute to attenuation of IRI (Terajima *et al.*, 2000; Kiemer *et al.*, 2002b).

Thus, ANP appears to protect liver cells by either directly inhibiting mediators of cell damage or to improve resistance of cells to cytotoxic products. Therefore, pretreatment of livers with ANP subsequently subjected to ischemia and reperfusion may be a new promising therapeutic intervention to protect livers against IRI.

5. The Atrial Natriuretic Peptide

The atrial natriuretic peptide (ANP) was first described by (de Bold *et al.*, 1981), and investigated for its actions on volume homeostasis by shutting off volume retentive stimuli in the kidney. In addition to its vasodilating properties, this peptide shows effects on other biological functions, e.g. of the immune system. In this context, ANP was suggested to possess anti-inflammatory potential in particular on macrophage function (Kiemer *et al.*, 1998; Kiemer *et al.*, 2000a; Kiemer *et al.*, 2002c) and to protect endothelial cells from TNF-α induced inflammation (Kiemer *et al.*, 2002d). Concerning cytoprotective action, ANP was shown to preserve kidney function after renal ischemia and reperfusion (Nakamoto *et al.*, 1987; Shaw *et al.*, 1987).

5.1 Natriuretic peptides

The natriuretic peptides (NP) are cyclic molecules that are derived from different precursor molecules encoded by separate genes. They all exhibit structural, if not functional, homology (Kone, 2001).

The first member of the natriuretic peptide family was discovered in 1981 when de Bold and coworkers injected atrial extracts into rats and detected increased diuresis, natriuresis, and hypotension (de Bold *et al.*, 1981). The responsible compound was identified as a peptide and the amino acid sequence and the corresponding cDNA were subsequently elucidated (de Bold *et al.*, 1983; Flynn *et al.*, 1983).

After ANP, further members of natriuretic peptides were identified: brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and the recently identified dendroaspis natriuretic peptide (DNP). Other related peptides include guanylin and uroguanylin (reviewed in (Levin *et al.*, 1998; Venugopal, 2001))

All NPs exhibit a cyclic structure of 17 amino acids that is formed by a disulfide bond between two cysteine residues (Currie *et al.*, 1984). This represents the prerequisite for the biological activity of the peptides (Misono *et al.*, 1984). Eleven of the cyclic amino acids are homologous in each NP, whereas the number of amino acids differ at the C- and N-terminal end. The amino acid sequence of ANP consists of 28 amino acids and is identical in all mammals except of one variation at position 110 (Rosenzweig *et al.*, 1991).

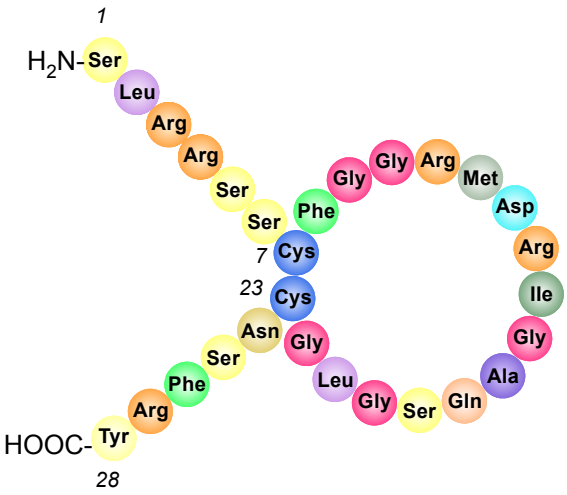

figure 4: Structure and amino acid sequence of ANP.

The biosynthesis of ANP is characteristical for peptide hormones. The ANP gene is transcribed into mRNA that codes for a 152 amino acid peptide, the so called pre-pro-ANP. By separation of a signal peptide at the N-terminus, pro-ANP is released and accumulated in granula (Rosenzweig *et al.*, 1991). Upon stimulation, such as stretching of the atria, pro-ANP is cleaved into the amino terminal fragment ANP 1-99 and the biologically active hormone ANP 99-126. Secretion of ANP from atrial myocytes into the circulation is performed by exocytosis (Nemer *et al.*, 1984).

5.2 Receptors and signal transduction

The natriuretic peptides exert their effects through interaction with high-affinity receptors on the surface of target cells. Three natriuretic peptide receptors (NPR) have been identified (reviewed in (Venugopal, 2001; Levin *et al.*, 1998)):

NPR-A and NPR-B are linked to the cGMP-dependent signaling cascade and mediate most of the effects of natriuretic peptides. The NPR-A receptor binds both ANP and BNP with preference for ANP. CNP is the natural ligand for the NPR-B receptor. Both receptors contain a kinase-like domain in their intracellular portion, followed by a guanylyl cyclase catalytic domain. Binding of the NP to their receptors activates guanylyl cyclase, leading to an elevation in intracellular cyclic guanosine monophosphate (cGMP).

The NPR-C receptor is structurally different from the other receptors and does not contain an intracellular guanylyl cyclase catalytic domain. It is involved in clearance of the peptides. All the NP are able to bind to it with equal affinity and are subsequently internalized and enzymatically degraded. Besides its regulatory function, this receptor is

discussed to mediate inhibition of adenylyl cyclase activity and activation of phospholipase C activity (Palaparti *et al.*, 2000).

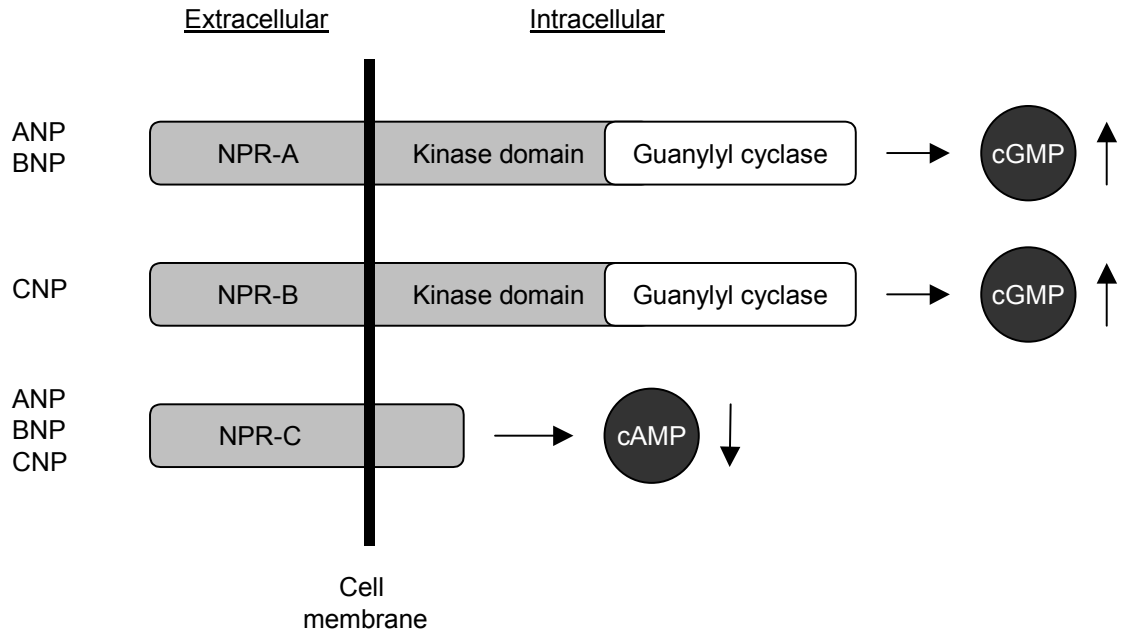

figure 5: Natriuretic peptide receptors (NPR). After extracellular binding at NPR-A and –B receptor, intracellular cGMP is elevated by a guanylyl cyclase domain. NPR-A binds ANP and BNP, whereas NPR-B binds CNP. NPR-C clears NPs and exerts other biological effects.

The receptors are widely distributed in mammalian tissue with predominance in the cardiovascular system, adrenal glands and kidney (Silberbach *et al.*, 2001). Additionally, all three receptors have been identified in the liver (Vollmar *et al.*, 1997).

5.3 cGMP-dependent signaling

After receptor binding and intracellular formation of cGMP by guanylyl cyclases, cGMP exerts its regulatory functions by interacting with various cGMP receptor proteins (figure 6):

1. In various cell types, the effects of cGMP are mediated by cGMP-dependent protein kinases (PKG) (Lohmann *et al.*, 1997; Pfeifer *et al.*, 1999).
2. In higher concentrations, cGMP is able to cross activate cAMP-dependent protein kinases (PKA) (Cornwell *et al.*, 1994).
3. A more common pathway by which cGMP utilizes the cAMP pathway is initiated by binding of cGMP to specific classes of phosphodiesterases (PDE), the enzymes responsible for the breakdown of cAMP and cGMP. Both a cGMP-stimulatable PDE (type III) and a cGMP-inhibitable PDE (type II) are present in

various cells, where they can cause a decrease or an increase of cAMP levels in response to cGMP (Lincoln *et al.*, 1993).

4. In some tissues cGMP is known to regulate ion channels by direct allosteric interaction. A cGMP-gated channel was detected in heart and also in kidney, where it may contribute to the cGMP mediated natriuresis (Biel *et al.*, 1998).

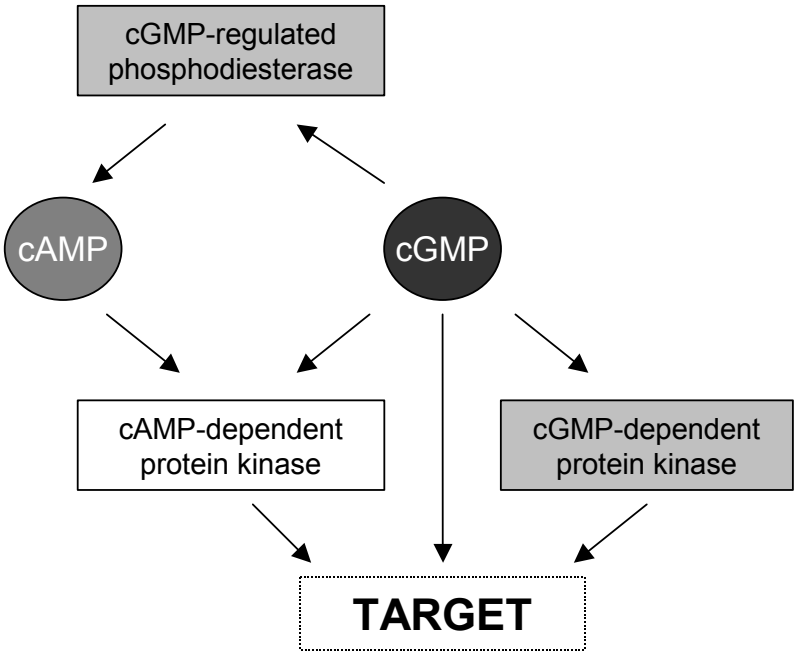

figure 6: Potential pathways by which cGMP may exert its physiological effects (for details see text).

5.4 Cyclic nucleotide dependent protein kinases

In this thesis, the contribution of PKG and PKA in ANP mediated hepatoprotection was investigated. Both enzymes are discussed to have beneficial effects in hypoxia related cell injury:

The calcium lowering potential of ANP activated PKG with subsequent vasodilation was proposed to protect ischemic kidneys (Shaw *et al.*, 1987; Nakamoto *et al.*, 1987) and hypoxic hepatocytes (Pella, 1991; von Ruecker *et al.*, 1989).

Cyclic adenosine monophosphate activated PKA and prevented livers from IRI in a model of cold ischemic and reperfused rat livers (Akbar *et al.*, 2001) and showed protective properties against apoptotic cell death in isolated cells (Li *et al.*, 2000).

cGMP-dependent protein kinases (PKGs) as well as the cAMP-dependent protein kinases (PKAs) belong to the large superfamily of protein kinases (Francis *et al.*, 1994; Francis *et al.*, 1999). These enzymes regulate the activity of numerous proteins by catalyzing the

transfer of the γ-phosphoryl group of ATP to the hydroxyl group of serine or threonine residues of an acceptor substrate protein.

5.4.1 cGMP-dependent protein kinases

Two isoenzymes of cGMP-dependent protein kinases (PKG I and II) have been identified in mammals (reviewed in (Pfeifer *et al.*, 1999; Vaandrager *et al.*, 1996; Francis *et al.*, 1994; Francis *et al.*, 1999)). Both isotypes have been cloned, and were shown to exhibit a sequence homology of more than 50%, and a similar structural organization. PKG I is predominantly a cytosolic protein, whereas PKG II is tightly bound to the plasma membrane. They represent homodimers consisting of monomeric subunits with a molecular mass of 77 kDa (PKG I) and 87 kDa (PKG II), respectively. These kinases are composed of a regulatory and a catalytic domain. Alternative splicing of the amino terminus results in two isofoms of PKG I, i.e. PKG Iα and PKG Iβ, which differ both in their length as well as in their affinity to cGMP. Their mode of action is as follows: cGMP binding inside the regulatory domain results in a conformational change. The substrate interacts with the substrate binding domain inside the catalytic domain and a phosphate residue of ATP is transferred onto serine or threonine of the substrate.

The tissue distribution of PKG I is highest in Purkinje cells of the cerebellum (Lohmann *et al.*, 1981), platelets (Waldmann *et al.*, 1986), and smooth muscle cells (Keilbach *et al.*, 1992). Likewise, cGK I is expressed in the kidney (Joyce *et al.*, 1986), in endothelial cells of blood vessels (Draijer *et al.*, 1995), neutrophil granulocytes (Lincoln *et al.*, 1993), and macrophages (Pryzwansky *et al.*, 1995). Among other sites, PKG II is highly expressed in intestinal epithelial cells (Markert *et al.*, 1995), in brain (el Husseini *et al.*, 1995) and kidney (Gambaryan *et al.*, 1996), pointing to a regulatory function of ion transport in these organs.

Interestingly, there is no unequivocal information available of PKG expression in the liver.

The physiological role of PKG activation comprises modulation of secretory processes in kidney and intestine. In the latter, the activation of PKG II by Escherichia coli enterotoxin stimulates chloride and water secretion leads to the clinical symptoms of diarrhea (Vaandrager *et al.*, 1997). In the kidney, PKG II is able to inhibit secretion and expression of renin, thereby affecting regulation of blood pressure, pointing to an ANP mediated effect (Wagner *et al.*, 1998). Additionally, the effect of ANP to increase glomerular filtration rate could be accounted for by the localization of PKG I in mesangial cells (Singhal *et al.*, 1989) and smooth muscle cells of the vasculature (Joyce *et al.*, 1986). Vasorelaxation by

decreasing intracellular Ca^{2+} is also involved in regulation of vascular tonus and blood pressure. Platelets express high amounts of PKG I and are regulated by nitric oxide (NO) *via* intracellular formation of cGMP (Moro *et al.*, 1996). Adhesion, activation and aggregation are inhibited by this pathway (Radomski *et al.*, 1987). In bones, PKG II was shown to have important influence in longitudinal bone growth (Yasoda *et al.*, 1998). Although identified in a lot of tissues, the functions of PKGs are still poorly understood and need further investigation.

5.4.2 cAMP-dependent protein kinases

cAMP-dependent protein kinase (PKA) is present in all mammalian tissues investigated, including the liver (Kosmopoulou *et al.*, 1994), and, in general, in higher concentrations than PKGs (Francis *et al.*, 1999). In contrast to PKG, the regulatory (R) and catalytic (C) domain of PKA is localized on different polypeptide chains. In its inactive form, PKA is available as a tetramer, consisting of two regulatory and two catalytic domains. Binding of cAMP leads to the dissociation of the tetramer and to the release of two active catalytic subunits that are responsible for transfer of the γ-phosphoryl groups (Shoji *et al.*, 1983; Takio *et al.*, 1984; Titani *et al.*, 1984).

Two major R subunit isoforms (R I and R II with 43 and 45 kDa), also referred to as PKA I and PKA II, have been identified as products of different genes and within these isoforms there are still further distinctions. Three isoforms of the C subunit (40 kDa) are known that form with the R subunits the complete enzyme. The PKA I complex is rather cytosolic compared with PKA II complex, which is almost exclusively particulate associated. Expression of R I and R II varies with the species and tissue distribution. R Iα is expressed in many tissues, and R IIβ is more selectively expressed (Francis *et al.*, 1999). The physiological importance of these variations is unclear (Doskeland *et al.*, 1993).

PKA in general is involved in an array of metabolic processes including gluconeogenesis, glycogenolysis and lipogenesis, as well as secretory processes, muscle contraction, learning, ion channel conductance, pro inflammatory cytokine production and action, differentiation, growth control, and apoptosis, for example (Houslay *et al.*, 1997).

6. The heme oxygenase system

The heme oxygenase-1 isozyme of the heme oxygenase enzyme family was investigated because of its emerging role in oxidant related cell injury (Immenschuh *et al.*, 2000).

6.1 Biological functions

The 32 kDa heme oxygenase (HO) is the enzyme responsible for the physiological heme degradation (Tenhunen *et al.*, 1968). In combination with the rate limiting enzyme of heme biosynthesis, δ-aminolevulinate synthase, HO regulates the cellular content of the pro-oxidant heme and produces catabolites with physiological functions (Maines, 1997). It decomposes protoheme IX that is released mainly from hemoglobin of senescent erythrocytes, by cleaving its α-methene bridge to generate biliverdin-IXα, divalent iron, and carbon monoxide (CO). In most mammalian species, biliverdin is subsequently reduced to bilirubin by biliverdin reductase (figure 7).

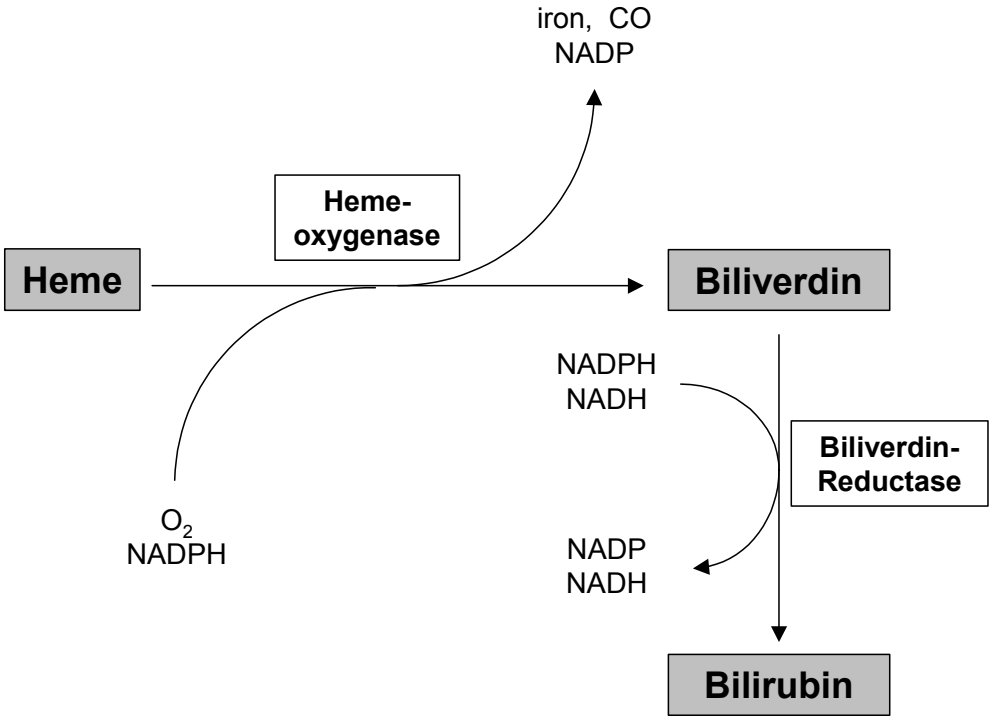

figure 7: The heme oxygenase enzyme reaction. Scheme of catalytic conversion of heme into bilirubin, carbon monoxide (CO), and iron.

Three genetically distinct isoforms are known in mammals (HO-1, -2, -3). HO-1 is widely distributed in tissues with highest occurrence in spleen and liver. It is highly inducible by various stimuli including, heavy metals, ROS, its substrate, protoheme IX, and heat, which lead to the term heat shock protein 32 (HSP-32). In the liver, HO-1 has been shown to be

constitutively expressed in Kupffer cells (Makino *et al.*, 2001). Induction has been reported predominantly for hepatocytes (Paxian *et al.*, 2001; Terajima *et al.*, 2000; Rizzardini *et al.*, 1998). HO-2 is constitutively active but not inducible. It is present in highest concentrations in the brain and testes of mammals. HO-3 is nearly devoid of catalytic capability and may function as a heme sensing or a heme binding protein (McCoubrey, Jr. *et al.*, 1997).

6.2 Heme oxygenase-1 in oxidant stress

Growing evidence supports an important role for HO-1 in protecting cells from oxidative stress (Choi *et al.*, 1996; Immenschuh *et al.*, 2000). The protection afforded by HO-1 seems to be due to a reduction of heme concentrations and even more importantly to the production of biliverdin and bilirubin, both potent antioxidants (Stocker *et al.*, 1987; Clark *et al.*, 2000). The generated iron, although itself a potential prooxidative mediator *via* production of ROS by Fenton chemistry, stimulates biosynthesis of ferritin, a regulator of intracellular iron state. Its iron sequestering properties were shown to prevent cells from iron mediated cytotoxicity (Vile *et al.*, 1993; Vile *et al.*, 1994). In addition, it has been demonstrated that HO-1 induction protects cells by augmented cellular iron efflux (Ferris *et al.*, 1999). The third reaction product, the signaling gas carbon monoxide (CO), has also been shown to be physiologically relevant. By formation of cGMP it is involved in the maintenance of vascular blood flow. In the liver, it has been demonstrated to protect the hepatic microcirculation under stress conditions (Suematsu *et al.*, 2000) and, moreover, to have vasodilatant properties in the cardiovascular system (Sammut *et al.*, 1998).

6.3 Heme oxygenase-1: significance in IRI

There is increasing evidence that induction of HO-1 in IRI of the liver exerts protective properties on organ function. Upregulation of HO-1 protected genetically fat rat livers from IRI. The organs showed significantly improved liver function, decreased hepatocyte injury, and extended animal survival in transplantation experiments (Amersi *et al.*, 1999). Additionally, HO-1 expression could be induced by preconditioning of livers with the chemotherapeutic doxorubicin, a compound known to generate free radicals. The preconditioned livers were subsequently more resistant to IRI than untreated rat livers, which was attributed to HO-1 induction (Ito *et al.*, 2000). Finally, hyperthermic preconditioning, i.e. sublethal heat exposure before a surgical intervention, rendered livers less susceptible to warm ischemia and was accompanied by increased HO-1 protein expression (Terajima *et al.*, 2000).

7. The isolated perfused rat liver: general aspects

For the evaluation of hepatic ischemia reperfusion (I/R) injury several experimental models have been established. Among them are cell culture models of parenchymal and non-parenchymal liver cells, *in vitro* liver perfusion models, and *in vivo* models of warm and cold ischemia and reperfusion (Clavien *et al.*, 1992) (Post *et al.*, 1996).

Cell culture experiments with isolated liver cells are suitable, for example, to investigate changes of ion homeostasis during ischemia-reperfusion (Brecht *et al.*, 1994). Microcirculatory disturbances, a major determinant of reperfusion injury, in contrast, can not be evaluated with cultured cells. Additionally, the missing interaction of liver cell populations is another major drawback of this method.

To study the pathomechanisms of hepatic reperfusion injury, mainly *in vivo* models of I/R were applied. The participation of microcirculatory disturbances (Menger, 1995) and Kupffer cell activation (Jaeschke, 1996) were clarified on the basis of these models. Furthermore, pharmacological strategies to protect from I/R injury are based on transplantation models. The disadvantage of such complex models is the difficulty to distinguish between hepatic and extrahepatic mechanisms affected by a specific intervention. Simplification of the experimental setting can thereby be beneficial.

The *ex vivo* model of the isolated perfused rat liver which is used in this investigation has to be seen in between the artificial cell culture system and the *in vivo* situation. Substantial information about I/R injury has been gathered from this technique. Extrahepatic factors of cell damage are missing and the resulting damage is solely caused by hepatic factors. A main advantage is the maintenance of liver architecture, rendering it a feasible model for the study of interactions between parenchymal and non-parenchymal cells (Jaeschke *et al.*, 1990). The isolated perfused rat liver is a well established model for studies on hepatic metabolism. Steady state conditions allow the calculation of metabolic flux rates. As the polarity of the cells is maintained, sinusoidal uptake, metabolism, and biliary excretion of substances can be studied (Vom Dahl *et al.*, 1997). In addition, substances can be investigated on their hepatoprotective or toxic properties. Major insight into the pathomechanisms of IRI in the liver were gathered from this model (Lemasters *et al.*, 1995), rendering it useful to investigate the mechanisms of ANP cytoprotection in IRI. Due to the application of blood-free perfusion buffers, however, the experiments can only be performed for about 3 to 5 h. This restricts the investigation of cell damage to the ischemic period and the early reperfusion phase.

B. Materials and Methods

1. The isolated perfused rat liver

1.1 Animals

For isolated liver perfusion and primary hepatocyte isolation male Sprague-Dawley rats weighing 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 received humane care and had free access to chow (Ssniff, Soest, Germany) and water up to the time of experiments. The study was registered with the local animal welfare committee.

1.2 Solutions

Krebs-Henseleit buffer (KH-buffer)

NaCl	118 mM
KCl	4.8 mM
KH_2PO_4	1.2 mM
$MgSO_4$ x 7 H_2O	1.2 mM
$NaHCO_3$	25 mM
$CaCl_2$	1.5 mM
pH 7.4	

1.3 Model of the isolated perfused rat liver

After anaesthetizing male Sprague-Dawley rats with Narcoren® (Merial, Halbergmoos, Germany, 50 mg/kg body weight, intraperitoneally), 250 IU heparin were administered *via* the vena femoralis to prevent clotting. At sustained blood circulation, the portal vein was cannulated with a 16 gauge polyethylene tube (Insyte-W, BD, Heidelberg, Germany) and the liver was perfused *in situ* with Krebs-Henseleit (KH) solution. The perfusion medium was pumped instantly through the liver with a membrane pump (Prominent® beta/4, Heidelberg, Germany) in a non-recirculating fashion (32 ml/min). For sufficient drain of perfusate the aorta abdominalis was incised and thorakotomie was performed to cannulate the vena cava inferior *via* the right atrium. To achieve complete perfusion of the organ, the vena cava inferior was ligated above the kidney vein and below the liver vein. Thereby, the venous liver perfusate was exclusively drained *via* the canula in the vena cava inferior. The liver was completely isolated from the cadaver to allow quick procedure for ischemic storage. For collection of bile fluid a polyethylene tube (length: 5 cm, inner diameter: 0.4 mm, total inner volume: 6 µl, Sims Portex Ltd., Hyte, UK) was placed in the

ductus choledochus.

The perfusate flow rate was kept at 3.0 – 3.5 ml x min^{-1} x g $liver^{-1}$. A hemoglobin and albumin free, bicarbonate buffered Krebs-Henseleit (KH) solution (pH 7.4) served as perfusion buffer, which was oxygenated by a tube oxygenator with carbogen gas (95% O_2 and 5% CO_2, Linde, Unterschleißheim, Germany) and kept at a temperature of 37°C (Sies, 1978). The tube oxygenator consists of an aluminum block, winded with a silicon tube and placed in a plexiglas chamber. Substances were infused via infusion pumps (Braun Melsungen AG, Melsungen, Germany). Bubbles were removed from the perfusion buffer with a bubble trap (volume 10 ml). Bile and perfusate samples were collected during perfusion and kept on ice until analysis. Portal pressure rates before the liver were monitored on a hydrostatic column.

A schematic illustration of the perfusion apparatus is shown in figure 8.

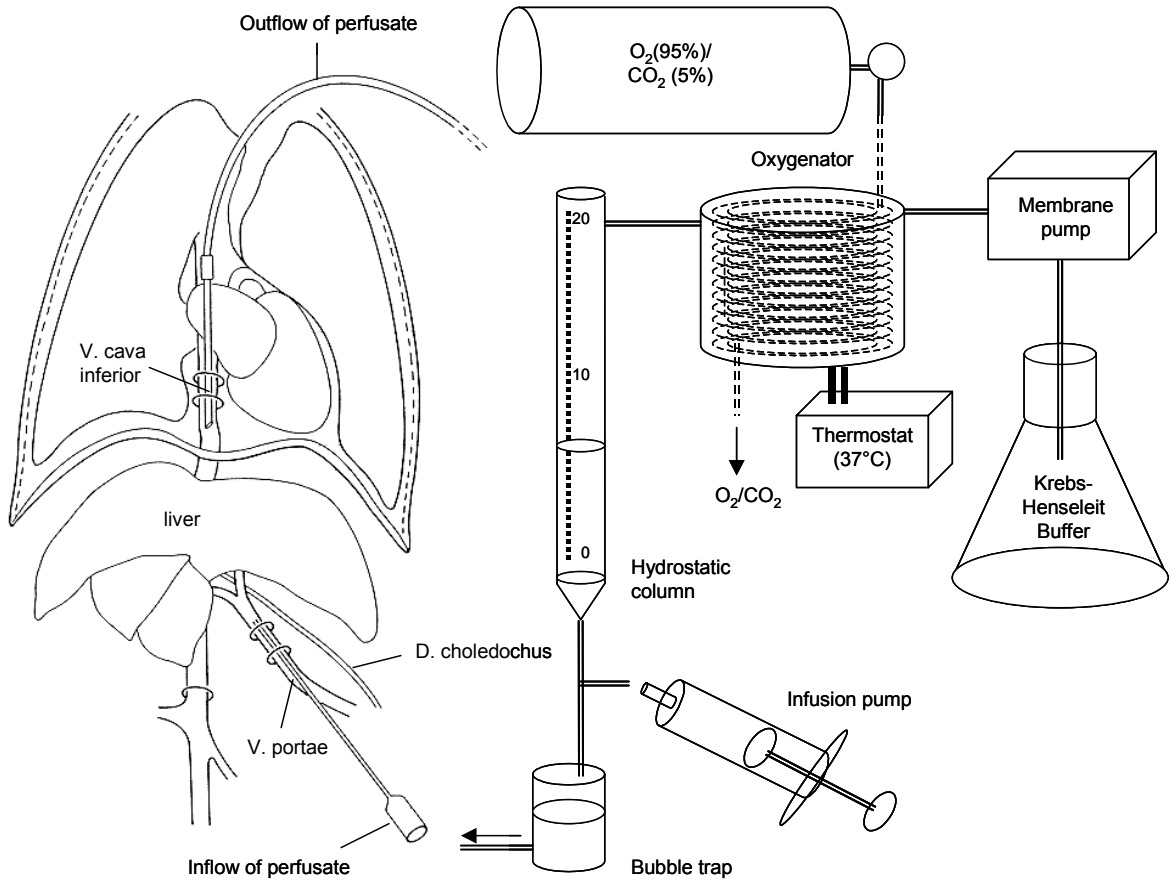

figure 8: The isolated perfused rat liver: schematic illustration.

1.4 Experimental setting

1.4.1 Continous liver perfusion

The perfusion model was validated according to Bilzer et al. (Bilzer, 1997). Livers were continuously perfused for up to 150 min. Lactate dehydrogenase activity was analysed to proof hepatocellular integrity (see 1.5.1). Bile production as indicator of liver function only slowly decreased and portal pressure remained constant during the perfusion period (see 1.5.2 and 1.5.3). Oxygen saturation of the perfusate was checked with a blood gas analyser (Radiometer ABL505, Copenhagen, Denmark). The results demonstrated a successful surgery and a functioning perfusion apparatus (data not shown).

1.4.2 Model of cold ischemia and reperfusion

After 30 min of perfusion with KH buffer, livers were perfused with 30 ml of cold (4°C) University of Wisconsin (UW) solution (Viaspan®, DuPont Pharma GmbH, Bad Homburg, Germany) for 1 min. The organs were then kept in 100 ml UW solution at 4°C for 24 h. Following the period of ischemia, livers were reperfused with KH buffer for 2 h at a flow rate of 3.0 – 3.5 ml x min^{-1} x g $liver^{-1}$. At the indicated times, i.e. before ischemia, at the end of ischemia and after 45 and 120 min of reperfusion livers were snap-frozen in liquid nitrogen and stored at –80°C (Herafreeze, Heraeus, Hanau, Germany) until further analysis. Unless stated otherwise, 4–5 independent experiments were performed.

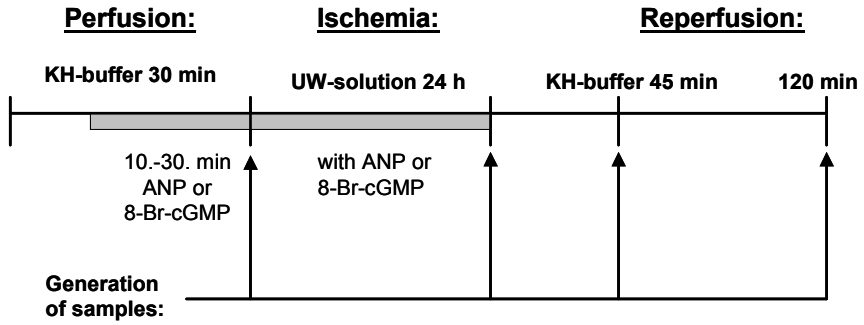

figure 9: Experimental setting.

1.4.3 Treatment protocols

For treatment of livers, substances were dissolved in the respective dissolution medium and filled into Perfusor® syringes (Braun Melsungen AG, Melsungen, Germany). They were placed into Perfusor® maschines and flow rate was set according to the desired end concentration in the perfusate.

Treatment was performed by infusing ANP and 8-Br-cGMP to the pre-ischemic perfusate for 20 min until ischemia and to the storage solution at the same concentration. Enzyme inhibitors, depending on their mode of action, were partially infused for longer times (table 1.1).

Substances	Vendor	End-concentration	Dissolution medium	Infusion time
ratANP 99-126	Calbiochem Mw 3062.5	200 nM	0.9% NaCl solution	10.-30. min and cold ischemia
8-Br-cGMP	Sigma Mw 446.1	50 µM	H_2O	10.-30. min and cold ischemia
ZnPP	Sigma Mw 446.2	1 µM	DMSO	10.-30. min and cold ischemia
Rp-8-Br-cAMPS	Biolog Mw 626	1 µM	H_2O	0.-30. min and cold ischemia
Rp-8-Br-pCPT-cGMPS	Biolog Mw 525.9	1 µM	H_2O	0.-30. min and cold ischemia

table 1.1: Substances for liver treatment

Control experiments for the ZnPP perfusion set were performed by infusion of the same DMSO concentration into the perfusate (0.3% (v/v)).

1.5 Parameters of liver function

1.5.1 Lactate dehydrogenase efflux

Lactate dehydrogenase (LDH) efflux into the perfusate is a sensitive indicator of cell damage in the isolated perfused rat liver (Sies, 1978). The activity measurement is based on the conversion of pyruvate to lactate by LDH. Reduction equivalents in form of NADH are thereby oxidized to NAD^+ (Bergmeyer HU, 1974) resulting in a decrease in NADH extinction.

Test preparation

K_2HPO_4 (50 mM)/KH_2PO_4 (50 mM), pH 7.5	500 µl
Pyruvate (60 mM)	10 µl
NADH (10 mg/ml)	10µl
Liver perfusate	500 µl

The extinction was continuously monitored by a plotter. Enzyme activity was calculated based on the decrease in NADH extinction (ε_{365}=3.34 $mM^{-1}cm^{-1}$).

1.5.2 Bile efflux

Bile was collected during the experiment and volumetrically quantified with a 100 µl

Hamilton™ microliter syringe (Bonaduz, Switzerland). The bile flow was calculated per min and g liver tissue.

1.5.3 Portal pressure

The portal pressure as parameter for hepatic circulation disturbances was monitored *via* a calibrated glass tube, connected to the portal catheter *via* the bubble trap. At the end of perfusion the liver was removed and the measured pressure was subtracted from the portal pressure during perfusion.

2. Liver cell culture

2.1 Animals

Animals were purchased and housed as described under 1.1.

2.2 Solutions

All solutions were prepared with double destilled H_2O and were subsequently autoclaved or sterile filtrated.

PBS (phosphate buffered saline)

Na_2HPO_4	1.48 g
KH_2PO_4	0.43 g
NaCl	7.20 g
H_2O	ad 1000 ml
Adjusting pH to 7.4	

Solution A

NaCl	115 mM
$NaHCO_3$	25 mM
KCl	5.9 mM
$MgCl_2$ x 6 H_2O	1.18 mM
NaH_2PO_4	1.23 mM
Na_2SO_4	1.2 mM
HEPES	20 mM
EGTA	0.5 mM

Solution B

NaCl	115 mM
$NaHCO_3$	25 mM
KCl	5.9 mM
$MgCl_2$ x 6 H_2O	1.18 mM
NaH_2PO_4	1.23 mM
Na_2SO_4	1.2 mM

$CaCl_2$ x 2 H_2O	2.5 mM
HEPES	20 mM
EGTA	0.5 mM
Collagenase H	0.05%
in sterile H_2O, adjusting to pH 7.35	

Percoll™ gradient

NaCl	136 mM
Na_2HPO_4 x 2 H_2O	10.2 mM
KCl	2.68 mM
KH_2PO_4	1.46 mM
$MgCl_2$ x 6 H_2O	0.5 mM
Perkoll™ solution	50% (v/v)

Hanks' balanced salt solution (HBSS)

$CaCl_2$ x 2 H_2O	0.95 mM
KCl	5.3 mM
KH_2PO_4	0.44 mM
$MgCl_2$ x 6 H_2O	0.49 mM
$MgSO_4$ x 7 H_2O	0.41 mM
NaCl	136.75 mM
Na_2HPO_4 x 2 H_2O	0.34 mM
HEPES	20 mM
Adjusting pH to 7.35	

Medium199 (Pan Biotech, Aidenbach, Germany) supplemented with:

Glucose	8.3 mM
Glutamin	2 mM
Dexamethason	2.5 µg/ml
Penicillin G	100 U/ml
Streptomycin	100 µg/ml
BSA	50 µg/ml
Insulin	125 U/l
HEPES	10 mM

Addition of 5% (v/v) foetal calf serum (FCS) for 4 h precultivation

Trypan blue solution

Trypan blue	0.08 g
NaCl	0.03 g
H_2O	ad 20 ml

2.3 Cultivation

Cultivation was performed in a cell incubator (Heracell, Heraeus, Hanau, Germany) at 37°C, 90% air humidity and 5% CO_2.

2.4 Isolation of primary hepatocytes

Isolation of primary rat hepatocytes was performed using the modified two step collagenase digestion method (Seglen, 1973) (Immenschuh *et al.*, 1998) (de Groot *et al.*, 1991). Collagenase resolves the extracellular matrix of the liver tissue and the following cell suspension is separated *via* a Percoll™ density gradient centrifugation. Hepatocytes have the highest density among liver cells and can therefore be separated at low centrifugation force.

Male Sprague-Dawley rats weighing 200 – 300 g were anaesthetized by intraperitoneal injection of Narcoren© (50 mg/kg body weight). All instruments were autoclaved or heat sterilized to reduce germ contamination. Additionally, the body was rinsed with ethanol 70% (v/v). 250 IU heparin were administered *via* the vena femoralis to avoid clotting. After opening the abdomen, the portal vein was cannulated with a 16 gauge polyethylene catheter (Insyte-W, BD, Heidelberg, Germany) and perfused with Ca^{2+} free solution A to resolve desmosomes by deprivation of Ca^{2+}. Perfusate flow was established by a roller pump (Watson Marlow 101U/R, Falmouth, UK) at 30 ml/min. The perfusate was oxygenated with carbogen (O_2 95%, CO_2 5%, Linde, Unterschleißheim, Germany) and was kept at 37°C. By incision of the aorta abdominalis, a sufficient drain for the perfusate was ensured. After thorakotomia, the vena cava inferior was cannulated *via* the right heart atrium. The vena cava inferior was ligated above the kidney vein and below the liver vein to prevent outflow of perfusate. After the liver was flushed blood free for 4 to 7 min with solution A, collagenase H (Roche Diagnostics, Mannheim, Germany) containing buffer B was perfused. The cannulas were connected *via* silicon tubes to obtain a recirculating system.

After further 6 to 8 min, the liver showed an increasing number of small rips, indicating the digestion of extracellular collagen matrix. The liver was carefully excised and rinsed with Ca^{2+}-containing medium B. The following working steps were performed in a laminar flow work bench (Herasafe, Heraeus, Hanau, Germany). The digested liver was placed into a Petri dish and solution B was added. Liver cells were isolated by teasing the liver capsule apart and carefully scratching out the cells with a spoon. The cell suspension was filtrated with a Falcon cell strainer (100 µM, BD Labware, Bedford, MA, USA) and sedimentation of cells was allowed for 10 min. The supernatant was removed and the cell suspension was stratified onto a Percoll™ 50% solution (Amersham Pharmacia Biotech, Upsala, Sweden) into a 50 ml centrifugation tube. After centrifugation at 650 x *g* for 10 min at 4°C the 3 layers in the supernatant were removed and the remaining pellet, consisting of

hepatocytes, was resuspended in solution B. Cell number and vitality was checked in a Neubauer counting chamber. Addition of trypan blue solution (500 µl trypan blue solution, 450 µl buffer B, 50 µl cell suspension) permitted counting of dead cells and calculation of cell vitality. After the isolation procedure, vitality was always larger than 80%. The hepatocytes were cultivated in collagen R coated (0.2 mg/ml, Serva, Heidelberg, Germany) 6-well cell culture plates (Peske, Aindling-Pichl, Germany) at a concentration of 10^6 cells/ml. The cells were seeded in 1.5 ml medium containing FCS. After 4 h, cells were made quiescent by changing to medium without FCS. To remove cell debris and detached cells, the plates were washed twice before each medium change with HBSS. Cells were used for experiments 48 h later, whereby medium was changed after 24 h and 26 h. After the indicated treatment time, the medium was removed, the plates were washed twice with PBS and stored at –80°C.

2.5 Isolation of Kupffer cells

Isolation of Kupffer cells was performed by Dr. A. K. Kiemer, Department of Pharmacy, University of Munich, Germany.

Rat Kupffer cells (KC) were isolated according to the method of Knook et al. (Knook *et al.*, 1976) with some modifications (Armbrust *et al.*, 1993). Briefly, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The liver was first perfused *in situ* after cannulation of the portal vein with 100 ml Gay´s balanced salt solution (GBSS), then digested by perfusion with pronase and pronase/collagenase-solutions. The obtained cells were suspended in a pronase/collagenase-solution, shaken carefully for 30 min and passed through a 100 µm sieve. The hepatocytes were separated by differential centrifugation and the remaining nonparenchymal cells were separated by a Nycodenz-gradient. The density gradient centrifugation was performed at 1500 x *g* for 15 min. The cells of the interphase were collected and separated according to size by counterflow elutriation using a Beckman-centrifuge (J 2-21, JE-6B rotor, Beckman Instruments, Munich, Germany). The obtained KC were sedimented, resuspended in culture medium (M 199, 15% FCS, 100 U penicillin/ml, 100 µg streptomycin/ml) and counted in a Fuchs-Rosenthal chamber after Trypan Blue-staining. Cells were then seeded at a density of 500 000 cells/well in 24-well or 2,25 x 10^6 cells/well in 6-well tissue plates and cultivated for 1 to 3 days. Two hours after plating, the cultures were washed to eliminate non-adherent cells. Cultures were kept in a 5% CO_2-atmosphere and saturated humidity at 37°C. KC purity was determined using a fluorescent isothiocyanate (FITC)-labeled antiserum against ED2 and fluorescence

microscopy and by measuring phagocytosis of coumarin-conjugated latex beads by FACS analysis (FACScan, Becton Dickenson, San Jose, CA, USA). Preparations of KC were found >90% pure as judged by flow cytometry.

3. Isolation of ribonucleic acids

3.1 Extraction of ribonucleic acids

total RNA (totRNA) was isolated using the guanidine thiocyanate/cesium chloride method according to (Chirgwin *et al.*, 1979).

The decomposition of RNA by the extraordinarily stable ribonucleases (RNAses) was prevented by addition of RNAse inhibiting substances (mercaptoethanol, guanidine thiocyanate). Moreover, solutions were prepared with double distilled and autoclaved water. All work was performed wearing plastic gloves. Glassware was sterilized for 3 h at 180°C and solutions and plastic ware were autoclaved for 20 min at 121°C and 2 bar.

Livers were homogenized in a guanidine thiocyanate solution and stacked onto a cesium chloride solution. By ultracentrifugation RNA is separated from DNA and proteins. Because of its higher density in comparison to other cell components RNA is collected as a pellet at the bottom of the centrifugation tube.

3.1.1 Solutions

Guanidine thiocyanate solution

Guanidine thiocyanate	47.24 g
Tris-HCl pH 7.5	ad 100 ml
Addition of 1% (v/v) mercaptoethanol shortly before use	

Tris/EDTA buffer (TE buffer)

Tris-HCl pH 7.5	10 mM
EDTA pH 8.0	1 mM

3.1.2 Guanidine thiocyanate/cesiumchloride-method

100 mg pieces of frozen livers were homogenized in guanidine thiocyanate solution with a Ultraturrax (Ika Labortechnik, Staufen, Germany) for 30 sec on highest stage. Besides homogenization, DNA is thereby sheared which results in a reduced viscosity of the solution. After addition of sodium lauroylsarcosine (final concentration 0.5%) the mixture was centrifuged for 5 min at 1,500 x *g*. The supernatant was stratified onto a 5.7 M cesium

chloride solution and centrifuged for 18 h at 40,000 x *g* in an ultracentrifuge (Sorvall Discovery 90, Hanau, Germany).

The supernatant was removed, the pellet was washed with ethanol 70% (v/v) and dried for 30 min at 45°C. The RNA was dissolved in TE buffer with SDS 0.1% (m/v) and precipitated by addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice cold ethanol 100% at –70°C. For further processing, the samples were centrifuged at 12,000 rpm (5 min, 4°C) and the resulting pellets were washed with ice cold ethanol 70% (v/v), dried, and dissolved in H_2O.

3.2 Measurement of nucleic acid concentration

The determination of nucleic acid concentration is based on the absorption maximum of nucleic acids at 260 nm. The absorption caused by the aromatic ring system, is measured at 260 nm (A_{260}) in quartz cuvettes of 1 cm of layer thickness. A solution containing 50 µg/ml double stranded DNA under these conditions has an absorption of 1. Single stranded RNA contains 40 µg/ml at an absorption of 1.

Aqueous RNA and DNA-solutions were determined in a Lambda Bio 20 photometer (PerkinElmer, Rodgau-Jügesheim, Germany). The purity of RNA was determined by measuring the A_{260}/A_{280} ratio. Pure RNA has an A_{260}/A_{280} ratio of 2.0. If RNA is contaminated with proteins, the ratio is significantly lowered (<1.8).

3.3 Gel electrophoretic separation of nucleic acids

Agarose electrophoresis is the standard method for separation and purification of DNA and RNA fragments. Because of their sugar phosphate backbone, nucleic acids are charged negatively and migrate towards the anode. The migration speed depends on their molecular weight and conformation. To resolve inter- and intra molecular hybridization and to linearize RNA, the denaturation of the molecules is necessary. This was performed by a denaturating glyoxal/DMSO gel electrophoresis, which leads to sharper bands in the following Northern blot analysis in comparison to formaldehyde gel (Thomas, 1980).

The bands of ribosomal eucaryotic RNA can be detected under UV light (254 nm) after staining with the intercalating fluorescent dye ethidium bromide (EtBr). The two bands corresponding to the 28S and 18S sedimentation coefficients can be used as a criterion for the integrity of RNA. The ideal ratio of 2:1 indicates a successful isolation procedure. Additionally, mRNA should be visible as a diffuse fluorescence in the low molecular weight area.

3.3.1 Solutions

Glyoxal-DMSO-phosphate (GDP)

Glyoxal 6 M	200 µl
DMSO	595 µl
NaH_2PO_4 0.1 M pH 7.0	117 µl
H_2O	300 µl

Loading buffer

Glycerol	50% (v/v)
NaH_2PO_4 pH 7.0	0.01 M
Bromphenol blue	0.4 % (m/v)

To control the migration progress of the electrophoresis bromphenol blue was added to the sample buffer. This dye has the property to migrate like fragments of 10-100 bp of size and marks the front of electrophoretic movement.

Ethidium bromide (EtBr) solution

NH_4-acetate 0.1 M	200 ml
NaOH 10 M	80 µl
EtBr 1 % (m/v)	80 µl

3.3.2 Agarose gel electrophoresis

A 1.2% (m/v) agarose gel (Seakem LE, Biozym, Hessisch-Oldendorf, Germany) was prepared with 0.01 M NaH_2PO_4 in a horizontal gel chamber (Easycast, Owl separation systems, Portsmouth, NH, USA). 20 µg of RNA were dried in a SpeedVac® Plus concentrator (Savant SC110A, Labsysteme Osvath, Geretsried, Germany) and denatured in 20 µl of GDP at 50°C for 1 h. To maintain the denaturation state the samples were kept on ice. After addition of 5 µl of loading buffer the total volume was pipetted into the gel slots and separated in 0.01 M NaH_2PO_4 pH 7.0 (80 V, 2.5 h, BioRad PowerPac 300). The formation of a H^+ gradient in the electrophoresis buffer has to be prevented since local pH values larger than 8.0 may lead to a dissociation of the glyoxal RNA binding. Therefore, the electrophoresis buffer was recirculated with a Millipore roller pump during electrophoresis.

3.3.3 Staining

The gel was stained in ethidium bromide solution for 30 min. Excessive staining solution was removed by washing the gel in H_2O for 15 min. Detection of intercalated dye was performed under UV light (254 nm) and imaged by a CCD camera system (Kodak image Station, Eastman Kodak Company, Rocester, New York, USA).

3.4 Separation of mRNA from totRNA

In contrast to ribosomal RNA (rRNA) mRNA possesses a sequence of numerous adenosine bases at its 3'-end (poly(A)$^+$-tail). This fact can be used for its isolation, which was performed with the PolyATract® mRNA Isolation system (Promega, Heidelberg, Germany): mRNA was coupled to biotinylated oligo-deoxythymidin primers (Oligo(dT). This complex was bound to paramagnetic streptavidin particles *via* the streptavidin-biotin interaction and separated from the total RNA by magnetic forces.

Total RNA at a concentration of 0.1-1.0 mg/500 µl H_2O was denatured at 65°C for 10 min. 150 pmol biotinylated Oligo(dT) primers and 20x SSC (see 4.5.1) were added and incubated at room temperature for 10 min to allow binding of the mRNA (polyA$^+$) to the oligo(dT) primers. After washing the paramagnetic streptavidin particles (SA-PMP) 3 times with 0.5x SSC, the SA-PMPs were resuspended in 0.5x SSC and added to the RNA/oligo(dT) mixture with subsequent incubation at room temperature for 10 min. The oligo(dT) adsorbed RNA is thereby bound to the SA-PMPs *via* biotin – streptavidin interaction. After four washing steps with 0.1x SSC the mRNA was obtained by resuspending the mRNA-biotin bound SA-PMPs in H_2O. The reduction of ionic strength resolves the mRNA from the biotin-SA-PMP complex, which is removed by a magnet. The resulting aqueous solution of RNA is finally centrifuged at 12,000 x g at 4°C for 5 min to eliminate all SA-PMPs. Quantification of the isolated mRNA was performed as described under 3.2.

4. Northern blot

HO-1 mRNA expression in rat livers was quantified by Northern blot analysis. totRNA was electrophoretically separated and transferred onto a nylon membrane. A radioactive cDNA probe complementary to the HO-1 mRNA sequence was hybridized to the expressed mRNA and was visualized by indirect autoradiography with intensifier screens (Phosphorimaging).

4.1 Bacteria

4.1.1 Strain

For plasmid amplification, the bacteria strain Escherichia coli DH5α™ (Gibco/Invitrogen, Karlsruhe, Germany) was used.

4.1.2 Growth media and antibiotics

Cultivation of bacteria was performed in LB-broth base (Lennox L Broth Base, Gibco/Invitrogen, Karlsruhe, Germany). After adjusting the pH-value to 7.5 with NaOH, the medium was autoclaved for 20 min at 1 bar and 121°C.

Medium

LB Broth Base	20 g
Glucose	1 g
H_2O	ad 1000 ml

For selection of successfully transformed bacteria containing plasmids with the β-lactamase gene, ampicillin-sodium solution (in 70% ethanol) was added to the medium in a final concentration of 100 µg/ml. Only transformed bacteria express β-lactamase, which hydrolyses the β-lactame circle of ampicillin and this allows them to grow in presence of high concentrations of antibiotics.

Selection of transformed bacteria was performed on ampicillin containing agar plates. 32 g LB agar (Lennox agar, Gibco/Invitrogen, Karlsruhe, Germany) and 1 g glucose were dissolved in 1 l of double distilled water, autoclaved and cooled to about 50°C. Ampicillin-sodium (100 µg/ml) was added and the solution was filled into Petri dishes (Peske, Aindling-Pichl, Germany) and stored at 4°C.

4.1.3 Cultivation

E. coli was cultivated under aerobic conditions at 37°C in 10 ml tubes or 250 ml cell culture bottles (Peske, Aindling-Pichl, Germany) under vigorous shaking (300 rpm) in a Unihood 550 incubator (Uniequip, Martinsried, Germany).

For short-term storage, LB-plates with bacteria colonies were kept at 4°C.

4.2 Vectors

4.2.1 Employed plasmids

For preparation of a [α^{32}P]-labeled cDNA probe for HO-1 Northern blot analysis, a pUC8 plasmid (2,665 bp), with a 883 bp HO-1 insert was used (kindly provided by Dr. Immenschuh, Giessen). This plasmid contains a β-lactamase gene for selection in ampicillin-LB-medium and allows isolation of the insert by cutting with the restriction enzymes HindIII (Boehringer Mannheim, Mannheim, Germany) and EcoRI (Promega, Heidelberg, Germany).

In situ hybridization of HO-1 was performed by *in vitro* transcription of a pBluescript II phagemid vector (2,961 bp) with a 800 bp HO-1 insert (kindly provided by Dr. Immenschuh, Giessen). Selective growth is allowed by the inserted ampicillin resistance gene. This plasmid includes restriction sites for Bam HI (MBI Fermentas, St. Leon Roth, Germany) and Hind III (Boehringer Mannheim, Mannheim, Germany) in its multiple cloning site. Cutting by Bam HI allows run off transcription of the antisense probe with T_3-polymerase, whereas Hind III cleavage leads to generation of the sense probe by T_7-polymerase.

4.3 Amplification of a cDNA-Northern blot probe

4.3.1 Preparation of competent cells

Competent bacteria are able to incorporate external DNA. Treatment with calcium chloride and rubidium chloride is one method to initiate this uptake.

E. coli DH5α were cultivated in LB-medium (20 ml) at 37°C up to an optical density ($OD_{600\ nm}$) of 0.3 to 0.5. Cells were centrifuged (5,000 rpm, 5 min, 4°C), the pellet was resuspended in 1 ml of ice cold solution A (3-[N-Morpholino]propansulfonic acid (MOPS) pH 7.0, 10 mM; rubidium chloride 10 mM), added up to 10 ml solution A and centrifuged again. The supernatant was discarded, the pellet resuspended in 1 ml solution B (MOPS, pH 6.5, 100 mM, calcium chloride 50 mM, rubidium chloride 10 mM) and after adding up to 10 ml with solution B, incubated on ice for 30 min. After centrifugation and gentle resuspensation in solution B, 10% glycerol was added and competent cells were storable at –70°C for several weeks.

4.3.2 Transformation of competent E. coli

Transformation of E. coli, the uptake of free external DNA into bacteria cells, was first described by Mandel and Higa (Mandel *et al.*, 1970).

100 µl of competent bacteria suspension were thawed on ice and incubated with 0.1 µg plasmid DNA for 30 min. The suspension was incubated at 42°C for 60 sec and transferred into 1 ml LB-medium. The mixture was again incubated at 37°C for 1 h, mixed and 20 µl or 200 µl, were plated onto LB-agar-ampicillin plates. The successfully transformed bacteria were able to grow overnight at 37°C in single colonies.

4.3.3 Mini-prep DNA isolation

Success of transformation was controlled by picking single colonies of bacteria grown on LB agar ampicillin plates and propagating them. The plasmids were isolated by lysing the bacteria with lysozyme, Triton X-100 (non-ionic detergent), and heat. Chromosomal DNA thereby clings to the cell membrane and can be separated together with cell debris from plasmid DNA by centrifugation. The solved plasmid DNA is then precipitated by isopropanol and washed with ethanol 70% (v/v) to remove in isopropanol insoluble salts. The identification of the plasmid DNA takes place by gel electrophoresis.

STET

Sucrose	8% (m/v)
Triton X-100	5% (m/v)
Tris-HCl pH 8.0	50 mM
EDTA	50 mM

One single bacteria colony was incubated in a sterile 15 ml centrifugation tube in 2 ml LB/ampicillin overnight under vigorous shaking (300 rpm, 37°C) in a Unihood 550 incubator (Uniequip, Martinsried, Germany). The next morning, 500 µl of the bacteria suspension were transferred to a sterile reaction tube and stored at 4 °C. The remaining bacteria suspension was centrifuged (100 x *g*, 5 min) and the supernatant removed. The bacteria pellet was resuspended in 200 µl STET. By addition of 16 µl lysozyme solution (10 mg/ml, sodium salt) and incubation at 75°C (60 sec) the bacteria were lysed. Centrifugation at 12,000 x *g* for 10 min separated the bacteria DNA (in the pellet) from the plasmid DNA (in the supernatant). Plasmid DNA was precipitated by addition of isopropanol 50% (v/v) (5min, RT) and after centrifugation at 12,000 x *g* for 10 min the pellet was washed with ethanol 70% (v/v). After a final centrifugation step (12,000 x *g*, 3 min), the pellet was air dried and dissolved in 40 µl TE buffer and stored at –20°C until electrophoresis.

4.3.4 Agarose gel electrophoresis

Plasmid DNA was identified by electrophoresis in an 1% (m/v) agarose gel in 1x TBE-buffer. 12 µl DNA solution were mixed with 3 µl commercial loading buffer (Promega, Heidelberg, Germany) and separated in 1x TBE electrophoresis buffer in a horizontal electrophoresis chamber at 80 V. 0.5 µg of known plasmid served as control.

10x TBE (Tris borate EDTA)

Tris	900 mM
Boric acid	889 mM

EDTA	25 mM

4.3.5 Midi-prep DNA isolation with Qiagen Plasmid Midi Kit

Larger amounts of highly purified DNA for the preparation of a DNA Northern blot probe were isolated with the Quiagen Midi Kit (Quiagen GmbH, Hilden, Germany). This kit is based on the principle of alkaline lysis with consecutive chromatographic purification *via* anionic exchange columns. Thereby, DNA is separated from polysaccharides, RNA, and other contaminants.

Bacteria were lysed with NaOH/SDS and proteins and DNA were denatured. The method is based on the fact that by neutralization with high concentrations of potassium acetate the relatively low molecular plasmid DNA again renatures, while proteins, DNA, and cell debris remain bound to salt/SDS complexes and can be separated by centrifugation. The following purification by anionic exchange columns removes salt- and protein impurities. By addition of isopropanol to the eluate the plasmid DNA is precipitated.

Buffer P1

Tris-HCl pH 8.0	50 mM
EDTA	10 mM
RNase A	100 µg/ml

Buffer QBT

NaCl	750 mM
MOPS pH 7.0	50 mM
Ethanol	15% (v/v)
Triton X-100	0.15% (m/v)

Buffer QC

NaCl	1.0 M
MOPS pH 7.0	50 mM
Ethanol	15% (v/v)

Buffer QF

NaCl	1.25 M
Tris-HCl pH 8.5	50 mM
Ethanol	15% (v/v)

STE (Sodium chloride Tris EDTA buffer)

NaCl	100 mM
Tris-HCl pH 8.0	10 mM
EDTA	1 mM

The successfully transformed E. coli DH5α bacteria were cultivated in 50 ml LB/ampicillin to an optical density (600 nm) of 1 to 1.5 and centrifuged at 1,000 x *g* at 4°C for 15 min. The pellet was resuspended in 10 ml of buffer P1 and lysed with 4 ml 200 nM NaOH/1% (m/v) SDS (5 min, RT) followed by incubation on ice for 15 min with 4 ml ice cold 3 M potassium acetate pH 5.5. The pellet was separated by centrifugation (20,000 x *g*, 30 min, 4°C) and the supernatant was cleared completely by centrifugation once more for 15 min. The supernatant was placed on a equilibrated Quiagen column (Quiagen-tip 100, with 4 ml QBT buffer) and the column was washed twice with 10 ml buffer QC. The eluate was discarded and the DNA bound to the column eluted with 5 ml buffer QF. The DNA in the eluate was precipitated with 0.7 volumes of isopropanol. The resulting pellet after centrifugation at 15,000 x *g* (4°C, 30 min) was washed with 70% (v/v) ethanol and air dried. After resolving in H_2O, concentration of DNA was determined according to 3.2.

4.3.6 Restriction endonuclease digestion

To obtain the inserted HO-1 DNA sequence for preparation of the radioactive probe the purified circular plasmid was cut with the restriction enzymes Eco RI and Hind III (see 4.2.1).

Reaction mixture	
Plasmid DNA	20 µg
Restriction enzyme	3 µl
Buffer	10 µl
H_2O	ad 100 µl

The reaction mixture was prepared on ice and incubated for 5 h at 37°C. To separate the restriction products an agarose gel electrophoresis was performed. The respective products were identified by comparison with a DNA ladder mix (GeneRuler™ DNA Ladder Mix, MBI Fermentas, St. Leon Roth, Germany).

4.3.7 Extraction of DNA from agarose gels

DNA fragments were extracted and purified from agarose gels with the QIAquick gel extraction kit from Qiagen (Hilden, Germany). Test principle is the adsorption of nucleic acids to a silica-gel membrane in presence of high salt concentration and a defined pH. Contaminants such as salts, enzymes, unincorporated nucleotides, agarose and dyes do not bind to the silica-gel membrane but flow through the column.

To extract the HO-1 fragment, the corresponding lane was cut out of the agarose gel and

3 volumes of buffer QG was added to 1 volume of gel slice. The gel was solubilized by incubating the mixture at 50°C for 10 min. The yellow color of the solution indicated the correct pH value (<7.5). One volume of isopropanol was added and the sample was mixed. To bind DNA to a QIAquick spin column, the sample was applied to a column in a collection tube and was centrifuged for 1 min at 14,000 rpm. The flow through was discarded and after addition of 0.5 ml of buffer QG the column was centrifuged again. After washing with 0.75 ml of buffer PE the flow through was removed and the column was centrifuged for 1 min to separate residual ethanol. To elute the DNA from the silica-gel membrane the column was placed in a clean reaction tube and 50 µl of H_2O were added to the column before centrifugation. Under these conditions (pH 7-8.5 and low salt) maximum elution efficiency is achieved. Until further investigation the DNA was stored at –20°C.

4.3.8 Sequencing of double stranded DNA

The generation of the correct plasmid insert was confirmed by sequencing the isolated product by the company Toplab, Martinsried, Germany. Sequencing by the method of Sanger et al. (Sanger *et al.*, 1977) revealed that the insert was identical with the 883 bp HO-1 sequence.

4.4 DNA labeling with radioactive nucleotides

4.4.1 Synthesis of radioactively labeled HO-1 cDNA

Radioactive labeling of a DNA probe for use as hybridization probe in HO-1 Northern blot analysis was performed with the random primed DNA labeling kit from Roche (Roche Diagnostics GmbH, Mannheim, Germany). This method is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled. [α^{32}P]-dCTP is incorporated into the newly synthesized complementary DNA strand which is synthesized from the 3'-end-terminus of the random hexanucleotide primer using Klenow enzyme (Feinberg *et al.*, 1983).

The DNA had to be denatured by heating for 10 min at 100°C and subsequent cooling on ice. The following was added to a reaction tube on ice according to the instructions of the manual:

Reaction mixture

cDNA template	0.01-2 µg
dNTP's (dATP, dGTP, dTTP)	1 µl
Hexanucleotides in 10x buffer	2 µl

[α^{32}P]-dCTP (Amersham)	50 µCi
Klenow enzyme	1 µl
H_2O	ad 20 µl

The mixture was incubated for 30 min at 37°C. The reaction was stopped by heating for 10 min at 65°C.

4.4.2 Synthesis of radioactively labeled 28S RNA oligonucleotides

Radioactive labeling of a 28S RNA oligonucleotide probe to subsequently validate HO-1 Northern blotting (Barbu *et al.*, 1989) was made by the T_4 polynucleotide kinase method. The T_4 polynucleotide kinase (Stratagene, Heidelberg, Germany) catalyzes the transfer of the terminal [γ^{32}P]-phosphate of ATP to the 5'-hydroxyl terminus of an RNA or DNA molecule (Richardson, 1965).

28S RNA oligo probe: 5'-AAC GAT GAG AGT AGT GGT ATT TCA CC-3' Barbu *et al*., 1989

Reaction mixture

28S RNA oligo probe	0.2 µg
T4 polynucleotide kinase buffer 10x	2 µl
[γ^{32}P]-ATP	5 µl (3000 Ci/mmol)
H_2O	10 µl
T_4 polynucleotide kinase	2 µl (1 µl + 1µl dilution buffer)

The mixture was incubated at 37°C for 30 min. The reaction was terminated by heating the mixture to 65°C for 5 min.

4.4.3 Removal of unincorporated dNTPs

Removal of radioactively labeled probes from non incorporated [α^{32}P]-dCTP or [γ^{32}P]-ATP was performed on a NucTrap® column (NucTrap® probe purification column, Stratagene, Heidelberg, Germany). The free nucleotides remain on the column and the eluate contains the DNA probe.

70 µl of STE (4.3.5) were added to the reaction mixture and placed onto a column which had been equilibrated with 70 µl STE. The solution was pressed through the column by the use of a syringe. This was repeated with 70 µl STE and finally with air. The eluate was collected in a reaction tube and the incorporation of radioactivity was measured in a β-counter (Beckmann LS6500, Multi-purpose Scintillation Counter, Irvine, CA, USA). 1 µl of the mixture was added to 5 ml of szintillation cocktail (Rotiszint, Roth, Karlsruhe, Germany) and was counted in a channel set for [^{32}P]. 2 x 10^6 cpm were required for

Northern hybridization.

4.5 Northern blot

For evaluation of HO-1 mRNA expression, Northern blotting was performed with a HO-1 cDNA probe. Northern blot results were validated, by hybridization with a 28S RNA oligonucleotide probe.

4.5.1 Solutions

20x SSC (Saline sodium citrate)

NaCl	175.3 g
Na-citrat	88.2 g
H_2O	ad 1000 ml
Adjusting to pH 7.0	

Hybridization solution

20x SSC	5 ml
50x Denhardt's solution (Biomol)	2 ml
Formamide 50% (v/v) deionized	10 ml
SDS 1% (v/v)	2 ml
denatured salmon sperm DNA (100 µg/ml)	200 µl
H_2O	800 µl

4.5.2 Transfer of electrophoretically separated RNA onto a nylon membrane

Electrophoretically separated but unstained RNA (3.3.2) was transferred onto a nylon membrane by vacuum blotting (VacuGene™XL Vacuum blotting system, Amersham Pharmacia Biotech, Freiburg, Germany). This method is characterized by short transfer times (45-60 min) and quantitative transfer with high band sharpness and resolution.

The nylon membrane (Nytran® 0.2 µm, neutral, Schleicher and Schüll, Dassel, Germany) was soaked in 20x SSC and placed into the blotting apparatus according to the instructions of the manufacturer. The membrane was covered with the agarose gel and 20x SSC was filled into the chamber. A vacuum of 50-55 mbar was established and kept for 45 min. After the transfer, the membrane was air dried and the RNA was UV-crosslinked to the membrane (Stratalinker® UV Crosslinker 1800, Stratagene, Heidelberg, Germany). By treatment with UV-light (254 nm), the uracil of RNA is covalently bound to amino residues of the nylon membrane.

4.5.3 Hybridization

Hybridization of immobilized RNA with radioactively labeled DNA was performed as described by (Vollmar, 1990) in a hybridization oven from Uniequip (Martinsried, Germany).

The blots were soaked in 20 mM Tris-HCl pH 8.0 for 5 min at 65°C to remove glyoxal traces. After washing in 6x SSC, the blots were rolled air bubble free into glass hybridization tubes and prehybridized at 42°C for 4 h in hybridization solution. After that time the radioactive cDNA probe was denatured for 5 min at 95°C and then added to the hybridization tubes. Hybridization was continued at 42°C overnight. The next day, the hybridization solution was removed and the blots were washed 2 times in 2x SSC/0.1% SDS at room temperature for 5 min. Afterwards, blots were washed 2 times in 0.2x SSC/0.1% SDS at room temperature for 5 min and 2 times in 0.2x SSC/0.1% SDS at 42°C for 15 min. Before air drying, the blots were rinsed with 2x SSC.

4.5.4 Detection

Hybridization of radioactive labeled probes and mRNA was visualized by indirect autoradiography with intensifyer screens (phosphorimaging). High energetic β-radiation is adsorbed by phosphate residues of intensifyer screens and is transformed into blue light. The screens were scanned in a Fujifilm BAS-1500 (Raytest, Straubenhardt, Germany) for densitometric evaluation of radioactive signals.

5. In situ hybridization

The cellular expression pattern of HO-1 mRNA was investigated by *in situ* hybridization with a radioactive HO-1 RNA probe.

In situ hybridization was performed by Dr. A. K. Kiemer in snap-frozen liver samples by a method described in detail by Milani et al. (Milani *et al.*, 1994).

5.1 Hybridization

After treatment with 0.2 N HCl and washing in H_2O, the slides were digested in pronase, rinsed in 0.1 M glycine/PBS, and fixed in 4% paraformaldehyde/PBS. Slides were then washed in PBS and acetylated. Washed slides were dehydrated in graded ethanols and air-dried prior to hybridization. Transcription and labeling of RNA probes were performed with [^{35}S]Uridine-5'-(-thio)-triphosphate (NEN, Boston, MA, USA). Slides were incubated

with hybridization mixture at 50°C overnight. Excess of probe was removed by washing in hybridization buffer (4 h, 50°C). Slides were then digested with RNase and washed in 0.1 M Tris-HCl pH 7.5/1 mM EDTA/0.5 M NaCl. After additional washing in 2x SSC and 0.1x SSC, slides were dehydrated in graded ethanols and air-dried.

5.2 Detection

Autoradiography was performed by dipping the dehydrated slides into Ilford G5 nuclear emulsion (Ilford, Mobberley Cheshire, UK) and exposing them for 28 days. Slides were developed and counterstained in hematoxylin-eosin. Sections from untreated and ANP-treated livers were processed in parallel, using the same batches of probes (sense and antisense HO-1) and reagents.

6. Reverse transcriptase polymerase chain reaction

The expression of cGMP-dependent protein kinases (PKG) in rat livers was evaluated by the reverse transcriptase polymerase chain reaction (RT-PCR) technique. This *in vitro* technique was developed by Mullis et al. (Mullis *et al.*, 1987) and allows the amplification of nucleic acid segments of defined length and sequence. For evaluation of mRNA expression, RNA had to be transferred into complementary DNA (cDNA) by reverse transcription first.

6.1 Reverse transcription

The reverse transcription was performed with viral reverse transcriptase from avian myeloblastosis virus (AMV-RT, and all other substances, Promega, Heidelberg, Germany). mRNA was used as preferred template for cDNA synthesis due to lower contamination compared to totRNA.

Reaction mixture

$MgCl_2$	5 mM
Tris-HCl	10 mM
KCl	50 mM
Triton X-100	0.1% (v/v)
DNTP	1 mM
RNase inhibitor	5% (v/v)
Oligo$(dT)_{15}$ primers	1% (v/v)
AMV-RT	15 U/1 µg mRNA

1 µg of mRNA was added to 10 µl reaction mixture. After incubation for 15 - 20 min at 42°C, the reaction was terminated by boiling for 5 min. The samples were placed on ice

and stored at –20°C.

6.2 Primers and reaction conditions

All oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany) and were HPLC purified.

Gene	Orientation	Sequence	Length	Reference
PKG I human	sense	5'-GGA GAC GTG GGG TCA CTG GTG-3'	377 bp	Lohmann, personal
	antisense	5'-AAA GAA GGT GTC CCC TCT TGC-3'		communication
PKG II rat	sense	5'-GTG GCC AGA TTC TCA ACC TC-3'	542 bp	Huber *et al.*, 1998
	antisense	5'-ACC TCG GGG GCC ACA TAC TCT-3'		
GAPDH rat	sense	5'-AGA TCC ACA ACG GAT ACA TT-3'		
	antisense	5'-TCC CTC AAG ATT GTC AGC AA-3'		

table 6.1: Primer sequences for RT-PCR.

	Pretreatment	Annealing	Extension	Denaturation	Posttreatment	Cycles
PKG I	70°C, 3 min	94°C, 40 sec	56°C, 40	72°C, 60 sec	72°C, 10 min	35
PKG II	70°C, 3 min	94°C, 40 sec	58°C, 40	72°C, 60 sec	72°C, 10 min	35
GAPDH	93°C, 60 sec	93°C, 30 sec	55°C, 30	73°C, 60 sec	73°C, 10 min	30

table 6.2: PCR reaction conditions.

RT-PCR was set up using the respective primers according to table 6.1 and amplification was performed as listed in table 6.2.

6.3 Polymerase chain reaction

Reaction mixture

BSA 1%	0.005% (m/v)
$MgCl_2$	1.5 mM
dNTP	1.0 mM
10x Buffer	1x
Primer sense	0.6 µg
Primer antisense	0.6 µg
H_2O	ad 100 µl
cDNA sample	2 ng
Taq DNA polymerase	2.5 U (was added after the first denaturation step)

The reaction mixture was prepared on ice in reaction tubes without the Taq DNA polymerase. For exclusion of unspecific amplification, samples without cDNA were additionally amplified. The thermocycler (Gene Amp PCR System 9700, PE Applied Biosystems, Foster City, CA, USA) was set up according to the cycle temperatures and times in table 6.2. After the first denaturation step (pre-treatment) the program was

interrupted in order to add the heat stable Taq DNA polymerase. This so called hot start method shows less unspecific products and reduces aggregation of primers to oligomers (Chou *et al.*, 1992). Early misspriming cannot serve as Taq DNA polymerase starting point. Misspriming is reversed after the initial denaturation step and primers can hybridize correctly at their annealing temperature. Thereafter, cycles of annealing, extension and denaturation of the DNA strand were repeated as indicated in table 6.2. The posttreatment allowed to finalize incomplete amplification.

6.4 Electrophoresis of amplification products

For evaluation of amplification products, samples were separated by gel electrophoresis. A 2.5% agarose gel was prepared with TAE buffer (see 4.3.4). Samples and DNA ladder (GeneRuler™ DNA Ladder Mix, MBI Fermentas, St. Leon Roth, Germany) for estimation of PCR product size were mixed with loading buffer (MBI Fermentas) and electrophoretically separated (80 V, TAE buffer, 2 h). Staining with ethidium bromide took place as described under 3.3.3.

Tris-acetate-EDTA buffer (TAE)

Tris-acetate	40 mM
EDTA	1 mM
Adjusting pH to 8.0	

7. Detection of proteins by Western blotting

Protein expression of Caspase-3 and HO-1 protein in rat livers was investigated by Western blot analysis.

7.1 Preparation of samples

7.1.1 Solutions

Lysis buffer:

NaCl	150 mM
Tris-HCl, pH 7,5	50 mM
Nonidet P 40	1%(v/v)
Deoxycholate	0,25% (m/v)
SDS	0,1% (m/v)

Lysis buffer was supplemented with the protease inhibitor mix Complete® (Boehringer Mannheim, Mannheim, Germany) before use according to the manufacturer's instruction.

5x Sample buffer

3.125 M Tris HCl pH 6,8	100 µl
Glycerol	500 µl
20% SDS	250 µl
16% DTT	125 µl
Pyronin Y 5 %	5 µl
Aqua demin.	20 µl

7.1.2 Preparation of cellular protein extracts from rat liver tissue

All work was performed in a 4°C cooling chamber. Livers were stored during the preparation procedure on dry ice. 100 mg of liver tissue was added to 1 ml of ice cold lysis buffer and homogenized by 10 strokes with a Potter S device (Braun Biotech, Melsungen, Germany). Afterwards, homogenates were centrifuged for 20 min at 4°C and 14,000 rpm. Clear supernatants were used for the following protein determination and protein electrophoresis.

7.1.3 Preparation of cellular protein extracts from rat hepatocytes

Hepatocytes were cultivated in 6-well cell culture plates. 200 µl lysis buffer containing Complete® protease inhibitor mix were added per well and cells were lysed by scratching with a cell scraper. After transferring to reaction tubes, cell suspensions were treated with eight strikes of ultra sonic to smash DNA and therefore reduce viscosity. Afterwards, homogenates were centrifuged for 20 min at 4°C and 14,000 rpm. Clear supernatants were used for the following protein determination and protein electrophoresis.

7.1.4 Preparation of samples for protein electrophoresis

Before protein electrophoresis, protein samples were denatured and were charged negatively by addition of 5x sample buffer and boiling for 5 min.

7.2 Measurement of protein concentration: Lowry-assay

Protein content for Western analysis was determined according to Lowry (Lowry *et al.*, 1951) by photometric quantification. The final colour is a result of the Biuret reaction with copper ion in alkali and the reduction of phosphomolybdic and phosphotungstic reagent by tyrosine and tryptophan present in the peptide chains.

Reagent A:

Na_2CO_3	10 g
NaOH 0.1 mol/L	500 ml

Reagent B:

Na/K tartrat	0.5 g
$CuSO_4$	0.25 g
Aqua demin.	50 ml

Reagent 1:

Alkaline copper solution	50 parts of reagent A plus 1 part of reagent B

Reagent 2:

Diluted Folin's reagent	Folin-Ciocalteu's phenol reagent - aqua demin. (1:1)

The protein solution was diluted with Aqua demin. as necessary and 200 µl of sample was transferred into a reaction tube. 1 ml of reagent 1 was added, mixed well and incubated for at least 10 min at room temperature. 100 µl of reagent 2 were added subsequently, mixed well and incubated for at least 30 min. Absorbance was measured at 500 nm in a Lambda Bio 20 photometer, Perkin Elmer. By creating standard dilutions with bovine serum albumin (0-500 µg/ml), the corresponding protein concentration was determined.

7.3 SDS-polyacrylamide gel electrophoresis (SDS-Page)

Separation of proteins was performed by the denaturating SDS-polyacrylamide gel electrophoresis (SDS-Page) (Laemmli UK, 1970). The anionic detergent sodium dodecyl sulfate (SDS) solubilizes proteins and leads to a constant net charge per mass unit. Therefore proteins are separated exclusively according to their molecular mass. Differences in molecular form are compensated by the loss of the tertiary and secondary structure because of the disruption of the hydrogen bonds and unfolding of the molecules. Disulfide bonds are cleaved by the reducing thiol agent dithiothreitol (DTT). Molecular weight standard (Santa Cruz Biotechnology, sc-2035, Heidelberg, Germany) was used to determine the molecular weight of the separated proteins.

Electrophoresis was carried out in a vertical apparatus from BioRad (Mini Protean II, Munich, Germany). Two gel runs were performed in parallel.

7.3.1 Solutions

For preparation of gels polyacrylamid (PAA)/bis-acrylamide stock solutions (Rotiphorese Gel 30) from Roth (Karlsruhe, Germany) were applied.

Resolving gel 10 %:

PAA solution 30%	6.25 ml
1.5 M Tris, pH 8.8	4.69 ml
10% SDS	0.188 ml
H_2O	7.625 ml

10 min degassing	
TEMED (Tetramethylethylenediamine)	18.75 µl
APS (ammonium persulfate) 10% (m/v)	93.75 µl

Stacking gel:

PAA solution 30%	2.125 ml
1.25 M Tris, pH 6.8	1.25 ml
10% SDS	0.125 ml
H_2O	8.75 ml
10 min degassing	
TEMED (cross linker)	25 µl
APS 10% (m/v) (radical starter)	125 µl

The solutions were degassed for 10 min by a vacuum pump to get anaerobic conditions for polymerization. After that, TEMED and APS were added.

Electrophoresis buffer:

Tris	3 g
Glycine	14.4 g
SDS	1 g
H_2O	ad 1000 ml

7.3.2 Electrophoresis

The stacking gel was placed about 2 cm above the resolving gel. The slot former was directly put into the stacking gel and complete polymerization was allowed overnight at 4°C. The next day samples with equal protein content were filled up to 35 µl and pipetted into the slots. Additionally, molecular weight standard (3 µl + 32 µl sample buffer) was added. Stacking was carried out at 100 V for 21 min. The proteins were resolved at 200 V for 36 min (power supply: Biometra, Göttingen, Germany).

7.4 Coomassie blue staining

To check for equal sample loading and effective blotting gels were stained with Coomassie blue solution.

7.4.1 Solutions

Staining solution:

Acetic acid (100%)	10% (v/v)
Ethanol (96%)	30% (v/v)
In H_2O	

Destaining solution:

Coomassie blue	0.3 %

Acetic acid (100%)	10% (v/v)
Ethanol (96%)	45% (v/v)
In H_2O	
Filtration	

7.4.2 Staining procedure

After Western blotting, SDS-page gels were stained for 15 min in Coomassie staining solution. Then, gels were destained in destaining solution 3 times for 15 min and finally stored in H_2O.

7.5 Immunologic detection by Western blotting

Proteins were transferred electrophoretically from the gel onto a polyvinylidenfluoride (PVDF) membrane (Immobilon-P, 0.45 µM pore size, Millipore, Bedford, MA, USA). To visualize the proteins of interest, a specific first antibody was bound to the immobilized proteins. By binding a second anti-immunoglobuline antibody labeled with horseradish peroxidase (HRP) to the first antibody, this complex can be identified *via* incubation with a substrate solution which starts an enzyme-substrate reaction: the oxidation of luminol in presence of H_2O_2 and peroxidase enzyme leads to a chemiluminescence reaction in the area where labeled antibody is bound. This light reaction was enhanced by a chemical enhancer (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Cologne, Germany) and imaged by a CCD camera system (Kodak image Station, Eastman Kodak Company, Rocester, New York, USA).

7.5.1 Solutions

Anodal buffer I, pH 10.4:

Tris	15 g
Methanol	100 ml
H_2O.	400 ml

Anodal buffer II, pH 10.4:

Tris	1.5 g
Methanol	100 ml
H_2O	400 ml

Cathodal buffer, pH 7.6:

ε-amino-n-caproic acid	2.6 g
Methanol	100 ml
H_2O	400 ml

TBS-T pH 8.0 (washing buffer):

Tris	3 g
NaCl	11.1 g
Tween 20	1 ml
H_2O	ad 1000 ml
Adjusting to pH 8.0	

7.5.2 Protein transfer by semidry blotting

Transfer of proteins onto the PVDF membrane was performed by semidry blotting between two horizontal graphite electrodes (Fastblot B43, Biometra, Göttingen Germany). The discontinuous buffer system leads to an equal and effective protein transfer with sharp signals. Additionally, this method is cheap, fast, and simple and requires only a small amount of buffer (Kyhse-Andersen, 1984).

The blotting membrane was cut to the size of the resolving gel and placed for 5 min in methanol, H_2O and anodal buffer II, respectively. Onto the anode, forming an air bubble free stack, 6 sheets of blotting paper (BioRad, Munich, Germany) were placed after moistening in anodal buffer I, then, 3 sheets treated with anodal buffer II. Onto this stack the membrane and the gel was laid and subsequently covered with 9 sheets of blotting paper, moistened with cathodal buffer.

Blotting was performed at a current of 0.8 mA per cm^2 of blotting surface and 115 V. After that, the membrane was dried for 30 min at 80°C.

To mask unspecific binding sites, the membrane was blocked overnight (4°C) in a 5% (m/v) solution of low fat milk powder (Blotto, BioRad, Munich, Germany) in TBS-T pH 8.0.

7.5.3 Used antibodies

7.5.3.1 HO-1 Western blot

For HO-1 Western blotting, a monoclonal mouse HO-1 antibody (Stressgen OSA-111, Victoria, Canada) served as first antibody, and a polyclonal anti-mouse (Cell signaling 7072-1, New England Biolabs GmbH, Frankfurt/Main, Germany) as secondary antibody conjugated to horseradish peroxidase (HRP)

7.5.3.2 Caspase-3

Caspase-3 protein was detected with a polyclonal rabbit antibody (Santa Cruz SC-7148, Heidelberg, Germany). Secondary antibody was an anti-rabbit antibody (Dianova, Jackson Immuno Research 111-035-144, Hamburg, Germany) coupled to HRP.

7.5.4 Incubation with antibodies and chemiluminescence detection

The antibodies were diluted 1:1000 in a solution of 1% Blotto in TBS-T pH 8.0. After incubating the membrane for 60 min with the primary antibody on a shaking platform, three washing steps for 10 min in TBS-T pH 8.0 followed. The second antibody was kept for 60 min on the blots and again three washing steps were performed. The two detection solutions (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Cologne, Germany) were mixed 1:1 according to the manufacturer's instruction and the membrane was incubated for 1 min. Chemiluminescence detection was performed on a Kodak image station.

8. In vitro phosphorylation by protein kinase A

Cyclic AMP dependent protein kinase A (PKA) catalyzes the transfer of γ-phosphate from adenosine triphosphate (ATP) to a serine or threonine residue in a protein substrate. This phosphorylation activity can be measured by *in vitro* phosphorylation of a short, biotinylated, synthetic and highly specific peptide substrate of defined sequence (kemptide: LRRASLG) with radioactively labeled ATP ([γ^{32}P]-ATP) (Pearson *et al.*, 1991).

The assay was performed with the Protein Kinase A Assay Kit from Calbiochem (Schwalbach, Germany) according to the manufacturer's instructions.

8.1 Solutions

Extraction buffer	
Tris-HCl pH 7.4	25 mM
EDTA	0.5 mM
EGTA	0.5 mM
Triton-X 100	0.05% (v/v)
Mercaptoethanol	10 mM
Leupeptin	1 µg/ml
Aprotinin	1 µg/ml

Reaction mixture	**(volume per sample)**
ATP solution 0.15 mM	2.5 µl
Biotinylated kemptide solution 0.25 mM	2.5 µl
Reaction buffer (200 mM $MgCl_2$, 400 mM Tris-HCl pH7.5)	2.5 µl
[γ^{32}P]-ATP (0.2 µCi/µl)	2.5 µl
H_2O	10.0 µl

Control mixture	**(volume per sample)**
ATP solution 0.15 mM	2.5 µl
Reaction buffer (200 mM $MgCl_2$, 400 mM Tris-HCl pH7.5)	2.5 µl

[γ^{32}P]-ATP (0.2 µCi/µl)	2.5 µl
H_2O	12.5 µl

8.2 Preparation of samples

8.2.1 Liver tissue

All work was performed in a 4°C cooling chamber. Livers were stored during this time on dry ice. 100 mg of liver tissue was added to 500 µl of ice cold lysis buffer and homogenized by 10 strokes with a Potter S device (Braun Biotech, Melsungen, Germany). Afterwards, homogenates were centrifuged for 20 min at 4°C and 14,000 rpm. Clear supernatants were used for the following PKA assay.

8.2.2 Primary hepatocytes

Primary hepatocytes were isolated and cultivated as described under 2.4. After 48 h of cultivation in 6-well plates, hepatocytes were stimulated with the respective substances and harvested after the incubation time by washing 2 times with cold PBS. Cells were lysed with 200 µl of extraction buffer per well and homogenized with a hand potter (5 strokes, Braun Biotech Melsungen, Germany). After centrifugation for 20 min at 4°C and 14.000 rpm the supernatants were used for the PKA assay.

8.3 Protein kinase A assay

Besides the preparation of a reaction mixture with ATP-solution, biotinylated kemptide, [γ^{32}P]-ATP, and reaction buffer for measuring phosphorylation of the peptide substrate, a control mixture without biotinylated kemptide was set up to determine unspecific phosphorylation. Background phosphorylation was determined by preparing a sample with reaction mixture but without protein lysate.

The first step was the incubation of the enzyme sample with the reaction mix containing biotinylated peptide substrate and [γ^{32}P]-ATP. This was performed at 37°C for 30 min. The reaction was terminated by adding stop solution (8.0 M guanidine hydrochloride). Free avidin in solution was supplemented for binding to the biotinylated [γ^{32}P]-peptide product and after 5 min of incubation, samples were transferred into centrifuge ultrafiltration units. These units contain a membrane that retains the product-avidin complex by affinity ultrafiltration but not the free [γ^{32}P]-ATP. After several washing steps the ultrafiltration units were directly placed into liquid scintillation vials (Greiner, Frickenhausen, Germany) and were counted after addition of 10 ml of scintillation liquid (Rotiszint, Roth, Karlsruhe,

Germany) in a multi-purpose scintillation counter (Beckmann LS6500).

Specific PKA activity was calculated as pmole phosphate incorporated per minute per sample amount as described in the instruction manual.

9. Fluorimetric enzyme activity assay

Caspases are a family of proteases that act in a cascade triggered by apoptotic signals. These enzymes are highly conserved cysteine proteases that specifically and efficiently cleave target substrates after the carboxy terminus of aspartate (Nicholson *et al.*, 1997).

The activation of the downstream caspase-3, considered as one of the major effector caspases (Hengartner, 2000), can be measured by applying the synthetic peptide substrate DEVD (asp-glu-val-asp). It is coupled to a fluorophor that is cleaved after the aspartate residue by the activated enzyme leading to increased fluorescence (Thornberry, 1994). Because other downstream caspases such as caspase-7 have similar substrate specifity, this assay does not exclusively demonstrate caspase-3 activity. Therefore, the measured activity is termed “caspase-3-like activity” regarding caspase-3 as the major effector caspase. To make detection possible, the DEVD substrate is labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin (AFC). The liberation of this fluorophore shows a blue to green shift in fluorescence at an extinction wavelength of 390 nm and an emission wavelength of 505 nm. AFC as fluorophore is highly sensitive and sufficiently stable to handle.

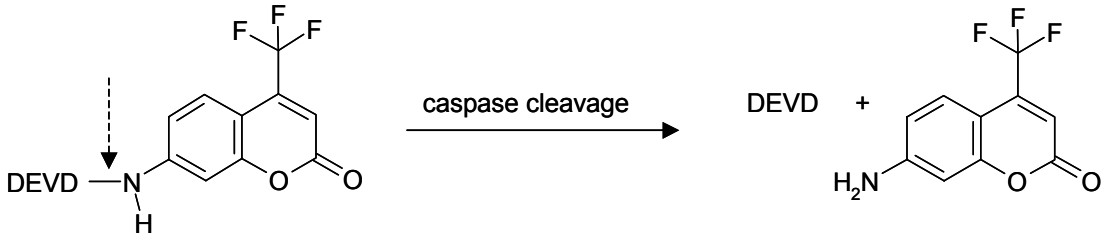

figure 10: liberation of AFC from DEVD-AFC by caspase cleavage (see text).

The reaction processes linearly at least over 2 h at substrate saturation. Because agents, such as air oxygen or traces of metal ions can oxidize the thiol group of the cysteine protease and therefore inactivate the enzyme, dithiothreitol (DTT), as a reducing agent, was added to the substrate buffer.

9.1 Solutions

Extraction buffer

HEPES pH 7.5	25 mM
$MgCl_2$	5 mM
EGTA	1 mM
Pefablock SC	1 mM
Pepstatin	1 µg/ml
Leupeptin	1 µg/ml
Aprotinin	1 µg/ml

Substrate buffer

Ac-DEVD-AFC (Biosource, Solingen, Germany)	50 µM
HEPES pH 7.4	50 mM
Sucrose	1% (m/v)
CHAPS	0.1% (m/v)
DTT	10 mM

9.2 Caspase-3-like acitivity assay

Cytosolic extracts from liver tissue were prepared by homogenization in hypotonic extraction buffer in a Potter S device (Braun Biotech, Melsungen, Germany). Homogenates were subsequently centrifuged (15 min, 14,000 rpm, 4°C), and supernatants were stored at -80°C. The fluorometric Ac-DEVD-AFC cleavage assay was carried out on microtiter plates (Greiner, Frickenhausen, Germany) according to the method originally described by (Nicholson *et al.*, 1995). Cytosolic extracts (10 µl, ~1 mg protein/ml) were diluted 1:10 with substrate buffer containing the fluorogenic substrate. Blanks contained 10 µl of extraction buffer and 90 µl of substrate buffer. Generation of free 7-amino-4-trifluoro-methylcoumarin (AFC) at 37°C was kinetically determined by fluorescence measurement (excitation, 385 nm; emission, 505 nm) using a fluorometer microplate reader (Fluostar, BMG GmbH, Offenburg, Germany). Enzyme activity was calculated using an external AFC (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) standard curve. The time point where highest enzyme activity was measured was set to 100%. Protein concentrations of the corresponding samples were estimated with the Pierce Assay (see below). Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

9.3 Protein determination: Pierce-assay (BCA-assay)

The resulting color of this photometric assay is based on the Biuret reaction of proteins with copper in alkali and the interaction of two molecules of bicinchinonic acid (BCA) with

one copper (I) ion (Cu^+). The purple complex is water soluble and has a strong absorbance at 562 nm (Smith *et al.*, 1985).

Reagent A	Na_2CO_3, $NaHCO_3$, BCA reagent and Na-tartrat in 0.2 mol/L NaOH
Reagent B	4% aqueous $CuSO_4$-solution

50 parts of reagent A were mixed with 1 part of reagent B. 200 µl of this working solution were added to 10 µl of sample, incubated at 37°C for 30 min and measured the absorbance at 562 nm in a Lambda Bio 20 photometer. By creating standard dilutions with bovine serum albumin (0-2000 µg/ml, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), the corresponding protein concentration was determined.

10. Histological analysis

10.1 TUNEL staining

DNA fragmentation was quantified by the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling method (TUNEL) (Gavrieli *et al.*, 1992).

TUNEL staining of paraffin embedded liver sections was performed by Dr. Meißner and Andrea Sendelhofert, Institute of Pathology, University of Munich, Germany.

At indicated time points liver samples were taken and slices were immediately fixed in buffered formalin solution (4% formaldehyde). After 48 hours samples were embedded in paraffin and cut into 6 µM sections. For evaluation of the number of apoptotic cells liver sections were stained with the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, New York, USA) as described in detail in the manufacturer's instruction. For quantitation, 10 high power fields were counted (1.96 mm²) using a Zeiss Axiolab microscope (Carl Zeiss Mikroskopie, Jena, Germany). This area contained approximately 4,000 hepatocytes. The ApopTag® staining results were evaluated in combination with morphological criteria. All histological evaluations were performed in a blinded fashion.

10.2 Immunohistochemistry

Immunostaining was performed by Dr. H. Meißner and Andrea Sendelhofert, Institute of Pathology, University of Munich, Germany.

Liver slices were fixed in buffered formalin solution. After 24 h samples were embedded in paraffin and cut into 2 µM sections. Paraffin was removed and samples were pretreated

by boiling in TRS 6 (Dako Hamburg, Germany) in the microwave. Endogenous peroxidase was blocked by treatment with aqueous H_2O_2 solution. For detection of HO-1, a monoclonal antibody from StressGen, Victoria, Canada, was used. The indirect peroxidase method was performed with the PicTure™–Plus Kit anti mouse (Zymed, San Francisco, USA) and red color resulted by use of AEC/Romulin AEC chromogen substrate (Biocare medical, Walnut Creek, USA). Blue staining of ED2 (antibody from Serotec, Raleigh, USA) as Kupffer cell marker was realized with the ChemMate™ APAAP Kit (Dako, Hamburg, Germany) based on the alkaline phosphatase-anti alkaline phosphatase method. As substrate for the alkaline phosphatase served the Alkaline Phosphatase Substrate Kit III (Linaris, Wertheim, Germany). Samples were counterstained in hematoxylin solution.

10.3 Hematoxylin/eosin (H/E) staining

HE staining was performed by Dr. H. Meißner and Andrea Sendelhofert, Institute of Pathology, University of Munich, Germany.

Paraffin embedded liver slices were incubated for 2 min in Papanicolaous solution 1a (Harris' hematoxylin solution), washed for 6 min in demin. water and then incubated for 3 min in a 0.5% eosin solution. After washing in demin. water (30 sec), the slides were immersed in increasing concentrations of ethanol (70%, 96%, 100% ethanol, 5 times each) and in xylol solution. The slices were mounted in Eukitt® and photographed (400-fold amplification, Zeiss Axioskop, Carl Zeiss Mikroskopie, Jena, Germany).

10.4 Trypan blue uptake of liver tissue

Experiments were performed by Dr. M. Bilzer (Department of Medicine II) and Dr. H. Arnholdt (Institute of Pathology), University of Munich, Germany.

Trypan blue uptake of necrotic cells was evaluated at the end of the 2-hour reperfusion period. Livers were perfused with trypan blue (0.2 mmol/L) for 10 min and fixed with formalin (1.5%) during a 5-min perfusion. The fixed liver tissue was embedded in paraffin. Slices of liver tissue were stained with eosin to visualize the cytoplasm.

11. Statistics

All data are expressed as mean and standard error, unless stated otherwise. The significance of differences between experimental groups was determined by one-way ANOVA with Bonferroni post-test or Student's t-test. $p<0.05$ was considered statistically significant. Analyses were performed with GraphPad Prism, Version 3.02, Graphpad Software Inc., San Diego, USA.

C. Results

1. Determination of apoptotic and necrotic cell death

For discrimination between apoptotic and necrotic cell death, commonly accepted biochemical methods combined with morphological criteria were used. As parameters for apoptotic cell death caspase-3-like activity in combination with caspase-3 protein processing and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) were performed. Morphological criteria were cell shrinkage, chromatin condensation and margination, and the occurrence of apoptotic bodies.

Necrotic cell death was characterized morphologically after hematoxylin and eosin (HE) staining and by determination of trypan blue uptake into injured cells. Cells were counted as necrotic when showing increased eosinophilia, vacuolization, cell disruption, loss of architecture, and karyolysis. These investigations were performed in a blinded fashion.

1.1 Evaluation of apoptosis

1.1.1 ANP and 8-Br-cGMP preconditioning reduce caspase-3-like activity and caspase-3 processing

Fluorometric DEVD-AFC assay showed a maximum of caspase-3-like activity after 24 h of cold ischemia. During reperfusion, caspase-3 like activity decreased to the basal activity levels of blood-free perfused control livers. Preconditioning with 200 nM ANP significantly reduced caspase-3 like activity by 53% after 24 h of cold ischemia and by 52% after 45 min of reperfusion in comparison to control livers (figure 11).

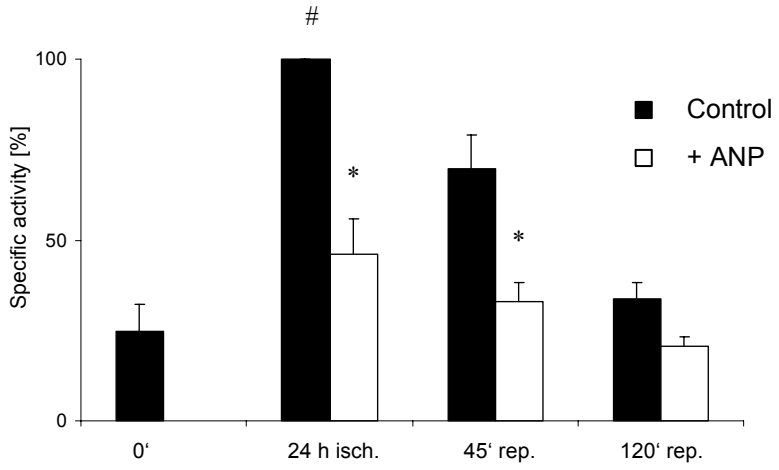

figure 11: ANP preconditioning reduce caspase-3-like activity.

*Liver were perfused for 30 min, stored in UW solution for 24 h (4°C) and were reperfused for 45 min and 120 min. At the indicated time points, untreated livers (control) and pre-treated livers (ANP 200 nM, 20 min) were snap frozen and prepared for fluorogenic measurement of caspase-3-like activity as described under "Materials and Methods". Columns show mean ± SEM of three independent experiments with n=4-5 organs per group. *p<0.05 significantly different from untreated control, #p<0.05 significantly different from 0' (Student's t-test).*

Similarly, pretreatment of livers with 50 µM 8-Br-cGMP showed a significant reduction of caspase-3 like activity after 24 h of cold ischemia. The extent of inhibition after cold ischemia was by 51% and after 45 min of reperfusion by 61% (figure 12). Control experiments confirmed that ANP and 8-Br-cGMP itself had no direct inhibitory effect on caspase-3-like activity (data not shown).

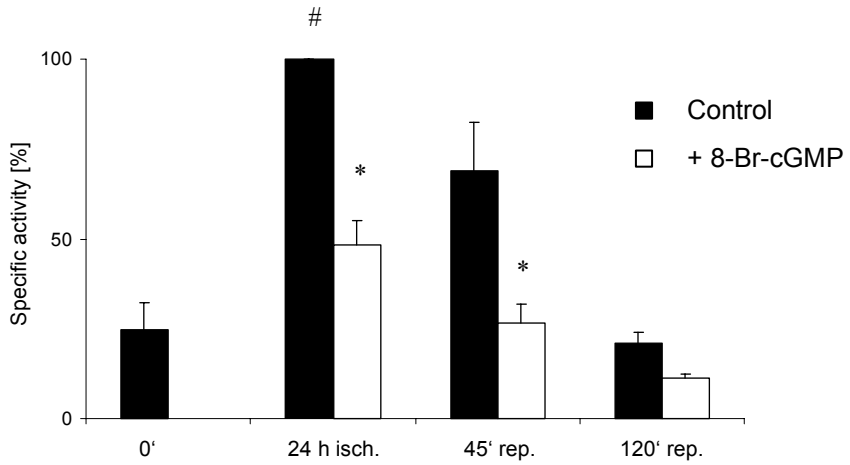

figure 12: 8-Br-cGMP preconditioning reduce caspase-3-like activity.

*At indicated time points (0': shortly perfused, no ischemia; 24 h of cold ischemia; 24 h of cold ischemia and 45 min and 120 min of reperfusion, each), control livers and pre-treated livers (8-Br-cGMP 50 µM) were snap frozen and prepared for fluorogenic measurement of caspase-3-like activity as described under "Materials and Methods". Columns show mean ± SEM of three independent experiments with n=4-5 organs per group. *p<0.05 significantly different from untreated control, #p<0.05 significantly different from 0' (Student's t-test).*

Western blot analysis was performed to detect caspase-3 protein cleavage. In pre-ischemic liver homogenates, caspase-3 precursor CPP32 was clearly detectable in contrast to its cleavage product p17, the proteolytic active subunit (figure 13). After 24 h of cold ischemia both the amount of precursor and proteolytic active subunit increased to a maximum, indicating highest enzyme activity. At 120 min of reperfusion, only small amounts of precursor and p17 cleavage product were detectable.

Preconditioning with ANP diminished the amount of caspase-3 precursor CPP32 and the active p17 subunit compared to control livers. This was apparent both after 24 h of cold

ischemia and after 120 min of reperfusion (figure 13).

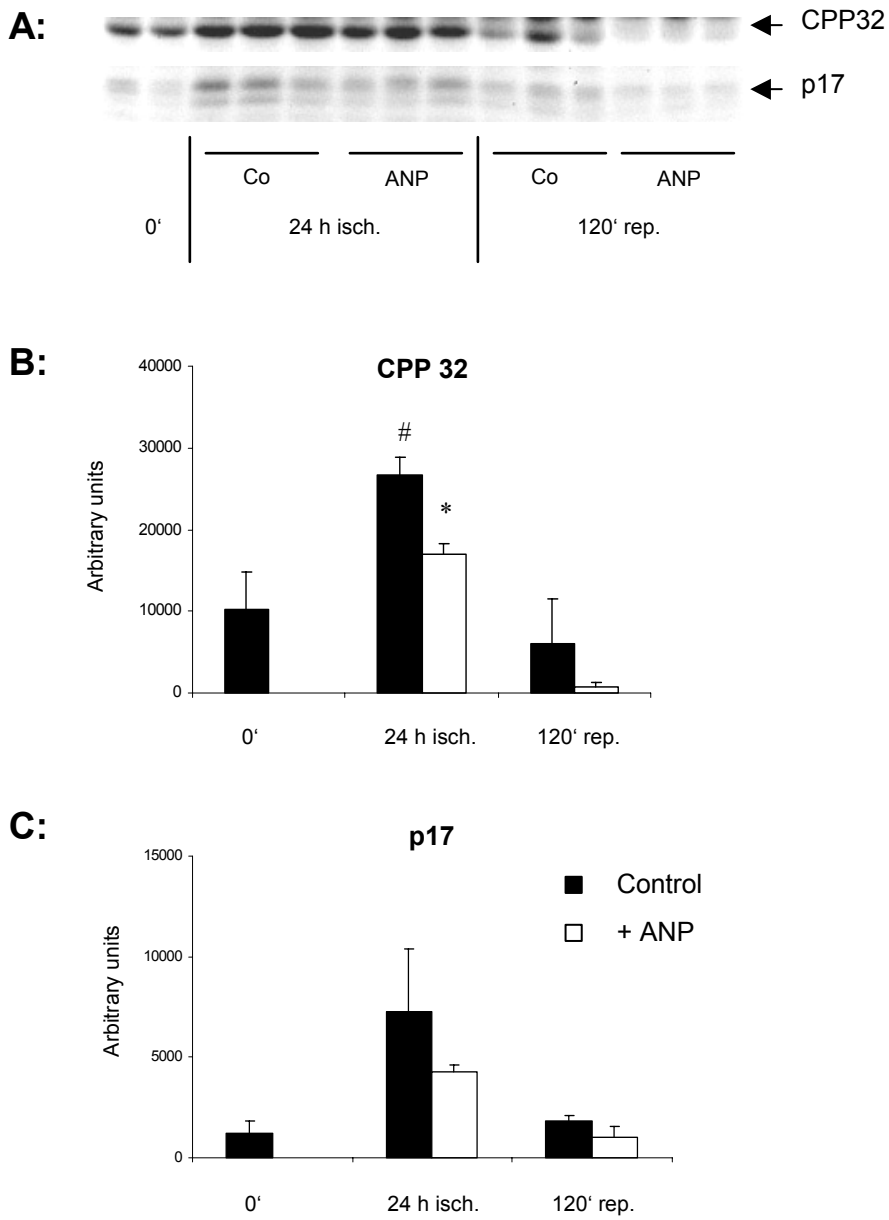

figure 13: Reduced processing of caspase-3 in ANP pre-treated livers.

*Liver were perfused for 30 min, stored in UW solution for 24 h (4°C) and were reperfused for 120 min. At indicated time points, untreated control livers and pre-treated livers (ANP 200 nM) were snap frozen. Western blot with caspase-3 antibody was performed as described under "Materials and Methods". CPP32: caspase-3 precursor; p17: proteolytic active subunit. Data show one representative blot out of three independent experiments (Western blot: panel A; densitometric analysis: panel B: CPP32, panel C: p17). *p<0.01 significantly different from untreated control, #p<0.05 significantly different from 0' (Student's t-test). Bars show mean ± SEM with n=2-3 organs per group.*

Preconditioning with 8-Br-cGMP (figure 14) revealed similar but less distinctive influence

on the amount of caspase-3 precursor CPP32. The active p17 subunit was not significantly decreased by 8-Br-cGMP compared to control livers.

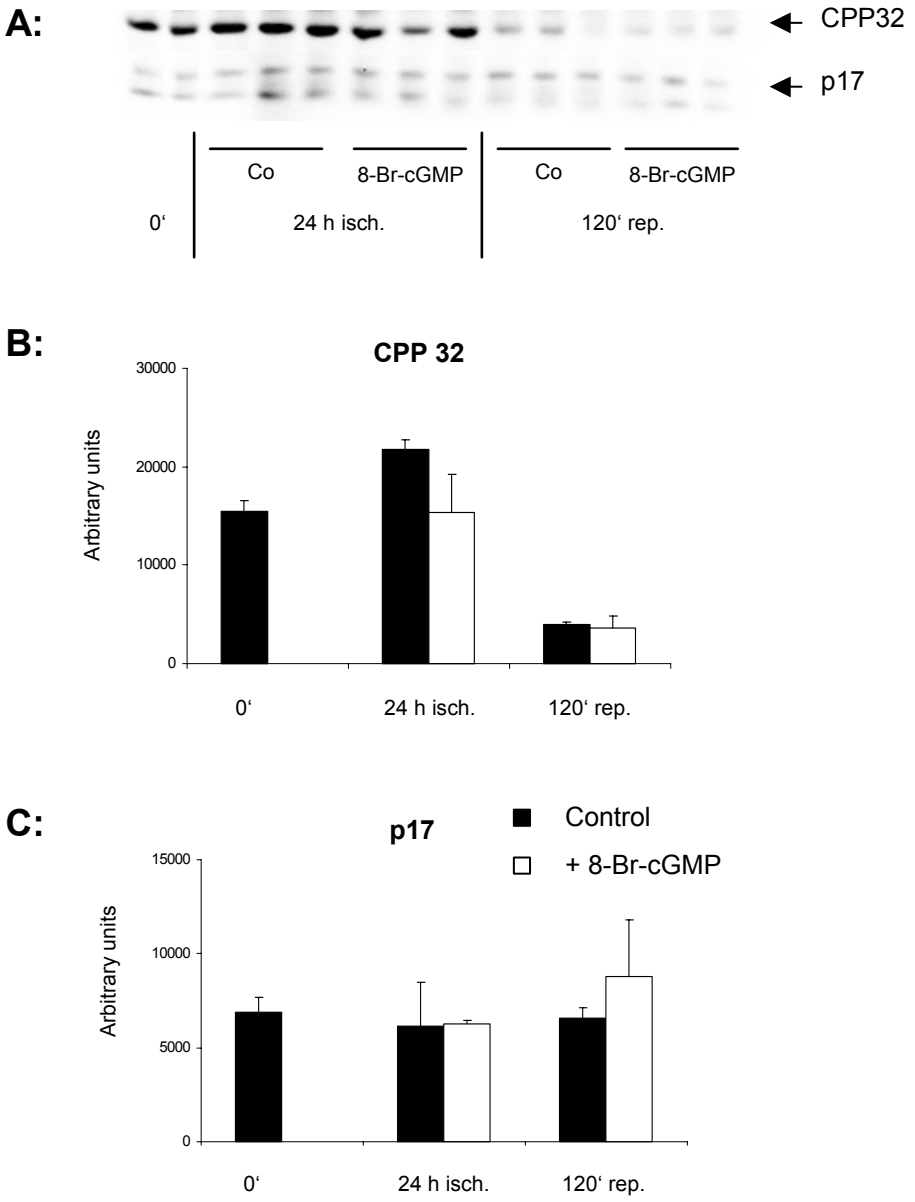

figure 14: Reduced processing of caspase-3 in 8-Br-cGMP pre-treated livers.

After 30 min of perfusion, livers were stored in UW-solution (4°C) for 24 h and were reperfused for 120 min. At indicated time points untreated control livers and pre-treated livers (8-Br-cGMP 50 µM) were snap frozen. Western blot with caspase-3 antibody was performed as described under "Materials and Methods". CPP32: caspase-3 precursor; p17: proteolytic active subunit. Data show a representative blot out of three independent experiments (Western blot: panel A; densitometric analysis: panel B: CPP32, panel C: p17). Bars show mean ± SEM with n=2-3 organs per group.

1.1.2 ANP and 8-Br-cGMP preconditioning decrease the proportion of TUNEL-positive cells

Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) of liver sections showed only few TUNEL positive stained hepatocytes and endothelial cells with characteristical morphology for apoptosis in control livers at the beginning of perfusion. After 24 h of cold ischemia, TUNEL positive hepatocytes increased maximally, decreased after 45 min of reperfusion, and again augmented at 120 min. Endothelial cells showed a similar pattern for TUNEL positive cells but to a much lesser extent.

Preconditioning with ANP (figure 15) reduced the number of TUNEL positive hepatocytes after 24 h of cold ischemia by 37%, but did not show a significant difference at the different reperfusion time points. Staining of endothelial cells was not altered by preconditioning with ANP.

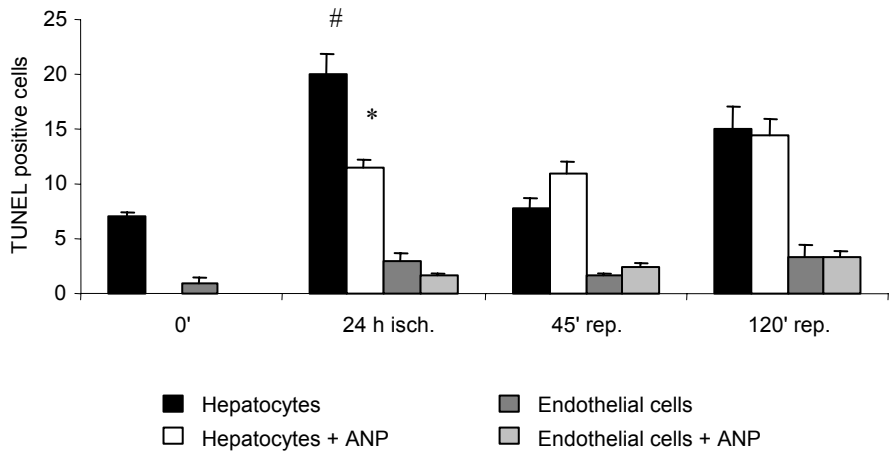

figure 15: ANP preconditioning decreases proportion of apoptotic cells after ischemia.

*Liver perfusion was performed (30 min), followed by storage in UW-solution for 24 h (4°C), and reperfusion for 45 or 120 min At indicated time points, untreated control livers and pre-treated livers (ANP 200 nM) were fixed in formalin, imbedded in paraffin, and prepared for TUNEL analysis as described under "Materials and Methods". Columns show mean ± SEM of four to five independent experiments. *p<0.05 significantly different from untreated control, #p<0.05 significantly different from 0' (Student's t-test).*

Preconditioning with 8-Br-cGMP (figure 16) similarly to ANP decreased the number of TUNEL positive hepatocytes after 24 h of cold ischemia by 28%. Again, there was no significant difference during reperfusion and no influence on staining of endothelial cells.

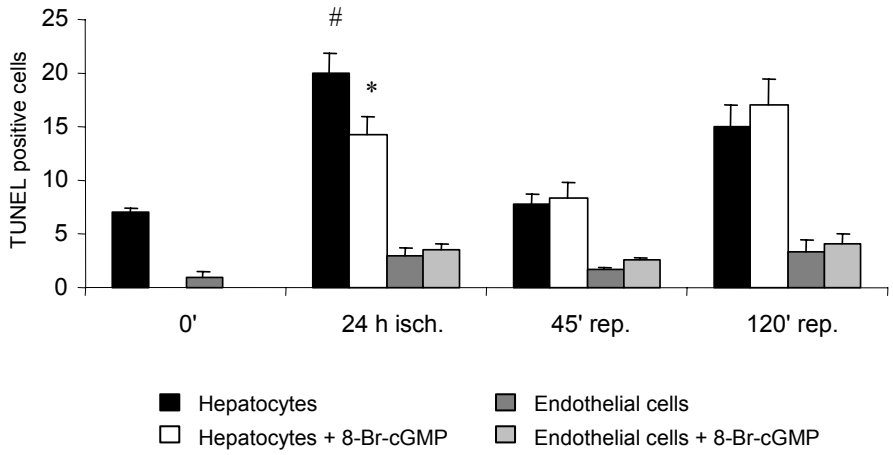

figure 16: 8-Br-cGMP preconditioning decreases proportion of apoptotic cells after ischemia.

*At indicated time points (0': shortly perfused, no ischemia; 24 h of cold ischemia; 24 h of cold ischemia and 45 min and 120 min of reperfusion) control livers and pre-treated livers (8-Br-cGMP 50 µM) were fixed in formalin, embedded in paraffin, and prepared for TUNEL analysis as described under "Materials and Methods". Columns show mean ± SEM of four to five independent experiments. *p<0.05 significantly different from untreated control, #p<0.05 significantly different from 0' (Student's t-test).*

1.2 Evaluation of necrosis

1.2.1 Decreased degenerative changes in ANP pre-treated livers

HE staining of cold preserved livers displayed significant evidence of cell injury, but only few necrotic cells. Hepatocytes showed large, clear vacuoles within the cytoplasm. Moderate swelling and loosening of intercellular attachments was visible with predominant location in the periportal area. ANP pre-treatment showed decreased degenerative changes with less vacuolization and improved cellular integrity (figure 17).

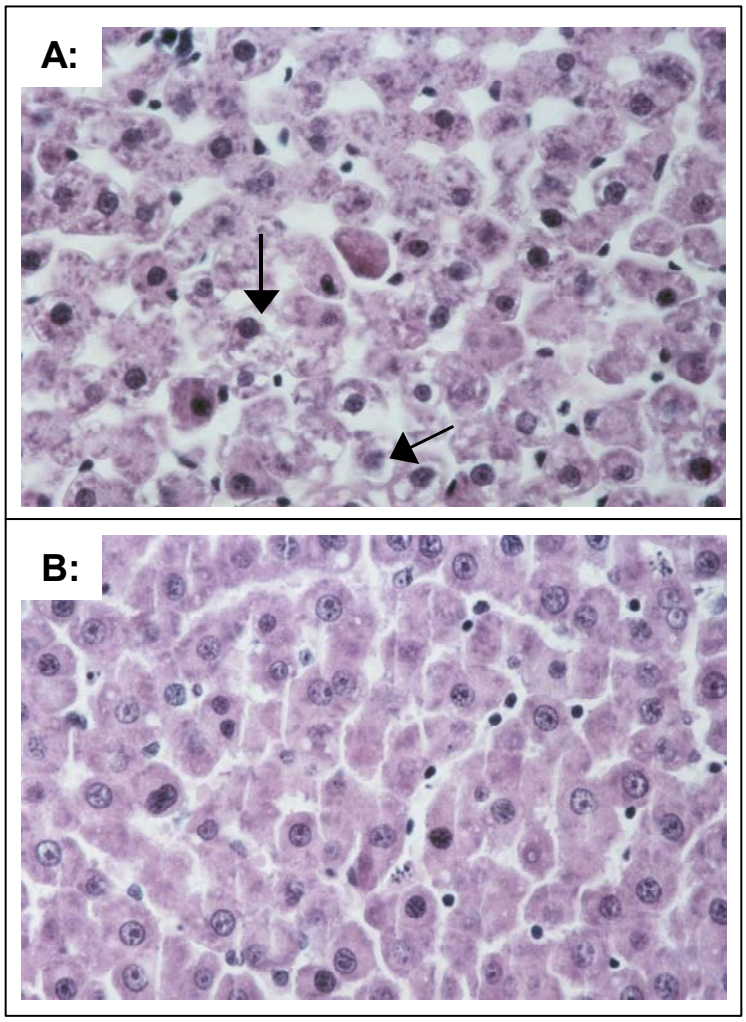

figure 17: Degenerative changes of hepatocytes are decreased by ANP pretreatment.

After 24 h of preservation in UW solution, liver slices were fixed in formalin solution (1.5%) and embedded in paraffin. HE staining was performed using a standard protocol. Panel A: Arrows show cytoplasmatic vacuolization as indicator of degenerative cell damage in control livers. Panel B: ANP pretreated liver slice with improved morphology. Original magnification: 400–fold.

1.2.2 Trypan blue uptake is significantly reduced by ANP preconditioning

Histological analysis of livers perfused with trypan blue at the end of the 2-hours reperfusion period revealed increased staining of liver cells, indicating necrotic cell death. Pre-treatment with ANP significantly reduced trypan blue uptake in hepatocytes in periportal but not in pericentral areas. Similarly, endothelial cells were protected by ANP preconditioning in periportal but not in pericentral areas (figure 18).

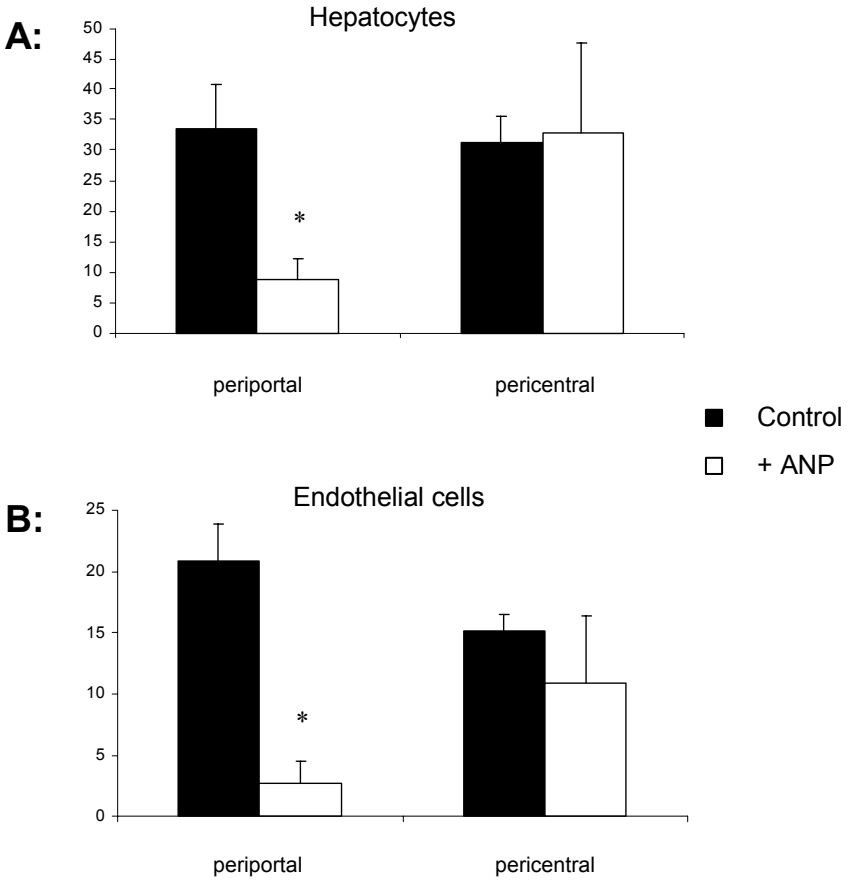

figure 18: Necrotic damage during reperfusion is reduced by ANP preconditioning in hepatocytes.

*After 24 h of preservation in UW solution and 2 h of reperfusion with KH buffer, untreated control livers and pretreated livers (ANP 200 nM) were perfused with trypan blue (0.2 mmol/L) for 10 minutes and fixed with formalin (1.5%) during a 5-minute perfusion as described under "Materials and Methods". Panel A: quantification of trypan blue uptake in hepatocytes in periportal and pericentral areas, panel B: endothelial cells. Columns show mean ± SD of four to five independent experiments. *p<0.001 significantly different from untreated control (Student's t-test)*

2. Signal transduction of ANP protection

2.1 Involvement of cGMP-dependent protein kinases

Due to controversial information concerning expression of cGMP-dependent protein kinases in the liver, the occurrence of cGMP-dependent protein kinases in the liver was investigated by RT-PCR. Experiments showed no expression of cGMP-dependent protein kinase I (PKG I) in the liver, whereas it could be clearly detected in the brain as positive organ (Tamura *et al.*, 1996; Sandberg *et al.*, 1989). Similarly, it was not possible to amplify the cGMP-dependent protein kinase II (PKG II). The intestine and the brain, organs with

high expression of PKG II (Jarchau *et al.*, 1994), served as positive control(figure 19, panel A). Isolation procedure of RNA and reverse transcription were verified by control amplification with the house keeping gene GAPDH (figure 19, panel B). These results suggested no presence of PKG in livers.

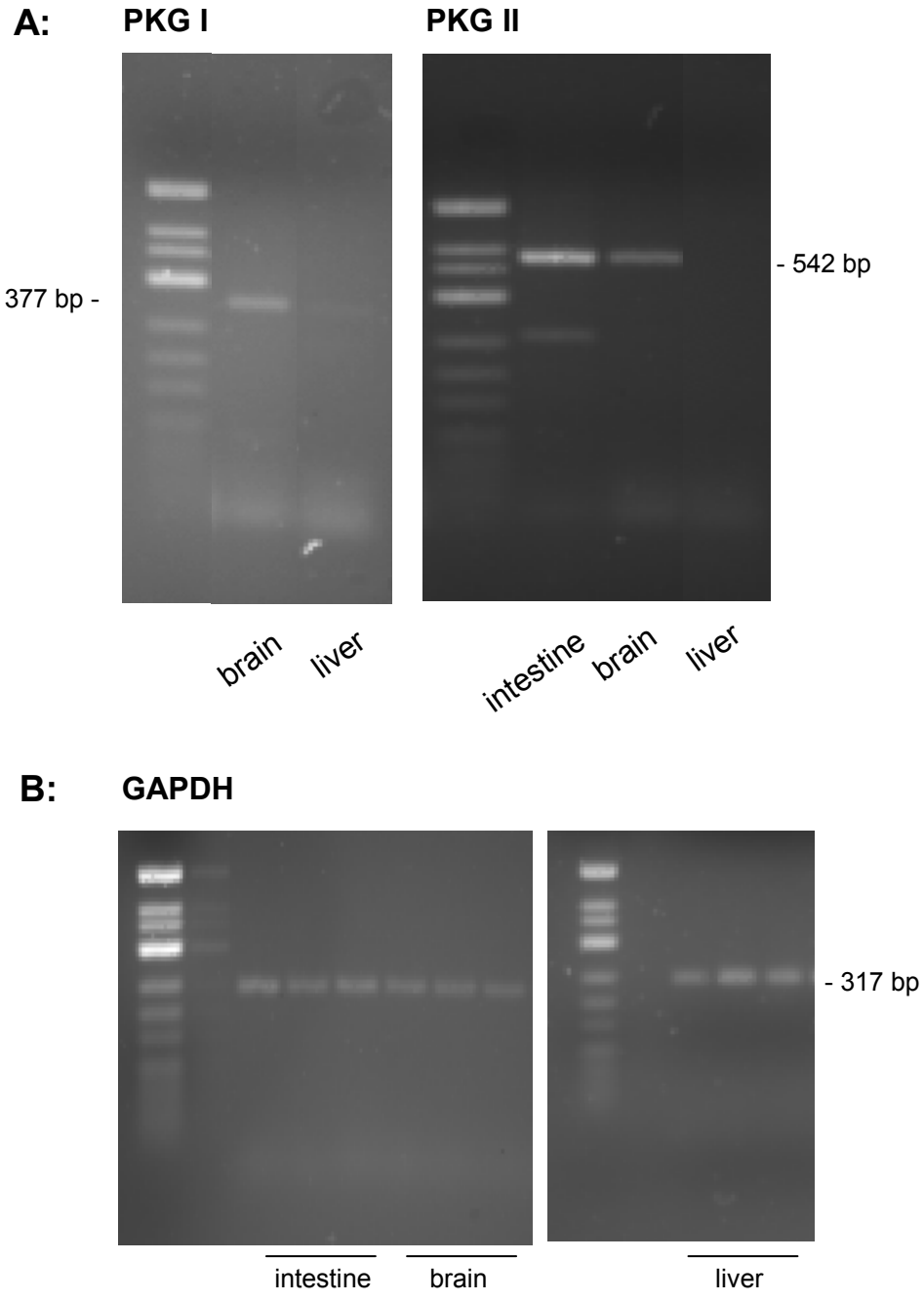

figure 19: Expression of cGMP-dependent protein kinases.

Livers were flushed blood free and prepared as described under "Materials and Methods". Intestine and brain as control organs were immediately removed and rinsed in PBS before snap freezing in liquid nitrogen. Panel A: RT-PCR experiments were performed with primers detecting cGMP-dependent protein kinase I (PKG I) and II (PKG II). Panel B: Amplification of

GAPDH as housekeeping gene was performed with primers detecting GAPDH. Data show one out of three independent experiments.

2.2 Effect of cGMP- and cAMP-dependent protein kinase inhibitors on the antiapoptotic property of ANP

Due to the knowledge of interaction of cGMP with the PKA pathway and to confirm the absence of PKG, the involvement of cAMP- and cGMP-dependent protein kinases was investigated on their action on apoptotic cell death by the use of specific inhibitors of these enzymes.

Due to their mode of action of binding to the cyclic nucleotide binding sites of the PKG and PKA enzymes, the compounds had to be infused into the rat liver before addition of ANP. The inhibitors alone had no direct influence on the caspase-3-like activity assay (data not shown).

2.2.1 Effects on caspase-3-like activity

The influence of the protein kinase inhibitors on apoptotic cell death was investigated after 24 h of cold ischemia, the time point with highest incidence of apoptosis. Preconditioning of livers lead to a significant reduction of caspase-3-like activity and confirmed the results shown in 1.1.1. Additional treatment with the cGMP-dependent protein kinase inhibitor Rp-8-pCPT-cGMPS (1 µM) did not change ANP action. These results suggested no involvement of PKG action in hepatoprotection conveyed by ANP supporting the observed missing of PKG in the liver. In contrast, infusion of the cAMP-dependent protein kinase inhibitor Rp-8-Br-cAMPS (1 µM) completely prevented ANP's antiapoptotic properties pointing to the involvement of PKA (figure 20). Perfusion of inhibitors alone did not significantly influence caspase-3-like activity (data not shown).

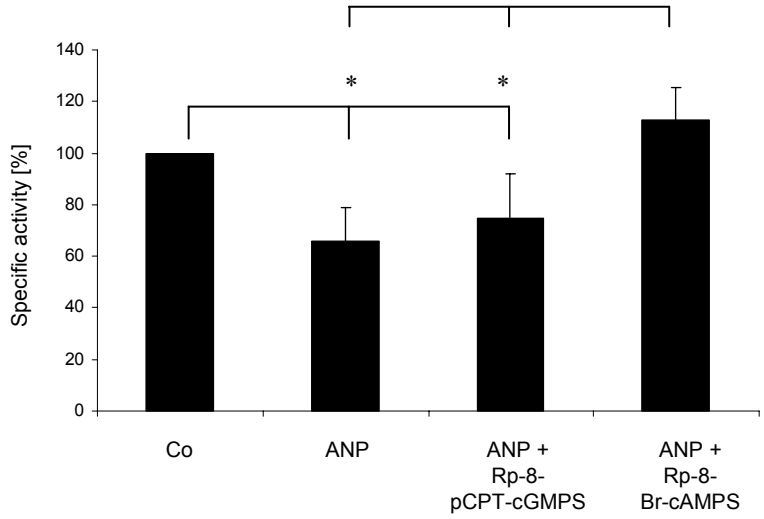

figure 20: Effect of cGMP- and cAMP-dependent protein kinase inhibitors on the antiapoptotic property of ANP.

*After 24 h of cold ischemia, control livers and pre-treated livers (ANP 200 nM) were snap frozen and prepared for fluorogenic measurement of caspase-3-like activity as described under "Materials and Methods". Rp-8-pCPT-cGMPS (1 µM): PKG inhibitor, Rp-8-Br-cAMPS (1 µM): PKA inhibitor. Data are expressed as percent of enzyme activity of untreated control livers at 24 h of cold ischemia, which was set as 100%. Columns show mean ± SEM of two independent experiments with n=4-5 organs per group. *p<0.05 significantly different from untreated control and ANP/Rp-8-pCPT-cGMPS treated livers (Student's t-test).*

2.3 Activation of cAMP-dependent protein kinase by ANP

The previous results suggested a role for PKA. In order to determine the capability of ANP to directly activate cAMP-dependent protein kinase A (PKA) it was investigated whether ANP in fact directly activates PKA in whole organs and in isolated hepatocytes.

2.3.1 Liver

In whole liver homogenates, 20 min perfusion with ANP lead to a slight increase of PKA activity compared to control livers (figure 21). After 24 h of cold ischemia, no difference in PKA activity between ANP treated and untreated livers was visible (data not shown). These result suggested early effect of ANP on PKA in liver. Due to big variability in the values measured the experiments were additionally performed in primary hepatocytes.

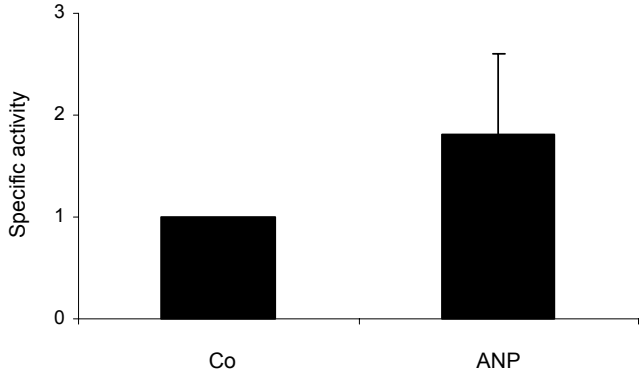

figure 21: Induction of PKA activity in liver.

Liver perfusion was performed (30 min) in the presence of ANP (20 min, 200 nM) or absence (control). PKA activity assay was performed as described under "Materials and Methods". Values expressed as x-fold increase of specific activity in comparison to untreated control. Columns show mean ± SEM of two independent experiments with n=3 organs per group.

2.3.2 Hepatocytes

Incubation of hepatocytes with ANP (200 nM) led to a significant increase in PKA activity (3.4-fold). Similarly, 8-Br-cGMP activated PKA but to larger extent (5.7-fold). The positive control Bt_2-cAMP increased PKA activity by 26-fold (figure 22).

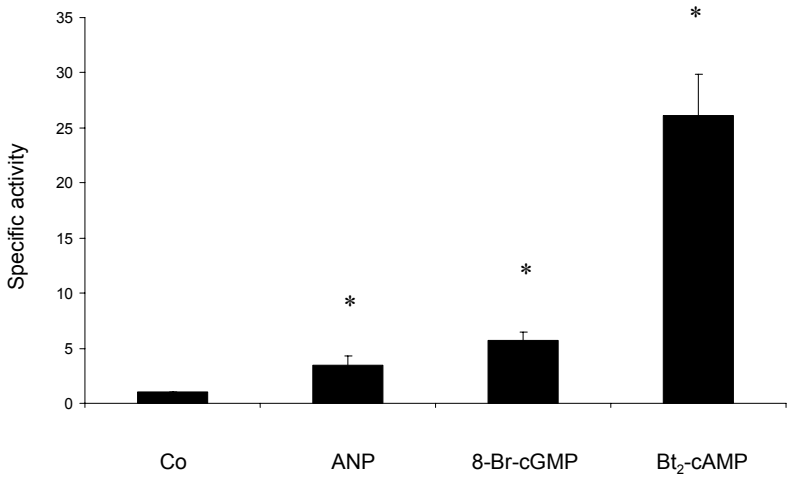

figure 22: Induction of PKA activity in hepatocytes.

*Hepatocytes were isolated as described under "Materials and Methods". Cultured hepatocytes were left unstimulated (Control) or treated with ANP (200 nM), 8-Br-cGMP (200 µM) and Bt_2-cAMP (250 µM) for 60 min. PKA activity assay was performed as described under "Materials and Methods". Specific activity is expressed as x-fold increase in comparison to untreated control. Columns show mean ± SEM of three independent experiments with n=3 samples per group. *p<0.05 significantly different from untreated control (Student's t-test).*

These results show, that an activation of PKA by ANP or 8-Br-cGMP could contribute to the observed antiapoptotic action, that is mitigated by simultaneous perfusion with an PKA inhibitor.

3. Heme oxygenase-1

Induction of the heme oxygenase-1 enzyme (HO-1) in IRI of the liver is discussed to have protective properties. A potential involvement of HO-1 in ANP mediated protection should be clarified by determining HO-1 expression in rat livers.

3.1 cGMP-independent induction of HO-1 by ANP preconditioning

In order to investigate whether HO-1 is upregulated in the system of ischemia and reperfusion, HO-1 Western and Northern blots of liver samples obtained pre- and post-ischemia and at two different time points of reperfusion (45 and 120 min) were performed. These experiments revealed no significant changes in HO-1 expression in the course of ischemia and reperfusion, neither in HO-1 mRNA nor in protein levels (figure 23).

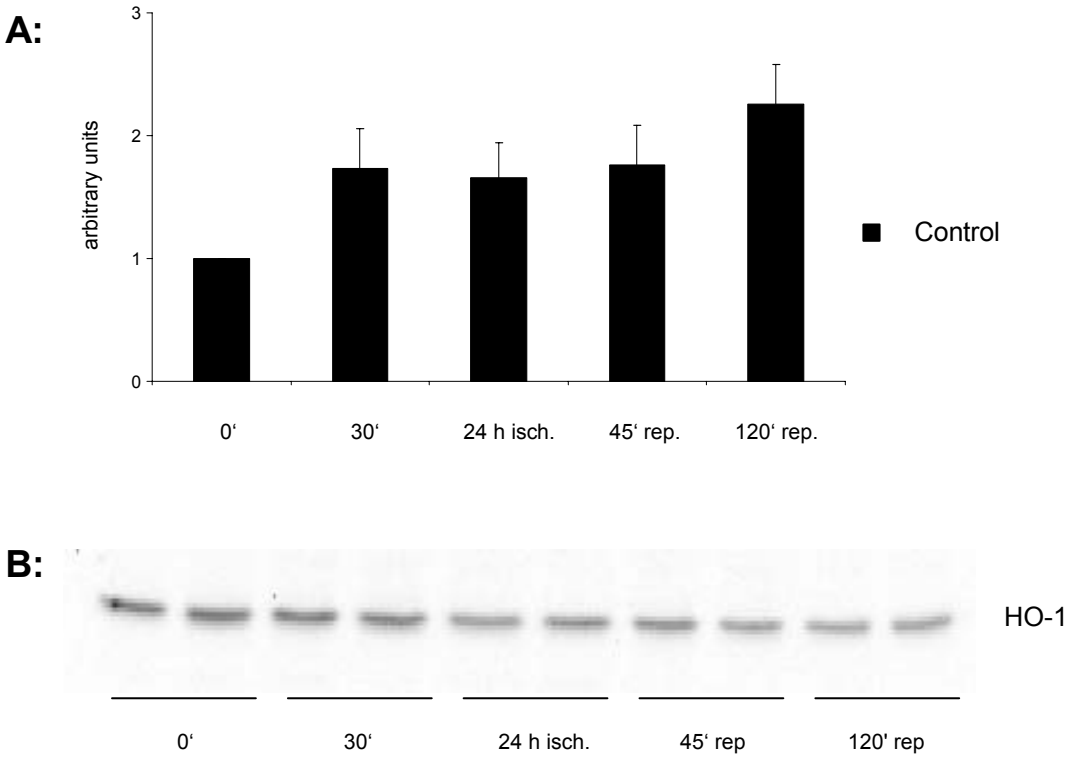

figure 23: HO-1 expression during ischemia and reperfusion.

Livers were perfused for 30 min, stored in UW solution for 24 h (4°C), and reperfused for 45 or 120 min. Panel A: HO-1 mRNA expression was determined by Northern blot. Normalization of RNA was performed by probing with a 28S oligonucleotide probe. Values for 0 min (0': livers flushed blood-free with KH buffer) were set as 1. Data show mean ± SEM of phosphorimaging values out of two independent blots with n = 2 – 4 organs per treatment group. Panel B: HO-1 protein expression was determined by Western blots. Experiments were performed as described under "Materials and Methods".

A significant increase in HO-1 mRNA levels in livers treated with ANP for 20 min was observed, whereas ANP did not affect post-ischemic HO-1 mRNA (figure 24).

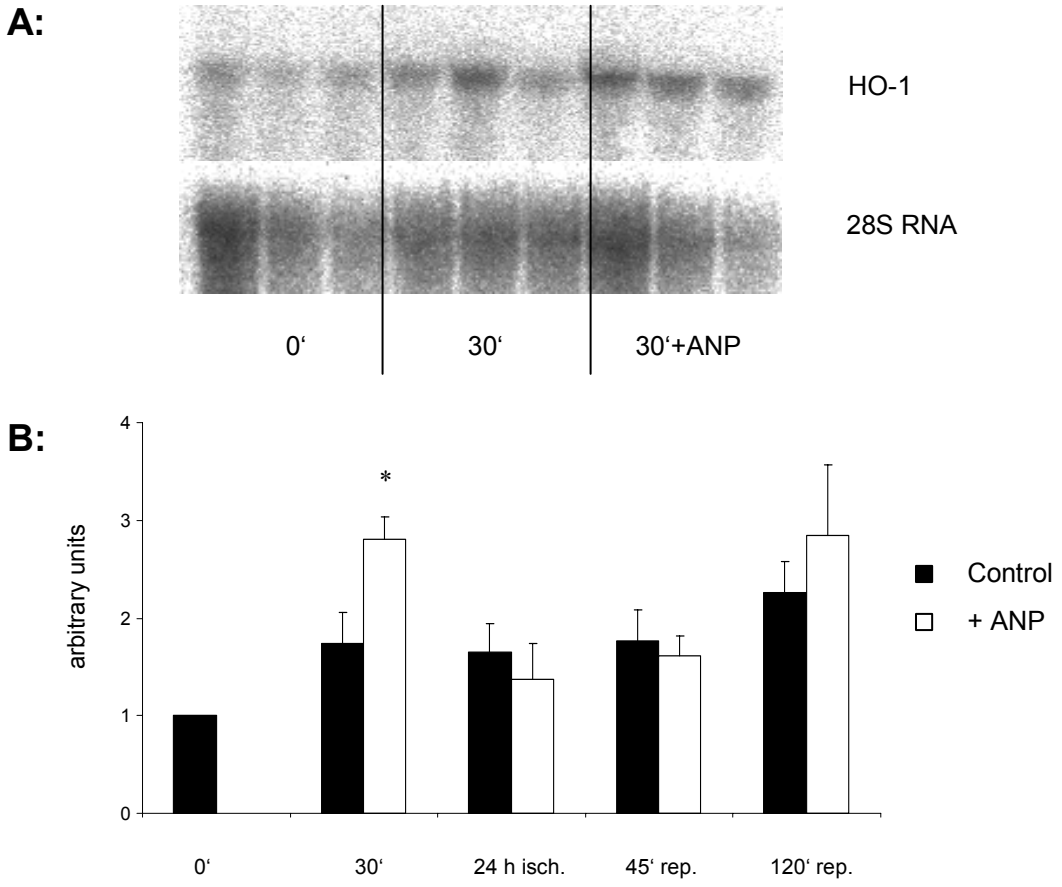

figure 24: Influence of ANP preconditioning on HO-1 mRNA expression.

*Panel A shows HO-1 expression of livers preconditioned with ANP (200 nM) for 20 min in comparison to untreated organs. Data show one representative out of 6 independent experiments. Panel B: Phosphorimaging analysis of Northern blots investigating HO-1 expression during ischemia and reperfusion. HO-1 signals were normalized by 28S signals whereby values for 0 min (0': livers flushed blood-free with KH buffer) were set as 1. Data show mean ± SEM of two independent blots with n = 2 – 4 organs per treatment group *p<0.05 represents significant differences to untreated organs (Student's t-test).*

HO-1 protein was slightly but not significantly elevated in ANP-preconditioned livers. ANP did not affect post-ischemic protein expression (figure 25).

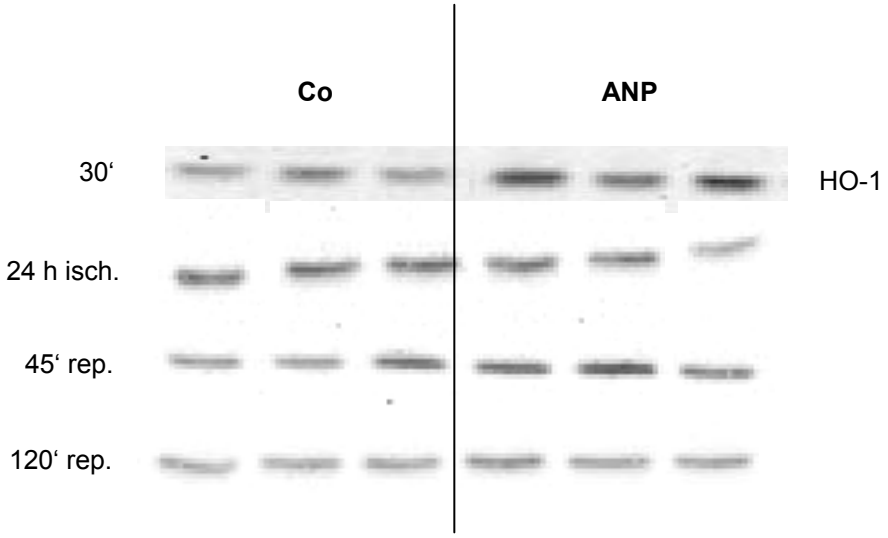

figure 25: Influence of ANP preconditioning on HO-1 protein expression.

Liver perfusion was performed (30 min) in the presence or absence of ANP (200 nM), followed by storage in UW-solution for 24 h (4°C), and reperfusion for 45 or 120 min. HO-1 Western blots were performed as described under “Materials and Methods”. Data show one representative out of 6 independent experiments. Data show mean ± SEM of two independent blots with n = 2 – 4 organs per treatment group.

In order to elucidate whether the effect of ANP on HO-1 mRNA is mediated *via* its guanylate cyclase coupled A receptor, HO-1 mRNA levels in livers preconditioned with the cGMP analogue 8-Br-cGMP (50 µM) were investigated. Interestingly, 8-Br-cGMP did not induce HO-1 (figure 26) suggesting that ANP mediates HO-1 induction independently of cGMP.

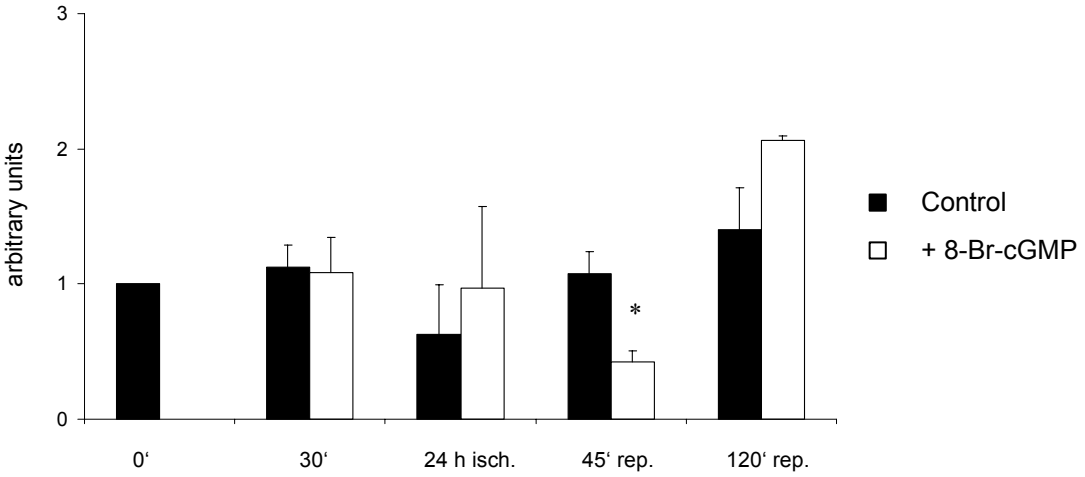

figure 26: Influence of 8-Br-cGMP on HO-1 mRNA expression.

Liver perfusion was performed (30 min) in the presence or absence of 8-Br-cGMP (50 µM), followed by storage in UW-solution for 24 h (4°C), and reperfusion for 45 or 120 min.

*Phosphorimaging analysis of HO-1 Northern blots was performed and signals were normalized by 28S signals whereby values for 0 min (0': livers flushed blood-free with KH buffer) were set as 1. Data show mean ± SEM of three independent blots with n = 2 – 4 organs per treatment group. *p<0.05 represents significant differences to untreated organs (Student's t-test).*

3.2 Localization of HO-1

The next point of interest was to elucidate the cell type responsible for expression of HO-1 in ANP-treated livers. *In situ* hybridization experiments showed that HO-1 mRNA in control organs is detectable exclusively in Kupffer cells (figure 27). HO-1 mRNA after ANP treatment was also only detectable in Kupffer cells.

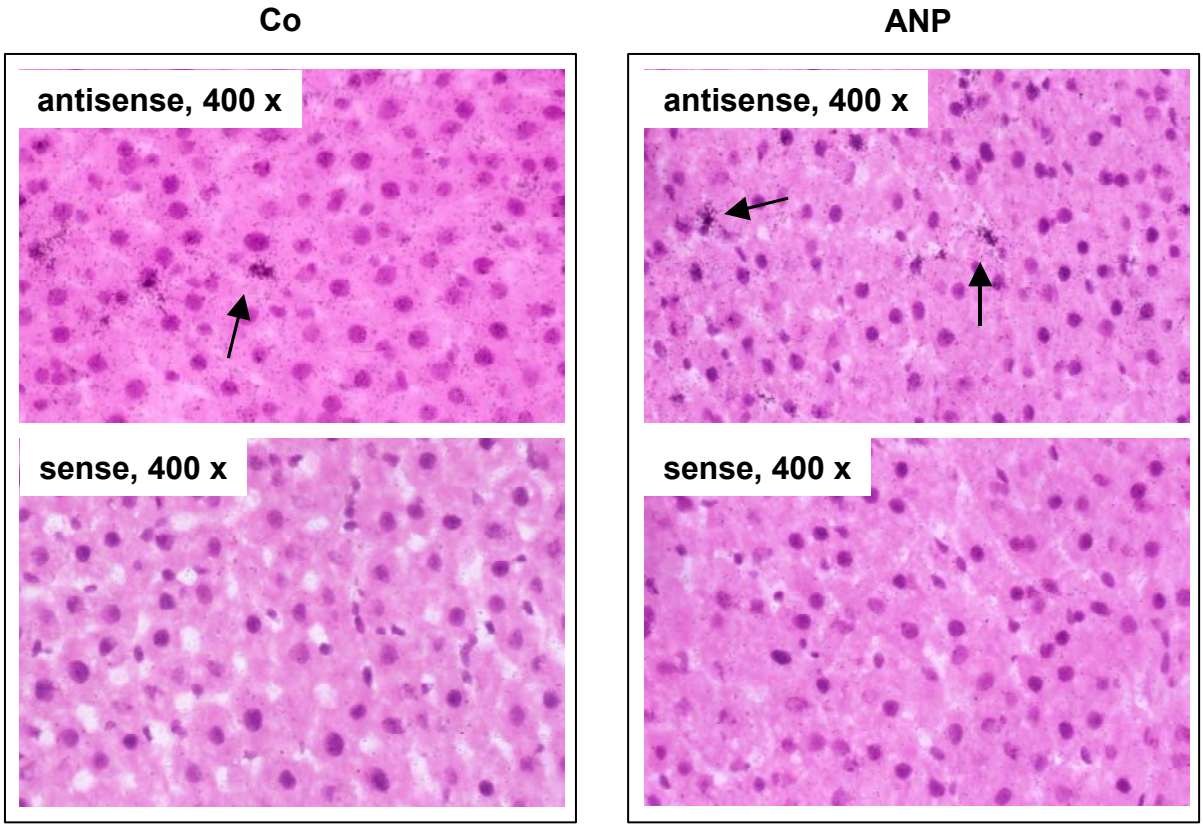

figure 27: Localization of HO-1 mRNA in liver tissue.

Livers were perfused with KH buffer for 30 min in the absence (control) or presence of ANP (200 nM), which was added after 10 min of perfusion. In situ hybridization with antisense and sense HO-1 RNA probes was performed in snap-frozen liver samples. Arrows mark cells which hybridized the HO-1 probe; due to their immunohistochemical features these cells were characterized as Kupffer cells. Original magnification 400–fold as specified in the figure.

Immunostaining revealed comparable results for HO-1 protein: double-staining with ED2 and anti-HO-1 antibody indicated that both basal as well as ANP-induced HO-1 protein was detectable in Kupffer cells but not in parenchymal cells (figure 28).

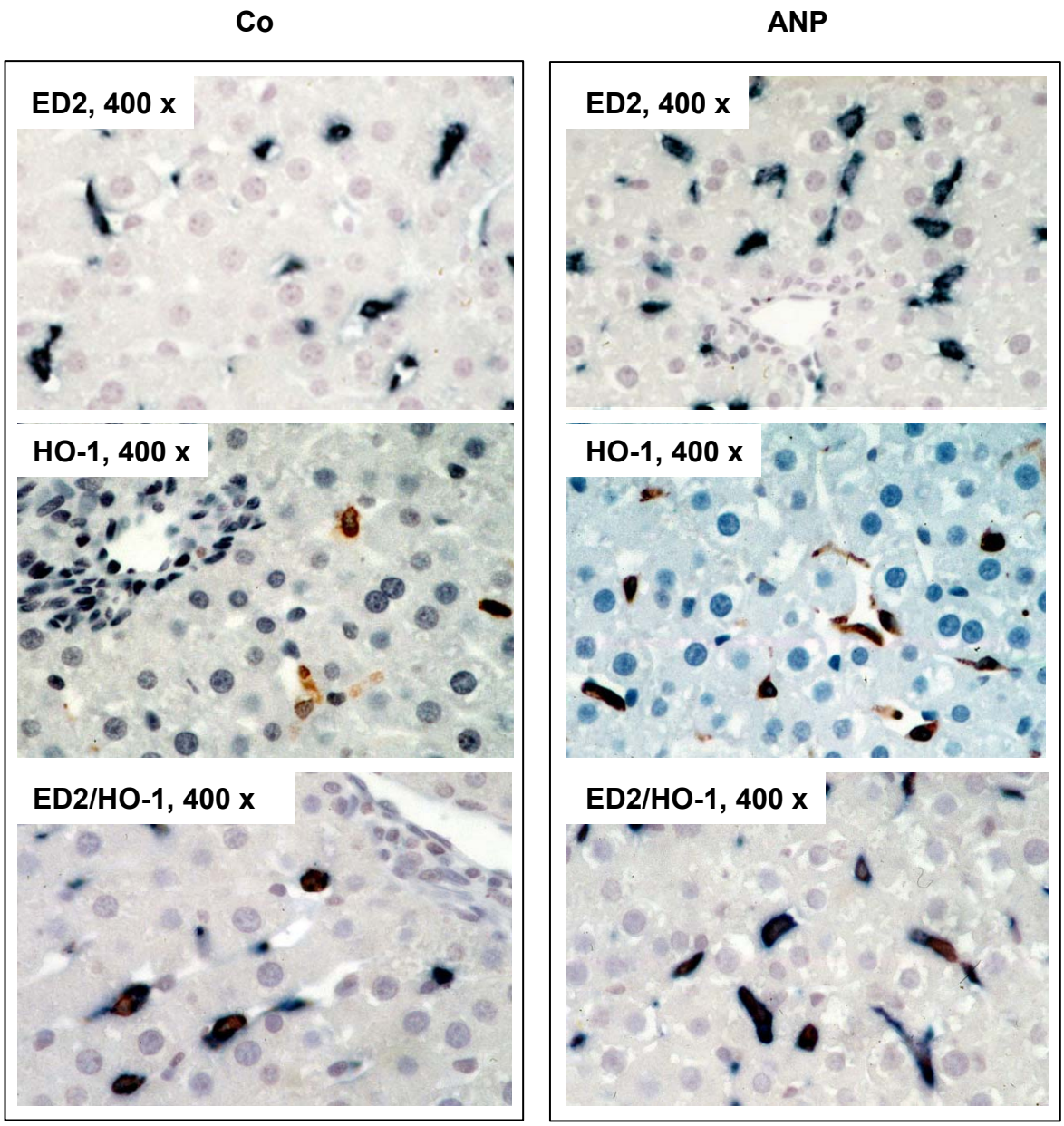

figure 28: Localization of HO-1 protein in liver tissue.

Livers were perfused with KH buffer for 30 min in the absence (control) or presence of ANP (200 nM), which was added after 10 min of perfusion. Immunohistochemistry: Liver samples were immunostained with an anti-ED2 antibody to identify Kupffer cells (blue color). HO-1 protein expression was visualized by an anti-HO-1 antibody (red color). Costaining was performed with both antibodies. Original magnification 400–fold as specified in the figure.

3.3 Induction of HO-1 in isolated Kupffer cells

The data obtained with *in situ* hybridization and with immunostaining indicated that HO-1 expression induced by ANP occurred only in Kupffer cells but not in parenchymal cells. In order to exclude that these data were due to a lack of sensitivity of these techniques for

hepatocyte HO-1 expression, additional experiments with isolated liver cells were performed.

Western blot experiments confirmed that ANP in fact induces HO-1 also in purified Kupffer cell preparations. Moreover, as shown in whole liver, ANP did not induce HO-1 expression in hepatocytes. 8-Br-cGMP was not capable to induce HO-1 in hepatocytes either. The positive controls dibutyryl-cAMP and glucagon markedly induced HO-1 in hepatocytes which proves that the hepatocytes used were capable to respond to HO-1-inducing stimuli (figure 29).

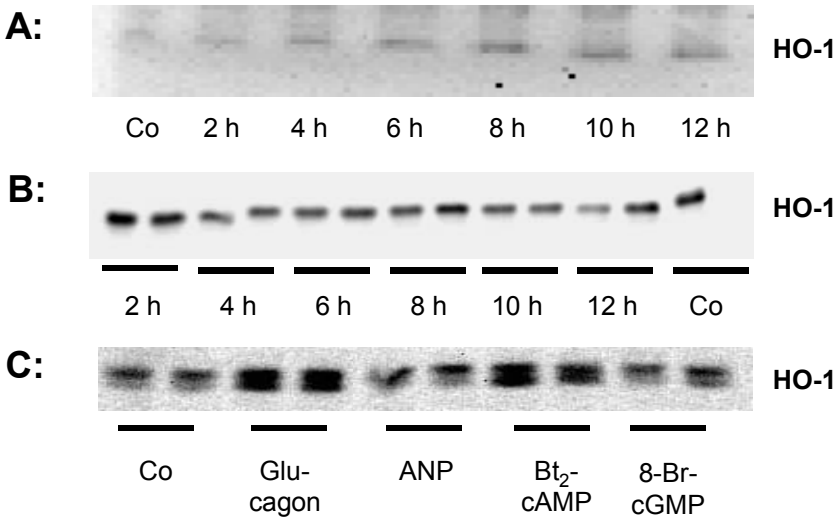

figure 29: Effects of ANP on HO-1 protein expression in Kupffer cells and hepatocytes.

Kupffer cells and hepatocytes were isolated as described under "Materials and Methods". Panel A: Isolated Kupffer cells were treated with ANP (200 nM) for the indicated time periods and HO-1 Western blots were performed. The figure shows one representative out of four independent experiments. Panel B: Isolated hepatocytes were treated with ANP (200 nM) for the indicated time and HO-1 Western blots were performed. The figure shows one representative blot out of 4 independent experiments. Panel C: Isolated hepatocytes were treated with ANP (200 nM), glucagon (50 nM) or dibutyryl-cAMP (Bt_2-cAMP 250 µM) for 6 h and HO-1 Western blots were performed. The figure shows one representative out of three independent experiments.

3.4 ZnPP perfusion

In order to demonstrate a causal relationship of ANP mediated mitigation of apoptosis and induction of HO-1, liver perfusions with the HO-1 activity inhibitor ZnPP were performed. Additional perfusion with ZnPP did not abrogate caspase-3-like activity inhibition by ANP. Interestingly, ZnPP perfusion by itself lead to a significant reduction of caspase-3-like activity. Control experiments revealed no direct interaction of ZnPP with the caspase-3-like activity assay (data not shown).

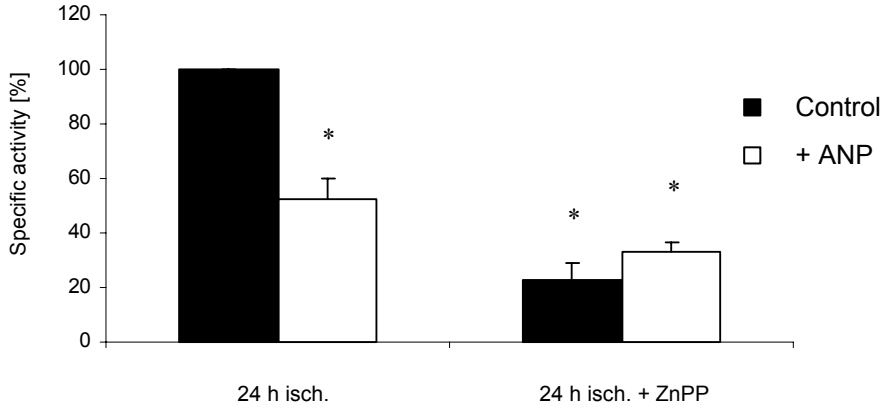

figure 30: Influence of ZnPP on caspase-3-like activity.

*Liver were perfused for 30 min and stored in UW solution for 24 h (4°C). Untreated control livers and pre-treated livers (ANP 200 nM and ZnPP 1 µM) were snap frozen and prepared for fluorogenic measurement of caspase-3-like activity as described under "Materials and Methods". Data is expressed as percent of enzyme activity of untreated control livers at 24 h of cold ischemia, which was set to 100%. Columns show mean ± SEM of two independent experiments with n=3-5 organs per group. *p<0.001 vs. untreated control (Student's t-test).*

D. Discussion

The cardiovascular hormone ANP has previously been demonstrated to protect livers against ischemia-reperfusion injury (Gerbes *et al.*, 1998). Aim of this work was the further characterization of protection and the elucidation of the associated signal transduction mechanisms.

1. Reduction of apoptosis and necrosis by ANP

Necrotic and apoptotic cell death was evaluated by applying common biochemical techniques (Miyoshi *et al.*, 1999). Caspase-3-like enzyme activation is known as an important characteristic of apoptotic cell death (Hengartner, 2000). Additionally to the results generated by measuring caspase-3-like activity, TUNEL analysis was performed to verify the occurrence of apoptotic cell death (Gavrieli *et al.*, 1992). The TUNEL assay detects DNA strand breaks by enzymatically labeling the free 3'-OH termini with modified nucleotides. These new DNA ends are generated upon DNA fragmentation and are typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferative nuclei, which have relatively insignificant numbers of DNA 3'-OH ends, are usually not stained. In some cases, cells exhibiting necrotic morphology may be stained (Grasl-Kraupp *et al.*, 1995). For this reason staining results were evaluated in conjunction with morphological criteria. Commonly accepted parameters of apoptosis, such as cell shrinkage, chromatin condensation and margination, and the occurrence of apoptotic bodies were therefore taken into account (Gujral *et al.*, 2001; Miyoshi *et al.*, 1999).

1.1 Reduction of apoptosis by ANP treatment

Apoptotic cell death was highest after 24 h of cold ischemia. This demonstrates an activation of the apoptotic cascade already during the ischemic period supporting the observations of Soeda et al. (Soeda *et al.*, 2001) and Rentsch et al. (Rentsch *et al.*, 2001). TUNEL analysis revealed that mainly hepatocytes were affected, whereas SEC showed only minor damage. This finding is in contrast to other groups, who regard SEC as the main target of reperfusion injury (Natori *et al.*, 1999), but similar results were shown by Rentsch et al. (Rentsch *et al.*, 2001).

Preconditioning with ANP led to a significant reduction of apoptosis after 24 h of cold ischemia and 45 min of reperfusion, respectively. Protection affected predominantly hepatocytes, whereas apoptosis of SEC was not influenced by ANP. Interestingly,

preconditioning of livers with 8-Br-cGMP resulted in a similar reduction of apoptosis. This analog of ANP's second messenger cGMP has previously been shown to mimic ANP action on liver protection, suggesting a cGMP mediated mechanism (Gerbes *et al.*, 1998). Preconditioning with either compound reduced apoptotic cell death after 24 h of ischemia by half, but this kind of cell death only comprised about 0.8% of total cells evaluated by TUNEL analysis. This points to a minor relevance of apoptosis in the model of IRI in the isolated perfused rat liver, which is supported in an *in vivo* model by Redaelli et al. (Redaelli *et al.*, 2002) but is in contrast to the findings of others (Gao *et al.*, 1998).

The finding that ANP reduces apoptotic together with necrotic cell death in the liver, represents a novel aspect of ANP action. ANP has been reported to induce apoptosis in cardiac myocytes of rats (Filippatos *et al.*, 2001), whereas ANP protected rat PC12 cells from apoptosis (Fiscus *et al.*, 2001). This suggests a highly cell-type specific regulation of apoptotic processes exerted by ANP with unclear mechanisms.

1.2 Caspase-3 upregulation during cold ischemia

Interestingly, Western blot analysis revealed a significant increase of caspase-3 precursor CPP32 during the ischemic period. Similarly, a transcriptional (Harrison *et al.*, 2001) and translational (Krupinski *et al.*, 2000) caspase-3 induction was demonstrated in a model of permanent middle cerebral artery occlusion, although at a temperature of 37°C. Indeed, the induction of transcriptional processes in cold ischemia has been shown for other genes before: despite substrate depletion and reduced metabolism endothelin-1 is upregulated in the preserved kidney (Wilhelm *et al.*, 1999). In addition, there is clear evidence for metabolic activity in cold ischemia as demonstrated by stimulation of stress-activated protein kinases by Crenesse et al. (Crenesse *et al.*, 2000).

Surprisingly, the amount of caspase-3 precursor CPP32 declined in the reperfusion period. This could be explained, at least partly, by the non-recirculating perfusion system leading to removal of apoptotic and wash out of necrotic cells.

1.3 ANP reduces early degenerative cell changes

HE staining of livers which had undergone ischemia without reperfusion did not identify necrotic cells, but revealed loss of cell integrity and increased vacuolization. These reversible, predominantly periportal changes are suggested to develop necrotic cell death in the reperfusion period. ANP revealed its protective potential already after 24 h of cold ischemia by clearly improving morphological appearance.

1.4 Reduction of necrosis by ANP treatment

Necrotic cell damage was clearly manifest after 2 h of reperfusion. Trypan blue is selectively taken up by injured cells and regarded as an indicator of necrotic cell death. In trypan blue perfused livers after 2 h of reperfusion, both periportal and pericentral regions showed necrotic changes in hepatocytes and SEC.

Similarly to apoptotic cell damage, preconditioning with ANP led to reduced necrotic damage after 2 h of reperfusion. Hepatocytes and SEC were protected in periportal areas whereas pericentral areas were not influenced.

Recently, Kiemer et al. reported that ANP stimulates phagocytic activity of Kupffer cells (Kiemer *et al.*, 2002a). Reduced phagocytosis by Kupffer cells is proposed to lead to accumulation of toxic metabolites released by dying cells and further aggravation of injury (Wanner *et al.*, 1999). By increasing Kupffer cell clearance capacity ANP might ameliorate liver injury by removing damaged cells.

ANP was moreover shown to prevent from Kupffer cell induced oxidant stress (Bilzer *et al.*, 1999a) as it similarly occurs during reperfusion. Due to the predominant localization of Kupffer cells in periportal liver areas (Gerok *et al.*, 1995), modulation of Kupffer cell function might account for the observed mitigation of periportal cell damage.

In summary, the results of this part of the study provide the following novel findings: 1. ANP is able to diminish apoptotic cell damage. 2. Cold ischemia induces procaspase-3. 3. ANP reduces ischemic degenerative cell changes. 4. Necrotic cell damage during reperfusion is decreased periportally by ANP.

2. Signal transduction of ANP protection

2.1 cGMP-dependent protein kinases

Information about the signal transduction related to the protective action conferred by ANP is still scanty. The intracellular formation of cGMP after ANP binding to its receptor was suggested to contribute to protection (Gerbes *et al.*, 1998). This also seems to be the predominant signaling pathway for ANP's renal and hemodynamic effects. But the mechanisms beyond cGMP, however are as yet unclear.

The cGMP-dependent protein kinases (PKGs) as target proteins of cGMP (Vaandrager *et al.*, 1996) were suggested to be involved in the observed effects. ANP's second messenger cGMP stimulates PKGs, which are able to reduce cytosolic Ca^{2+} concentration

(Lincoln *et al.*, 1993) leading to vasorelaxation and positively influencing intracellular ion homeostasis. It was proposed that the Ca^{2+} reducing properties of ANP *via* PKG could contribute to the protection (Pella, 1991; von Ruecker *et al.*, 1989). The prerequisite for a protection by this mechanisms is the expression of PKGs in the liver.

The presence of PKGs in the liver was demonstrated by Ecker et al. (Ecker *et al.*, 1989) by immunohistochemistry. Because of the fact that platelets contain large amounts of PKG protein (Waldmann *et al.*, 1986), it has to be noted that positive immunohistochemistry can also result from remaining platelets or other PKG containing blood constituents. In contrast, other groups reported low to no expression of PKG mRNA in the liver, making the presence and significance of these kinases in the liver still questionable (Jarchau *et al.*, 1994; Tamura *et al.*, 1996; Sandberg *et al.*, 1989). In addition, in preparations of rat liver endoplasmatic retikulum no PKG activity was evident (Kosmopoulou *et al.*, 1994), further suggesting the absence of PKG in the liver.

This study can not verify the expression of both major isoforms of the PKGs in the rat liver. RT-PCR experiments revealed no amplification in the liver, whereas control organs showed the expected amplicons. Based on these results, the involvement of PKGs in ANP mediated protection seems unlikely.

A functional relationship between ANP protection and PKG activation function could also be excluded since perfusion of ANP treated rat livers with a specific inhibitor of PKG, Rp-8-pCPT-cGMPS, did not abrogate hepatoprotection, further supporting the missing of a functional role of PKG. This inhibitor shows high lipophilicity, but is still water soluble and phosphodiesterase stable. It is a competitive inhibitor for both PKG I and PKG II, and is sufficiently selective for PKG over PKA (Schwede *et al.*, 2000).

2.2 cAMP-dependent protein kinases

Under some conditions also PKA can act as a target molecule for cGMP (Cornwell *et al.*, 1994). For example, testosterone production by ANP results in isolated mouse Leydig cells from an activation of PKA by cGMP (Schumacher *et al.*, 1992).

In order to demonstrate the existence of this pathway in our system, livers were perfused with ANP and a cAMP-dependent protein kinase (PKA) inhibitor, Rp-8-Br-cAMPS. Treatment with both compounds was able to abrogate the antiapoptic properties pointing to a PKA mediated mechanism. The inhibitor used has similar chemical properties as the PKG inhibitor but shows preference in inhibiting the PKA I to the PKA II isoform.

The activation of PKA has been shown to be antiapoptotic in numerous studies. For example, PKA expression is increased in a wide range of cancers (Alper *et al.*, 1999) and antisense RNA of PKA produces a marked decrease in proliferation in cancer cell lines. Activation of PKA leads to inhibition of apoptosis in a number of cell types, including neutrophils (Parvathenani *et al.*, 1998; Rossi *et al.*, 1995), smooth muscles (Orlov *et al.*, 1999), and HL60 promyeloid leukemia cells (Jun *et al.*, 1998). Interestingly, the antiapoptotic effects are independent of the apoptotic stimuli used. The inhibitory action of PKA lies upstream of caspase-3 activation (Parvathenani *et al.*, 1998; Orlov *et al.*, 1999), and it is suggested that PKA phosphorylates and therefore inactivates the proapoptotic protein Bad at ser^{112} (Harada *et al.*, 1999). Importantly, all studies suggesting an antiapoptotic role focused on the PKA I isoform, which is preferentially inhibited by the inhibitor used in the present experiments.

Moreover, antiapoptotic action of PKA in the liver was demonstrated by Akbar et al. in cold stored liver grafts. Supplementation of the organ preservation solution with dibutyryl-cAMP mitigated apoptotic cell death after cold storage and led to increased phosphorylation of Bad at ser^{112} (Akbar *et al.*, 2001). Postischemic enzyme leakage of alanine aminotransferase was reduced whereas hepatic bile production was enhanced.

This was further supported by Li et al. who reported that cGMP- and cAMP analogs reduce TNF-α/Act-D mediated apoptosis in primary hepatocytes (Li *et al.*, 2000). The inhibition of caspase activation and cytochrome-c release was attributed to both PKA-dependent and -independent mechanisms.

Furthermore, our results from *in vitro* stimulation of hepatocytes by ANP underscore this hypothesis. Thereby, ANP and 8-Br-cGMP were both able to increase PKA activity.

In summary, this part of the study demonstrates that cGMP-dependent protein kinases seem to be absent in the rat liver and therefore do not contribute to ANP mediated protection. Rather, a participation of cAMP-dependent protein kinases is discussed to be responsible for the beneficial effects of ANP.

2.3 Further potentially involved pathways

There are further approaches to explain the ANP action: activation of the MAPK pathways is discussed to contribute to cytoprotection in IRI (Fung, 2001). Induction of p38 MAPK in hepatocytes has been demonstrated to attenuate apoptotic cell death (Roberts *et al.*, 2000; Amersi *et al.*, 2002). In this context it is interesting to know that we were able to demonstrate a strong increase of p38 MAPK activity by preconditioning of rat livers with

ANP (unpublished data).

ANP has recently been shown to activate the heat shock transcription factor (HSF) and the heat shock protein 70 (HSP70) (Kiemer *et al.*, 2002b), both mediators of the heat shock response (Jäättelä, 1999). The antiapoptotic properties of HSP70 might contribute to the observed ANP effects (Beere, 2001; Ikeyama *et al.*, 2001).

3. Heme Oxygenase-1 expression

Induction of heme oxygenase-1 is discussed to mediate cytoprotection in IRI (Amersi *et al.*, 1999). Therefore, the expression of HO-1 in ANP pretreated livers was investigated in order to elucidate a potential induction by ANP.

3.1 HO-1 expression during ischemia and reperfusion

The presented data show no change in the expression of HO-1 during ischemia and reperfusion in the system of cold ischemic storage and reperfusion of isolated rat livers. In contrast to this observation, induction of HO-1 during reperfusion has been reported (Yamaguchi *et al.*, 1996; Sonin *et al.*, 1999). These investigations, however, were performed after warm ischemia with reperfusion times as long as 4 or 6 h (Sonin *et al.*, 1999). Therefore, the shorter time scale of the experimental setting might be responsible for the lack of increased HO-1 expression.

Moreover, cold ischemia might induce other mechanisms than warm ischemic storage. There is little information on HO-1 after cold ischemic liver storage as yet. One work was performed in fatty rat livers which underwent cold ischemic storage. It showed no alteration in HO-1 expression compared to untreated control organs during up to 2 h of reperfusion (Amersi *et al.*, 1999) and therefore supports our data.

3.2 ANP induces HO-1 in rat livers

The observation that ANP induces HO-1 is in line with published work showing HO-1 induction by ANP in cultured bovine aortic endothelial cells (Polte *et al.*, 2000) and renal cells (Polte *et al.*, 2002). However, to our knowledge, there are no investigations concerning effects of ANP on HO-1 in liver so far.

It was recently shown by Kiemer et al. (Kiemer *et al.*, 2002b) that ANP represents a pharmacological inducer of the heat shock response in rat livers including the activation of the heat shock transcription factor (HSF) (Jäättelä, 1999). Due to the characteristic of HO-1 as a heat shock protein involving a HSF-binding heat shock element in its promotor

region, transcriptional induction by ANP might be mediated *via* this pathway (Maines, 1997; Shibahara *et al.*, 1989).

It might seem amazing that HO-1 is upregulated as early as 20 min after ANP treatment. Very early cardiac induction of HO-1 mRNA after only 6 min of administration of an NO donor has also been described in the literature (Katori *et al.*, 2000). Assuming that upregulation of HO-1 is mediated by activated HSF, this rapid elevation is understandable since an increase in the binding of HSF to the promotor region of the gene involves message stabilization as a major factor in accumulation of the transcript (Maines, 1997; Raju *et al.*, 1994).

3.3 Induction of HO-1 in Kupffer cells

Interestingly, ANP induces the expression of HO-1 in Kupffer cells but not in parenchymal cells. Most data from the literature report parenchymal induction of HO-1 (Paxian *et al.*, 2001; Terajima *et al.*, 2000; Rizzardini *et al.*, 1998). After hemorrhagic shock (Hoetzel *et al.*, 2001; Paxian *et al.*, 2001) and hyperthermia (Terajima *et al.*, 2000) in rats and after LPS treatment of mice (Rizzardini *et al.*, 1998), HO-1 was reported to be induced predominantly in hepatocytes. However, localization of HO-1 induction in the liver seems to be dependent on the type of stimulus. In human cirrhosis HO-1 induction mostly occurred in Kupffer cells and was minor in hepatocytes (Makino *et al.*, 2001). Bauer et al. reported rat HO-1 induction in sinusoidal lining cells after LPS challenge (Bauer *et al.*, 1998). These data suggest that specific stimuli induce signaling cascades leading to a cell-type specific regulation of HO-1.

Compared to other papers reporting induction of HO-1 in hepatocytes (Paxian *et al.*, 2001; Terajima *et al.*, 2000; Rizzardini *et al.*, 1998), our data show a rather weak yet significant induction of HO-1. The lack of induction of HO-1 in hepatocytes might explain the moderate increase in HO-1 in our system.

3.4 Induction of HO-1 by ANP is independent of cGMP

It has been reported before that ANP induces HO-1 in cultured bovine endothelial cells (Polte *et al.*, 2000). In this cell system, however, ANP worked via its guanylate-cyclase-coupled A receptor. In contrast to these data our findings reveal that ANP-mediated induction of HO-1 is exerted independent of cGMP.

In this context it should be noted that some effects of ANP seem to be independent of cGMP, such as attenuation of proliferation in astroglial cells (Levin *et al.*, 1991), reduction

of endothelin expression in endothelial cells (Hu *et al.*, 1992), or inhibition of COX-2 expression in macrophages (Kiemer *et al.*, 2002c). The ANP “clearance” receptor was suggested to be responsible for these effects (Levin *et al.*, 1991; Hu *et al.*, 1992; Kiemer *et al.*, 2002c). This receptor has been shown to be expressed in liver tissue (Vollmar *et al.*, 1997).

This result showing ANP-mediated HO-1 expression is the first report to describe cGMP-independent action of ANP in the liver, suggesting a pleiotropic biological profile of ANP in liver tissue.

3.5 Perfusion with ZnPP

Zinc protoporphyrin (ZnPP) inhibits the catalytic activity of both HO-1 and –2. Several groups have demonstrated with this compound a causal relationship between HO-1 induction and cytoprotection (Amersi *et al.*, 1999).

In this study, ZnPP was not able to abrogate the ANP mediated inhibition of caspase-3-like activity, suggesting that ANP’s hepatoprotective action is not mediated *via* the HO-1 pathway. Surprisingly, this compound itself lead to a significant reduction of caspase activity, implying the existence of side effects conferred by this compound.

In summary, it is demonstrated that preconditioning of rat livers with Atrial Natriuretic Peptide induces early HO-1 expression. Interestingly, this induction occurs independently of cGMP and localization was confined to Kupffer cells but was not detectable in hepatocytes. Induction of HO-1 does not seem to mediate the antiapoptotic action of ANP.

E. Summary

This work characterizes the protective effects of ANP preconditioning in ischemia-reperfusion injury of the isolated perfused rat liver. It was of particular interest to evaluate the influence of ANP on the mode of cell death occurring during cold ischemia and reperfusion and to elucidate the involved signal transduction pathways.

Apoptotic cell death was mainly seen after cold liver storage, whereas necrosis was predominant in the reperfusion period.

It could be demonstrated for the first time that preconditioning with ANP was able to reduce both apoptotic as well as necrotic cell death. After cold ischemia, in particular hepatocytes were protected against apoptosis. After reperfusion, protection against necrosis comprised hepatocytes and sinusendothelial cells predominantly in the periportal liver areas.

As target molecules for ANP action, the cGMP-dependent protein kinases did not seem to be responsible for the conferred cytoprotection. In the liver, no expression of these kinases could be detected and a functional connection could not be derived. In contrast, the cAMP-dependent protein kinases were identified to promote survival. This was further supported by the ability of ANP to directly activate cAMP-dependent protein kinases in livers and hepatocytes.

An early transcriptional induction of HO-1 by ANP independent of cGMP could be demonstrated. The induction of heme oxygenase-1 by ANP might not be responsible for the observed hepatoprotection, since inhibition of HO-1 activity did not abrogate the ANP effect. Interestingly, cell-type specific evaluation detected that induction of HO-1 in livers by ANP is exclusively restricted to Kupffer cells.

In summary, this thesis gives new insights into the actions of the cardiovascular hormone ANP in IRI of the rat liver. This data helps to understand the mechanisms of how ANP mediates cytoprotection by illuminating effects and potential pathways, an important prerequisite for a rational application in therapy.

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F. Appendix

1. Abbreviations

A	Ampère
ANP	Atrial natriuretic peptide
APS	Ammonium persulfate
ATP	Adenosine-5’-triphosphate
BNP	Brain natriuretic peptide
Bp	Basepair
Bq	Bequerel
BSA	Bovine serum albumine
cAMP	Cyclic Adenosine-5’-monophosphate
cDNA	Complementary DNA
cGMP	Cyclic Guanosine-5’-monophophate
Ci	Curie (1 Ci=3.7x10^7 Bequerel)
CNP	C-type natriuretic peptide
Co	Control
cpm	Counts per minute
Da	Dalton
dATP	2’-deoxyadenosine-5’-triphosphate
dCTP	2’-deoxycytosine-5’-triphosphate
dGTP	2’-deoxyguanosine-5’-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleosidtriphosphate
DTT	Dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(aminoethylether)-tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol

FCS	Fetal calf serum
GDP	Glyoxal DMSO phosphate
GTP	Guanosine-5’-triphosphate
h	Hour
HRP	Horseradish peroxidase
IRI	Ischemia reperfusion injury
kDA	Kilo Dalton
L	Liter
LDH	Lactate dehydrogenase
m	Milli
M	Molar
MeOH	Methanol
min	Minute
MOPS	3-[N-Morpholino]propansulfonic acid
MRNA	Messenger ribonucleic acid
n	Nano
NADH	Nicotinamide adenine dinucleotid
NPR	Natriuretic peptide receotor
NTP	ATP, CTP, GTP or TTP
OD	Optical density
PAA	Polyacrylamide
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
Rp-8-Br-cAMPS	8-Bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer
Rp-8-pCPT-cGMPS	8-(4-Chlorophenylthio)guanosine-3',5'-cyclic

	monophosphorothioate, Rp-isomer
rpm	Rotations per minute
RT	Room temperature
SA-PMP	Strepavidin paramagnetic particles
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of mean
SSC	Saline sodium citrate buffer
STE	Sodium chloride, Tris, EDTA buffer
TAE	Tris, acetate, EDTA buffer
TBE	Tris, borate, EDTA buffer
TBS-T	Phosphate buffered saline solution with Tween
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor alpha
totRNA	Total RNA
Tris	Tris-hydroxymethyl-aminomethan
TUNEL	Terminal deoxynuceotidyl transferase mediated dUTP nick end labeling
U	Unit
UTP	Uridine-5’-triphosphate
V	Volt
W	Watt
ZnPP	Zinc protoporphyrin IX
% (m/v)	Mass per volume per cent
% (v/v)	Volume per cent
µ	Micro
8-Br-cGMP	8-Bromoguanosine-3', 5'-cyclic monophosphate

2. Alphabetical order of companies

Alexis Biochemicals	Grünberg, Germany
Amersham	Braunschweig, Germany
Beckmann Instruments	Munich, Germany
Biolog	Hamburg, Germany
Biometra	Göttingen, Germany
BioRad Laboratories	Munich, Germany
Biosource	Nivelle, Belgium
Biozym Diagnostics	Oldendorf, Germany
BMG GmbH	Offenburg, Germany
Boehringer	Mannheim, Germany
Braun	Melsungen, Germany
Calbiochem	Schwalbach, Germany
Cell Signaling	Frankfurt/Main, Germany
Charles-River GmbH	Sulzfeld, Germany
Dianova	Hamburg, Germany
DuPont	Bad Homburg, Germany
Eastmen Kodak Company	Rocester, USA
Eppendorf	Maintal, Germany
Gibco/Invitrogen	Karlsruhe, Germany
Greiner	Frickenhausen, Germany
Hamilton	Bonaduz, Switzerland
Heraeus	Hanau, Germany
Ika Labortechnik	Staufen, Germany
Intergen	Purchase, USA
Labsysteme Osvath	Geretsried, Germany
Linde	Unterschleißheim, Germany
MBI Fermentas	St. Leon Roth, Germany
Merck-Eurolab	Munich, Germany
Merial	Halbergmoos, Germany

Millipore	Eschborn, Germany
MWG-biotech	Ebersberg, Germany
NEN	Cologne, Germany
Owl separation systems	Portsmouth, USA
PAN	Aidenbach, Germany
PE applied biosystems	Foster City, USA
Perkin-Elmer	Rodgau-Jügesheim, Germany
Peske	Aindling-Pichl, Germany
Pharmacia Biotech	Heidelberg, Germany
Pierce	Rockford, USA
Promega	Heidelberg, Germany
Prominent	Heidelberg, Germany
Qiagen	Hilden, Germany
Raytest	Straubenhardt, Germany
Roche Diagnostics	Mannheim, Germany
Roth	Karlsruhe, Germany
Santa Cruz	Heidelberg, Germany
Schleicher & Schüll	Dassel, Germany
Serva	Heidelberg, Germany
Sigma	Taufkirchen, Germany
Sims Portex Ltd.	Hyte, UK
Ssniff	Soest, Germany
Stratagene	Heidelberg, Germany
Stressgen	San Diego, USA
Toplab	Martinsried, Germany
Uniequip	Martinsried, Germany
Watson Marlow	Falmouth, UK

3. Publications

3.1 Abstracts

Gerwig T, Kiemer AK, Vollmar AM, Gerbes AL.

ANP Präkonditionierung induziert die Hämoxygenase-1 in der isoliert perfundierten Rattenleber.

Zeitschrift für Gastroenterologie, 2001,6 ,487

Workshop für klinische und experimentelle Leberchirurgie und Transplantation, Wilsede, Germany

Gerwig T, Meißner H, Arnoldt H, Bilzer M, Kiemer AK, Vollmar AM, Gerbes AL.

ANP preconditioning protects against hepatic ischemia-reperfusion injury by attenuating necrotic and apoptotic cell death.

Hepatology supplement. 2001, 34: 202 AASLD, Dallas

52. Annual Meeting of the American Association for the Study of Liver diseases, Dallas, USA

Förnges A, Gerwig T, Vollmar AM, Kiemer AK.

Protection against hepatic ischemia-reperfusion injury by ANP preconditioning is not mediated by inhibition of matrix metallo proteinases.

Naunyn-Schmiedeberg´s Arch Pharmacol Suppl. 2002, DGPT, Mainz

43. Tagung der Deutschen Gesellschaft für klinische Pharmakologie und Toxikologie in Mainz, Germany

Kiemer AK, Kulhanek S, Gerwig T, Gerbes AL, Vollmar AM

The Atrial Natriuretic Peptide activates p38 MAP Kinase and attenuates apoptotic cell death during ischemia-reperfusion injury of rat livers.

Hepatology supplement. 2000, 32: 253 AASLD, Dallas

51. Annual Meeting of the American Association for the Study of Liver diseases, Dallas, USA

3.2 Original publications

Gerwig T, Meißner H, Arnholdt H, Bilzer M, Kiemer AK, Vollmar AM, Gerbes AL.

ANP preconditioning protects against hepatic preservation injury by attenuating apoptotic and necrotic cell death

submitted, 2002

Kiemer AK, Gerwig T, Gerbes AL, Meißner H, Vollmar AM.

Kupffer-cell specific induction of heme oxygenase 1 (hsp32) by the Atrial Natriuretic Peptide is independent of cGMP

submitted, 2002

Kiemer AK, Vollmar AM, Bilzer M, Gerwig T, Gerbes AL.

Atrial Natriuretic Peptide reduces expression of TNF-a mRNA during reperfusion of the rat liver upon decreased activation of NF-kB and AP-1.

Journal of Hepatology 2000; 33: 236-246

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