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Characterisation of Anti-Apoptotic Signalling Pathways in Hepatocytes activated by α-Lipoic Acid and Atrial Natriuretic Peptide

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Erklärung

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Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

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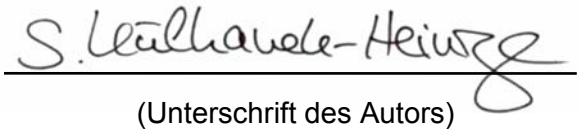

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dedicated to my family

B INTRODUCTION

1 BACKGROUND AND OVERVIEW

The apoptotic cell death of hepatocytes describes a prominent pathomechanism of diverse liver diseases, like alcoholic liver disease, endotoxine-induced liver failure or ischemia reperfusion injury (IRI). Thus, the inhibition of apoptosis might represent an approach of prevention or treatment of liver injury.

Two naturally occurring substances, the enzyme cofactor α-lipoic acid (LA) and the cardiovascular hormone atrial natriuretic peptide (ANP), have been described to possess hepatoprotective potential.

In prokaryotic and eukaryotic cells LA is essential for the activity of a variety of enzyme complexes that catalyse oxidative decarboxylations. The substance exhibits antioxidative, redox regulating and metal chelating properties, and is therefore approved in Germany for the treatment of diabetic polyneuropathy. Interestingly, it has also been described by our group to protect against hepatic IRI and liver cirrhosis. Therefore, we aimed to determine whether LA has potential to regulate hepatocyte apoptosis. Since LA appears to possibly mediate protection other than due to its antioxidative action, we were especially interested in investigating LA-induced antiapoptotic signalling pathways involved in hepatoprotection.

ANP has been demonstrated to protect from hepatic IRI, comprising a marked attenuation of post-ischemic apoptotic cell death. The signal transduction pathways mediating this inhibition of ischemic cell death is poorly understood. Since ANP strongly activates p38 MAPK, a protein kinase suggested to mediate protection of ischemic livers, its involvement in ANP-mediated protection was hypothesised.

Aim of the present work was to elucidate the signalling pathways involved in the antiapoptotic actions of the two substances, LA and ANP, in primary rat hepatocytes and isolated perfused rat livers, respectively. Therefore, the following questions should be answered:

1 **Elucidation of signal transduction processes induced by α-lipoic acid:**

(a) Does LA-preincubation prevent TNF-α-/ActinomycinD-induced hepatocyte apoptosis?

(b) Does LA mediate this protection *via* antioxidative or Fe-chelating properties?

(c) Does LA-preincubation activate the PI3-K/Akt pathway and is the possible downstream target BAD involved in this cytoprotective signalling?

2 **Elucidation of signal transduction processes induced by the cardiovascular hormone ANP:**

(a) Is p38 MAPK involved in the antiapoptotic signalling of ANP?

(b) Which cGMP-dependent proteins are involved in the ANP-mediated signal transduction? Involvement of the two protein kinases PKG and PKA.

(c) Which possible downstream target of PKA might mediate ANP's antiapoptotic action?

2 LIVER CELL DEATH/APOPTOSIS

Liver cell injury and liver cell death is a prominent feature in all liver disease processes. Recent studies suggest also apoptotic cell death to contribute to hepatic organ failure. Thus, aim of the present thesis was to elucidate ANP- and LA-mediated signal transduction pathways protecting against hepatic apoptosis.

2.1 MORPHOLOGICAL FEATURES OF APOPTOSIS AND NECROSIS

The original description of apoptotic cell death was based on morphology (1). A cell undergoing apoptosis is characterised by cell shrinkage, nuclear condensation, chromatin margination, and fragmentation of both the nucleus and the cytoplasm into apoptotic bodies (1). These apoptotic bodies are enclosed by an intact plasma membrane, thus preventing leakage of cellular material into surrounding tissue and subsequent inflammation (1). The morphological changes are a result of the activation of caspases and endonucleases, which induce the breakdown of structural proteins, repair enzymes, and DNA, respectively (2). Caspases are a family of proteases playing a fundamental role in the execution of apoptosis. These enzymes are highly conserved cysteine proteases that specifically and efficiently cleave target substrates after the carboxy terminus of aspartate (3). Functionally, apoptosis eliminates excess and unneeded cells during development as well as damaged cells during normal tissue turnover.

In contrast to apoptosis, another cell death termed “necrosis” occurs after injury or excessive contact with cytotoxic agents, and typically induces inflammation (1). Characteristic features of necrotic cell death, more recently renamend oncotic necrosis, involve cell swelling, vacuolation, karyolysis, and cell content release, thus affecting cells in large areas of the tissue in combination with a substantial inflammatory response (4).

Recent studies in the liver displayed a detrimental role of apoptotic cell death in diverse liver diseases. Additionally, it is hypothesised that inhibition of hepatic apoptosis might protect against secondary necrotic cell death. Thus, the elucidation of apoptotic signalling pathways is essential for the development of possible therapeutic interventions.

2.2 SIGNALLING MECHANISMS IN HEPATOCYTE APOPTOSIS

In general, execution of hepatocyte apoptosis can be distinguished into two different pathways: a variety of mediators such as TNF-α, Fas ligand (FasL), and TRAIL (TNF-α-related apoptosis-inducing ligand) are able to activate an extrinsic pathway of apoptosis in hepatocytes. TNF-α binds to its receptor TNFR1, which causes receptor oligomerisation and the association of the adapter molecules TRADD (TNF-α receptor-associated death domain) and FADD (Fas-associated death domain) (FIGURE 1). TRADD and FADD promote the binding of Procaspase-8, thus leading to its proteolytic cleavage to activated Caspase-8. Additionally, Caspase-8 can cleave Bid, a BH3 domain-only Bcl-2 family member, to activated tBid (truncated Bid). Subsequently, tBid translocates to the mitochondria and induces the release of soluble proteins from mitochondria and thus the intrinsic/mitochondrial pathway (5). The soluble proteins include Cytochrome c, AIF (apoptosis inducing factor), and Smac, which reside in the intermembrane space between the mitochondrial inner and outer membrane (6-9).

The release of intermembrane proteins follows the TNF-α-induced onset of mitochondrial permeability transition (MPT). MPT occurs from the opening of the permeability transition pore, which is permeable for proteins up to 1,500 kDa (10). This opening induces depolarisation, uncoupling, and swelling of the mitochondria. This swelling leads to a rupture of the outer membrane, thus facilitating the release of proteins from the intermembrane space. Besides this MPT-mediated mechanism for cytochrome c release, other models exist: tBid might form *via* interaction with Bax or Bak channels in the outer membrane, thus enabling the release of cytochrome c and other even larger proteins from the intermembrane space. The nature and composition of these channels, however, is to date poorly understood (7;11;12). Bcl-2 and Bcl-X_L can block the Cytochrome c release or the pore formation and thus apoptosis. These protective effects of Bcl-2 can be inhibited by another member of the Bcl-2 family, namely BAD. BAD is a proapoptotic member of the Bcl-2 gene family. Unphosphorylated, it binds Bcl-2 and Bcl-X_L, thus inhibiting their antiapoptotic potential (13). Released Cytochrome c interacts with Procaspase-9, Apaf-1 and ATP to form the apoptosome, thus activating Caspase-9 and other downstream caspases (e.g Caspase-3). Activated Caspase-3 then initiates the final execution stages of apoptosis, including cell shrinkage, surface blebbing, phosphatidyl serine externalisation onto the outer plasma membrane, nuclear condensation, and chromatin margination.

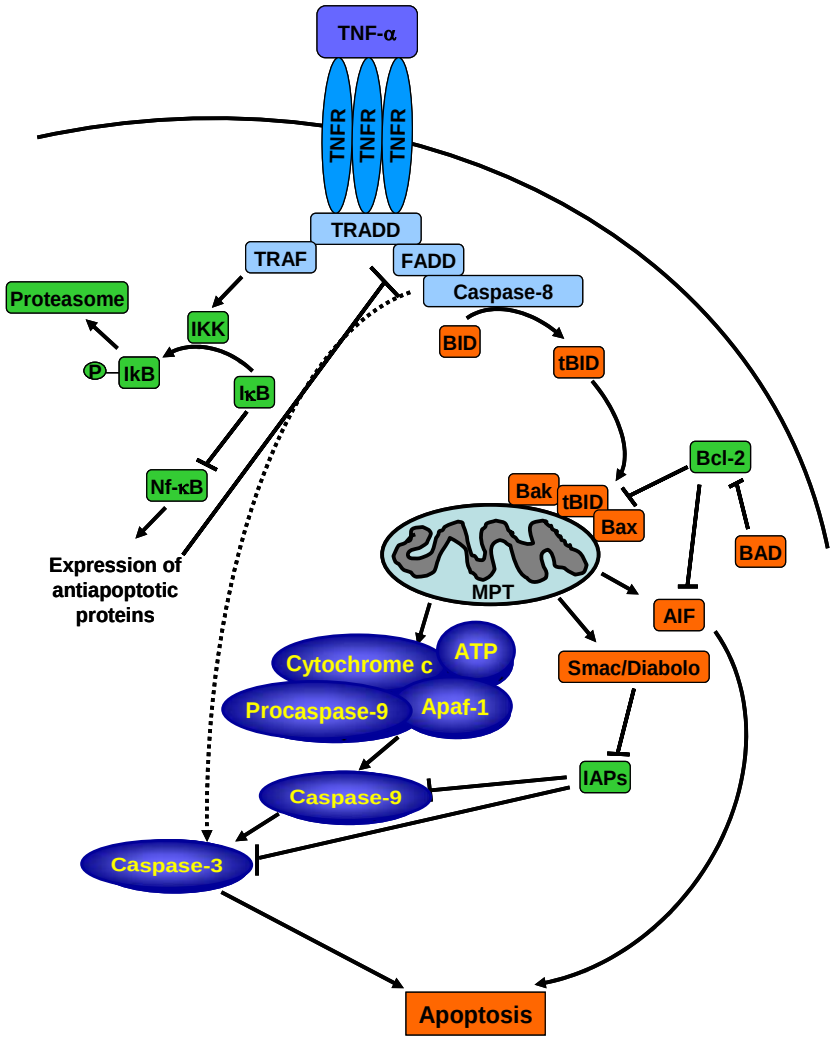


FIGURE 1: Schema of the TNF-induced signalling pathways in hepatocytes (adapted from (2)): for details see text.

2.3 SIGNALLING MECHANISMS PROTECTING FROM APOPTOSIS

2.3.1 AKT

General aspects

Akt/PKB, nowadays viewed as a “survival” kinase, was originally characterised after isolation of two genes termed akt1 and akt2, identified as the human homologues for the viral oncogene v-akt, which is responsible for a type of leukaemia in mice (14). Later, two groups discovered that v-akt and its human homologue encode a protein kinase with homology to both PKC and PKA, and therefore it was called PKB (15;16). To date, three

members of the Akt family have been isolated, namely Akt1, Akt2 and Akt3 (PKB α,β,γ) (17). Although being products of different genes, they are all closely related to each other, with up to 80% of amino acid homology. The three genes are expressed differentially, with a broader expression for Akt1/2 and a more restricted expression for Akt3. Each isoform exhibits a pleckstrin homology (PH) domain of approximately 100 amino acids in the N-terminal region and a kinase domain very similar to that of PKA and PKC (15;18). The kinase domain has a threonine residue (Thr308/Akt1), whose phosphorylation is required for Akt activation. Alongside, the kinase domain is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site with a Serin residue (Ser473/Akt1). Thr308 and Ser473 phosphorylation occurs in response to growth factors and other extracellular stimuli, and is essential for maximal Akt activation (19).

Akt activation

Phosphatidylinositol-3 kinases (PI3-K) constitute a lipid kinase family characterised by their ability to phosphorylate inositol ring 3'-OH groups in inositol phospholipids (20). Typically, ligands, such as growth factors, bind to receptors with receptor protein tyrosine kinase (RPTK) activity resulting in the association of PI3-K with the receptor through one or two SH2 domains, followed by a subsequent allosteric activation of the catalytic subunit (21). This activation leads to the production of the second messenger PIP_3 (phosphatidylinositol-3,4,5-triphosphate) within a few seconds. Then, PIP_3 recruits different signalling proteins containing a PH domain to the membrane. Akt interacts with these phospholipids *via* its PH domain, and is thus translocated to the inner membrane, where another PH domain-containing protein, namely PDK1 (protein serine/threonine kinase 3'-phosphoinositide-dependent kinase 1) is located. The interaction of Akt's PH domain with PIP_3 is thought to provoke conformational changes in Akt, resulting in exposure of its two main phosphorylation sites (Thr308, Ser473). Likewise, PH domains may mediate the binding of Akt and PDK1 through their heterodimerisation. PDK1, which is thought to be constitutively active, phosphorylates Akt at Thr308, leading to the stabilisation of the active conformation (FIGURE 2).

Thr308 phosphorylation is necessary for the kinase activation, but for full kinase activation phosphorylation of the Ser473 residue located at the hydrophobic C-terminal region is also required. The kinase phosphorylating this residue has not been identified

yet, even though several findings suggest a role of the protein kinase ILK (integrin-linked kinase) in this activation process (22-24).

In a later step, active Akt is translocated to the nucleus through an unknown mechanism, where many of its substrates are localized (25).

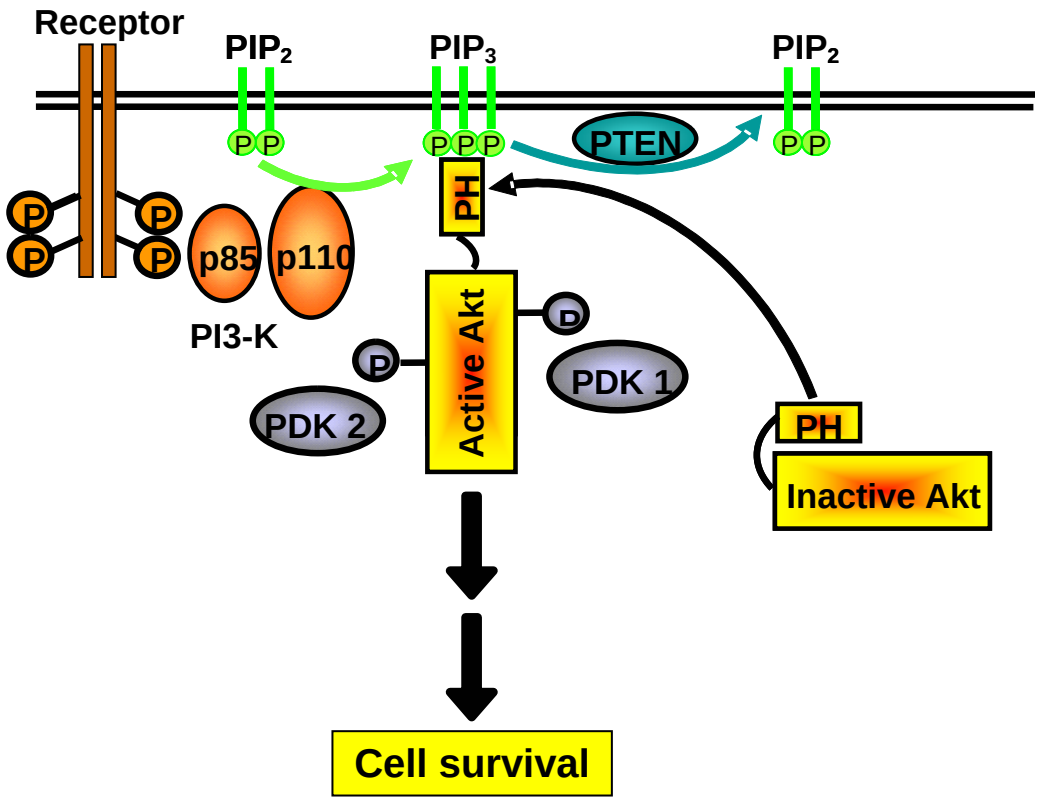


FIGURE 2: Akt activation cascade: Activation of growth factor receptor protein tyrosine kinases results in autophosphorylation of tyrosine residues. PI3K is recruited to the membrane by directly binding to phosphotyrosine consensus residues of growth factor receptors or adaptors through one or both SH2 domains in the adaptor subunit. This leads to allosteric activation of the catalytic subunit. Activation results in production of the second messenger PIP_3. The lipid product of PI3-K, PIP_3, recruits a subset of signalling proteins with PH domains to the membrane, including PDK1 and Akt. PTEN is a PIP_3 phosphatase, which negatively regulates the PI3-K/Akt pathway. Once activated, Akt mediates the activation and inhibition of several targets, resulting in cellular survival, growth, and proliferation through various mechanisms.

Downstream targets of Akt

Once activated, Akt modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression, and cellular growth (for review see: (26-28). In the following, it should be dwelled upon the role of Akt in cell death regulation: activated Akt phosphorylates members of the forkhead family of transcription factors (FH), resulting in an association of FH members with 14-3-3 proteins, thus decreasing their transcriptional activity, which is required for promoting apoptosis. Target genes of the FH family are thought to be extracellular ligands, including Fas ligand, TRAIL and TRADD, and intracellular components for apoptosis, like the proapoptotic Bcl-2 family members Bim or Bcl-6 (29).

Moreover, Akt can phosphorylate and activate the IκB kinase (IKK), thereby activating the transcriptional activity of NF-κB and thus the transcription of survival genes (30). The transcription factor NF-κB is a key regulator of the immune response and deregulation of its activity is involved in the development of diseases such as autoimmune disease and cancer. Mostly, activation of NF-κB depends on phosphorylation and degradation of IκB by IKK. Another transcription factor activated *via* Akt by phosphorylation is CREB (cAMP response element-binding protein) (31). Again, this phosphorylation activates the transcription of CREB-regulated survival genes.

Further, Akt has been described to phosphorylate and inactivate the proapoptotic factors BAD and Procaspase-9. Akt-induced phosphorylation of Procaspase-9 at Ser196 prevents Cytochrome c-mediated cleavage of the pro-caspase and thus inhibits the activation of Caspase-9, an initiator and effector of apoptosis (32). BAD phosphorylated at Ser136 dissociates from Bcl-2/Bcl-X_L and interacts with the 14-3-3 protein instead, a family of ubiquitous highly expressed adapter proteins. Phosphorylated BAD is unable to heterodimerise with Bcl-2/Bcl-X_L, and thus these antiapoptotic proteins are then free to promote cell survival (FIGURE 3) (33).

Activation of Akt has been shown to protect a variety of cells from apoptosis, but the underlying mechanism is still unclear (for review see (33). However, resuming the current literature concerning Akt-mediated cell survival, it seems that phosphorylation of BAD and its subsequent dissociation from Bcl-X_L is the most important signalling event linked to the antiapoptotic effect of Akt.

2.3.2 BAD

One important downstream target of Akt is the proapoptotic protein BAD (see above). BAD is a distant member of the Bcl-2 gene family that promotes cell death in mammalian cells (34). Several members of the Bcl-2 family (including Bcl-2, Bcl-X_L, MCL-1, A1, BAG-1) promote survival while other members (including BAD, Bcl-X_S, Bax, Bak) promote apoptosis. Bcl-2 family proteins form homo- and heterodimers, and the balance between particular dimers is thought to be critical for the maintenance of cell survival or the induction of apoptosis (for review see: (35-39)).

It is still unknown how the activity of Bcl-2 family members is regulated through specific signal transduction molecules activated by survival signals. One possibility is that growth factor-regulated protein kinases phosphorylate Bcl-2 family members, thus regulating their functions. One likely target of those phosphorylations is BAD (40). The function of BAD is modulated by phosphorylation at two sites, Ser112 and Ser136 (41-43). Also BAD phoshphorylation at Ser155 is described: nicotine- and growth factor-induced survival in lung cancer cells/Hek-293 cells is mediated *via* phosphorylation at Ser155 (44;45). The authors propose, three kinasese to phosphorylate BAD at different Ser residues: Erk1/2 phosphorylates Bad at Ser112, Akt at Ser136, and PKA at Ser155. Unphosphorylated BAD induces apoptosis *via* the formation of heterodimers with Bcl-2/Bcl-X_L and the concomitant generation of Bax homodimers. Phosphorylated BAD binds to 14-3-3 proteins, and is thus sequestered from Bcl-X_L (FIGURE 3). Then, free Bcl-X_L can promote cell survival.

Several studies report an Akt-mediated protection against apoptosis by BAD phosphorylation in different cell systems: *Datta et al.* displayed that Akt promotes survival of neurons *via* BAD phosphorylation (46). Furthermore, Akt is reported to inhibit apoptosis in different tumour cells through BAD inactivation (47;48). BAD resides in the cytosol and is phosphorylated at Ser112 and Ser136 after cells are stimulated with the cytokine IL-3, thus preventing apoptosis in hematopoietic cells (43). *Hong et al*. observed in the antiapoptotic liver a slight BAD phosphorylation (Ser112), even though a causal link between Akt activation and BAD phosphorylation was not further investigated (49).

With BAD being one of the main downstream targets of Akt, Erk and PKA, aim of the

present study was to elucidate Akt- and PKA-dependent BAD-phosphorylation in hepatocyte apoptosis.

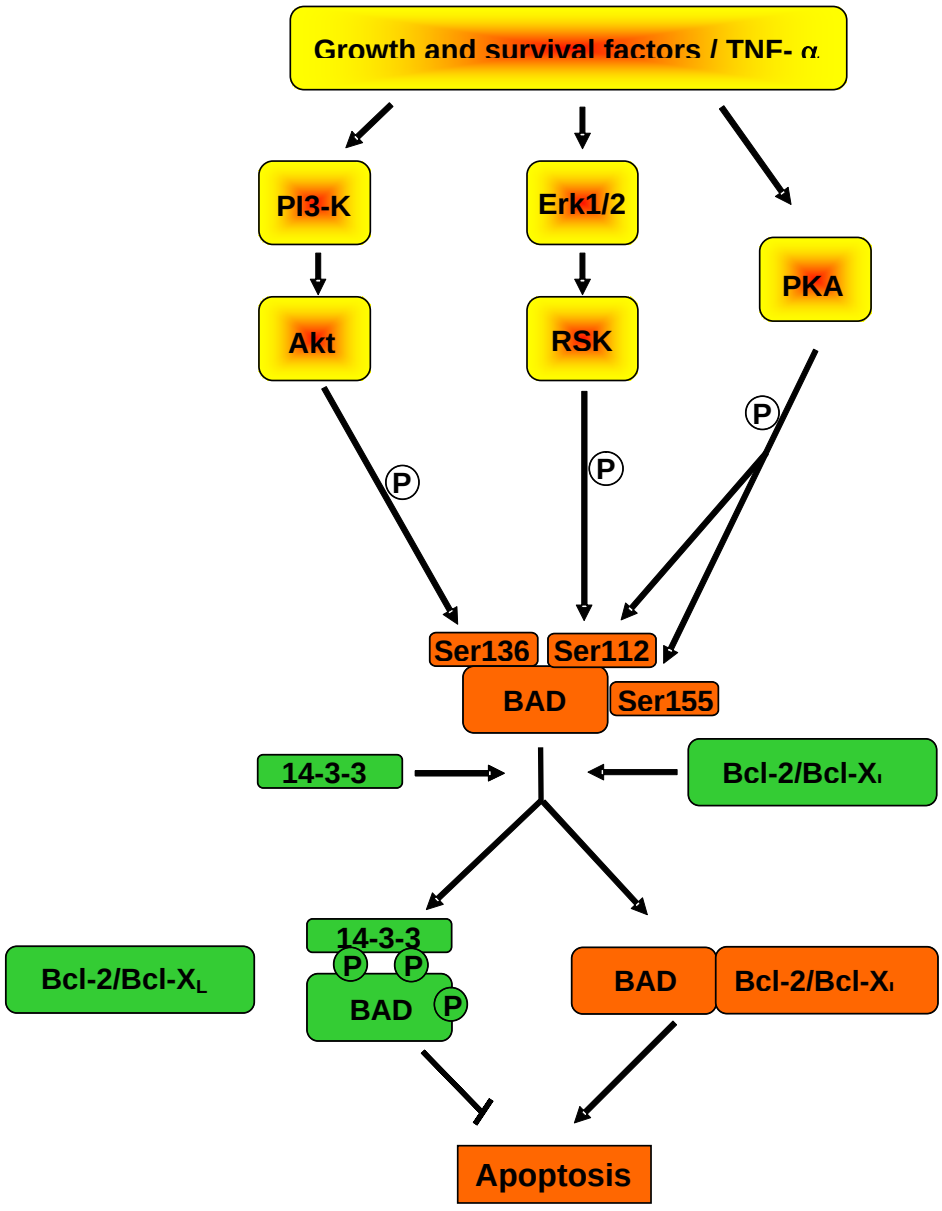


FIGURE 3: Ways to activate/inactivate BAD *via* phosphorylation (for details see text).

3 ISCHEMIA-REPERFUSION INJURY OF THE LIVER

3.1 GENERAL ASPECTS

In 1963 Thomas Starzl performed the first human liver transplantation (50). Since then this transplantation technique evolved into an established therapy for end stage liver diseases and acute liver failure. As a result of increasing experience, optimisation of surgical techniques and immunosuppression, the actuarial survival rate improved considerably and is to date 85-90% after one year (51). Also advanced organ preservation accounts for this development: the commonly used University of Wisconsin (UW) solution (52) facilitates preservation times up to 20 h and hence replaced the former, less effective Euro-Collins solution (53).

Despite the recent improvements in liver preservation and surgical techniques, hepatic ischemia reperfusion injury (IRI) remains an important clinical problem, precisely because hepatic IRI also occurs in liver surgery, hemorrhagic shock-resuscitation and heart failure. Moreover, due to the shortage of organs for transplantation, the use of fatty livers having a low tolerance to hypoxia and being predisposed to reoxygenation damage, greatly increases the risk related to reperfusion injury (54;55). Over the past years, the acknowledgement of the mechanisms underlying IRI appreciably improved. The accepted theory is that IRI is caused by a combination of cellular changes occurring during ischemia and the following reperfusion period, where blood flow and oxygen supply are restored.

Several factors contribute to hepatic ischemia: the lack of oxygen during the ischemic period causes the loss of mitochondrial respiration. Thus, Ca^{2+}-induced activation of ATPases leads to ATP depletion, thus even impairing the cellular energy status during ischemia (56;57). Due to this reduced energy status, energy-dependent metabolic pathways and transport processes collapse, finally resulting in disruption of ion homeostasis, affection of cell volume regulation and activation of proteases (56;58;59). These proteases, including aspartate proteases, matrix metalloproteases, Ca^{2+}-requiring calpains, and endonucleases (60-62) proteolytically cleave and disrupt membrane/cytoskeletal proteins and chromatin. Ca^{2+}-dependent phospholipases alter membrane fluidity and function (63). Sinusoidal endothelial cells (SEC) seem to be more susceptible to cold ischemia than hepatocytes (64) and contribute to increased hepatic

injury by detaching from the extracellular matrix at the beginning of the reperfusion period (65).

Although ischemic stress itself primes cells for damage and will eventually cause cell death, cell injury often does not manifest until the ischemic liver is reperfused (66). During reperfusion, blood supply is restored, thereby activating a complex network of hepatic and extrahepatic mechanisms leading to an aggravation of liver cell damage. Following reoxygenation, Kupffer cells (KC), the central mediators of hepatic pathomechanisms, are rapidly activated (67;68). Once activated, the liver macrophages generate reactive oxygen species (ROS), proinflammatory cytokines, chemokines, and other mediators contributing to postischemic tissue injury, to systematic inflammatory response syndrome and to multiorgan failure (69). Besides, the macrophages contribute further to inflammation *via* secretion of Platelet activating factor (PAF) (44) and proinflammatory cytokines such as interleukins and TNF-α (70-72). Together with activated complement factors (73), these inflammatory mediators activate and recruit neutrophils into the postischemic liver (74;75), which generate even more ROS (76;77), and release additional proteases and other degradative enzymes (78). In addition to the inflammatory response, vasoconstriction of sinusoids induced by Endothelin-1 (79) promotes heterogenous closure of many vessels, which prolongs ischemia in certain areas of the liver even after reperfusion and leads to the development of delayed perfusion failure (80;81).

Taken together, improved protection against IRI might decrease the rate of preservation-related complications and, moreover, should thereby increase the number of organs available for liver transplantation. Therefore, elucidation of the involved pathomechanisms is essential for developing protective strategies.

3.2 APOPTOSIS OR NECROSIS: WHICH CELL DEATH OCCURS DURING HEPATIC IRI?

Several studies have reported evidence for the occurance of apoptosis in hepatic IRI. According to these observations, 50-70% of endothelial cells and 40-60% of hepatocytes seem to undergo apoptosis during reperfusion (82-84). For instance, it is reported that prolonged ischemic intervals lead to a burst of various cytokines including TNF-α (74),

and that TNF- α initiates apoptosis in hepatocytes and sinusoidal endothelial cells (SEC) (85;86). Other studies confirm that SECs undergo apoptosis during cold I/R (82) and both, SECs and hepatocytes die apoptotic after warm I/R (84). Furthermore, several studies display that inhibition of apoptosis significantly prevents cell death and improves animal survival after prolonged periods of ischemia (83;86-90).

However, a controversy emerged over the past years on whether apoptotic or necrotic cell death accounts for the severe parenchymal injury observed during reperfusion (91;92). Some investigations claim that the major part of parenchymal injury is caused by massive necrosis (91).

One possible explanation for the occurance of both types of cell death during IRI is the so called necrapoptosis/aponecrosis theory developed by *Lemasters JJ*: the author postulates that a process can start with a common death signal or toxic stress and then cumulate in either necrotic or apoptotic cell death (FIGURE 4) (93). Thus, it is possible that both pathways are present after ischemic injury and that apoptosis and necrosis might overlap after reperfusion injury. Which type of cell death is performed depends on other modifying factors, such as the decline of cellular ATP levels, degree of steatosis or onset of mitochondrial permeability transition (MPT) (94-96). After the onset of MPT, mitochondrial uncoupling and activation of the mitochondrial uncoupler-stimulated ATPase leads to profound ATP depletion and thus to necrosis (96). Moreover, the onset of MPT leads to large-amplitude mitochondrial swelling followed by the release of Cytochrome c. Released cytochrome c can interact with Apaf-1, Pro-caspase-9, and ATP forming the apoptosome which is required for Caspase-9 and subsequent Caspase-3 activation. This Cytochrom c-dependent caspase activation requires ATP (6). Accordingly, apoptosis is an energy-dependent mechanism. Consequently, MPT can lead to both, apoptosis *via* Cytochrome c release and necrosis *via* ATP depletion.

Thus, if reperfusion leads to both MPT onset and ATP depletion, apoptotic signalling is blocked at the level of the apoptosome, and necrosis occurs as a direct result of the failure of ATP regeneration. By contrast, if glycolytic substrate is available, profound ATP depletion is prevented, and necrosis does not occur (97-99).

Therefore, if apoptosis is repressed at a certain point before both MPT onset and ATP depletion start, this inhibition might also prevent the cells from necrotic cell death.

Finally, aim of the present study was to elucidate the signalling pathways of two different therapeutic approaches reducing hepatocyte apoptosis, which consequently might also protect against necrotic cell death.

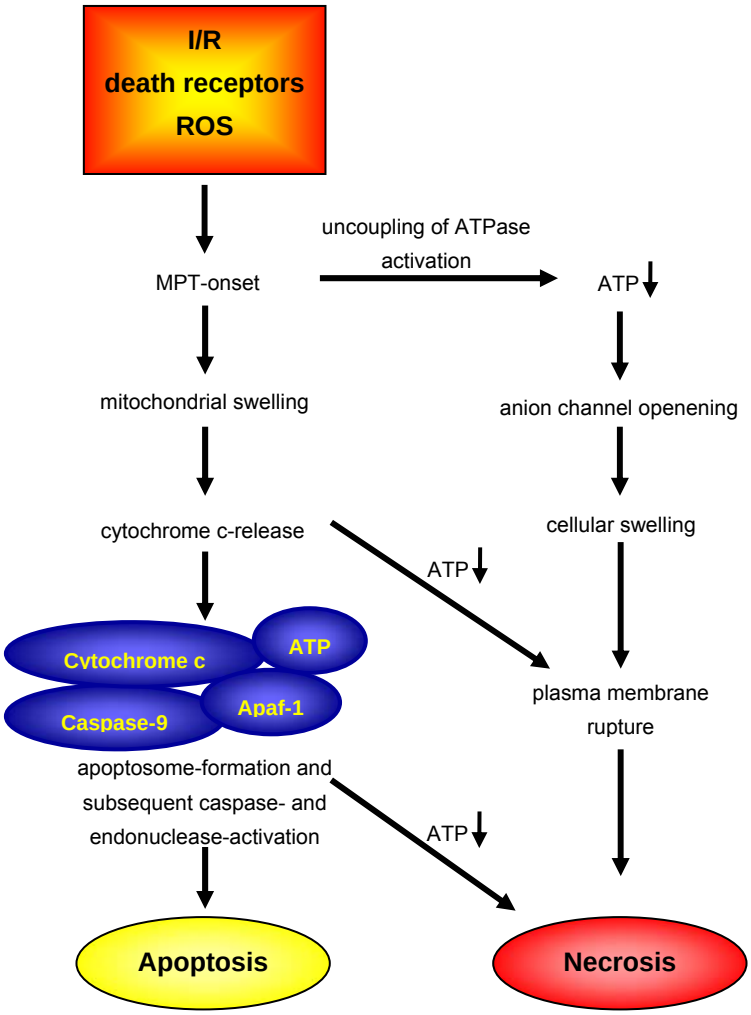


FIGURE 4: Scheme of MPT-dependent mechanisms in necrapoptosis (adapted from (2)): for details see text.

4 TNF-α-/ACTINOMYCIND-INDUCED APOPTOSIS IN ISOLATED HEPATOCYTES

TNF-α-/ActinomycinD-induced apoptosis is a useful model for the investigation of the signalling pathways involved in this cell death. Since apoptosis plays an important role in a variety of liver diseases, such as IRI, we used this model to investigate the signalling pathways involved in LA-induced inhibition of hepatic apoptosis.

TNF-α is a multifunctional cytokine involved in inflammation, immunity, antiviral response, and a variety of diseases, like IRI (100), and induces apoptosis in different cell types. In the liver, TNF-α-mediated cell death is thought to contribute to viral, alcoholic and fulminate hepatitis, and to injury from hepatotoxins (74;101-105). Although TNF-α can act *via* two different receptors, TNFR (TNF receptor)1 and TNFR2, TNF-α-induced apoptosis is solely mediated by TNFR1 (106). Binding of TNF-α to TNFR1 results in trimerisation of the C-terminal cytoplasmic "death domain" of the receptor and recruitment of some intracellular proteins involved in apoptotic signal transduction (FIGURE 1) (107-109).

Hepatocytes are normally resistant to the cytotoxicity mediated *via* TNFR1 (100;110-112). Even though TNF-α triggers a signalling pathway leading to apoptosis, it can also activate key molecules able to block this pathway. The transcription factor NF-κB thereby plays a central role: it is activated by TNF-α and inhibits the apoptotic response induced by this cytokine and other stimuli (FIGURE 1) (113-115). Since TNF-α-induced apoptosis is enhanced by blocking protein synthesis, it appears that NF-κB upregulates the synthesis of antiapoptotic genes. Thus, pretreatment of mouse hepatocytes with TNF-α prevents subsequent TNFR-mediated apoptosis by a rapid defense mechanism induced by the activation of NF-κB (116). Recently, *Marchetti et al.* described a PI3-K-dependent NF-κB activation by TNFR2, leading to persistent NF-κB activity being essential for neuronal survival (117). However, other mechanisms contributing to hepatocyte survival have not yet been fully elucidated. In addition to activation of the NF-κB pathway, TNF-α has recently been shown to activate other antiapoptotic signalling pathways. Among the growth factor signalling molecules, PI3-K has been shown to be important for cell survival (118). Recent studies indicate that protective

effects of PI3-K are mediated mainly by one of its downstream targets, namely Akt (119). Activation of the PI3-K/Akt pathway does inhibit liver cell death induced by a diversity of apoptotic stimuli (120).

5 INTERVENTIONS TO PROTECT AGAINST LIVER DAMAGE

5.1 LA

5.1.1 HISTORY

In 1937, *Snell et al.* reported about a nutritional factor extracted from potatoes, which was required for the growth of Lactobacillus. This factor was named “potatoe growth factor” (121). Later studies in yeast extracts described an “acetate replacing factor” and a “pyruvate oxidation factor”. Then, in 1949, it was proposed that probably all these factors were the same compound. Later, in 1951, pale yellow crystals of this compound were purified and called “α-lipoic acid” (122). To date, α-lipoic acid is also known as 6,8-thioctic acid, 1,2-dithiolane-3-pentanoic acid, or 1,2-dithiolane-3-valeric acid (FIGURE 5). α-lipoic acid (LA) is an eight-carbon disulfide containing a chiral carbon, therefore existing in two enantiomeric forms, the naturally occurring R-enantiomer (R-LA), and the unnatural S-isomer (S-LA).

LA was initially classified as a vitamin, but was later found to be synthesised by plants, animals and humans (123;124). Although octanoic acid and a sulphur source are known to be precursors of LA, the complete biosynthesis has not been elucidated yet (124-126).

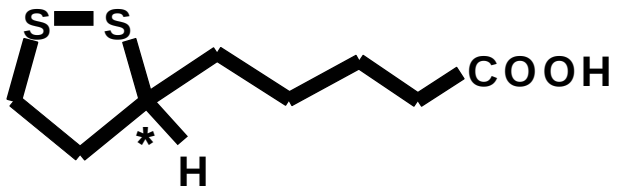


FIGURE 5: α-lipoic acid

5.1.2 PHYSIOLOGICAL FUNCTIONS

The disulfide-containing cofactor R-α-lipoic acid (R-LA) is an extremely widely distributed protein-bound cofactor which is essential for the activity of a variety of enzyme complexes that catalyse oxidative decarboxylations in prokaryotic and eukaryotic cells (127;128). In humans, it is part of several 2-oxo acid dehydrogenases that take part in energy formation. As lipoamide, it is covalently bound to the amino group of lysine residues and functions as prosthetic group in the pyruvate dehydrogenase complexes (127;128). This multienzyme complex is composed of multiple copies of three enzymes: the pyruvate (α-keto acid) dehydrogenase (E_1), the dihydrolipoyl acetyltransferase (E_2), and the dihydrolipoyl dehydrogenase (E_3). These three enzymes catalyse five reactions that oxidatively decarboxylate their substrates. R-LA binds acyl groups and transfers them from one part of the enzyme complex to another. In this process, LA is reduced to dihydrolipoic acid (DHLA) by dihydrolipoyl acetyltransferase, which is subsequently reoxidised by dihydrolipoyl dehydrogenase under the formation of NADH. Generally, LA and DHLA act as a redox couple, carrying electrons from the substrate of the dehydrogenases to NAD^+.

Besides the pyruvate dehydrogenase, LA is also part of the glycine cleavage system, a multienzyme complex located in the hepatic mitochondrial matrix. This complex catalyses the oxidation of glycine to CO_2 and ammonia, forming NADH and 5,10-methylenetetrahydrofolate.

5.1.3 PHARMACOKINETICS OF LA

5.1.3.1 THE BIOAVAILABILITY OF LA

After oral application of 1 g R-LA, R-LA is rapidly absorbed, and concentrations up to 1.154 ng/ml of free R-LA appear in the blood plasma (129;130). The absolute biovailability of LA was calculated to be between 20% and 38%, depending on the isomer and formulation, and its half live in plasma is approximately 30 min (131).

LA is presumably eliminated in the liver. Anyhow, only one study exists examining the particular kinetic of LA uptake in rat liver: LA is absorbed in low concentrations (< 75 µM) by a saturable, in higher concentrations (> 75 µM) by a nonsaturable kinetic. Therefore,

LA uptake at low concentrations is carrier-mediated, at higher concentrations this uptake primarily occurs *via* diffusion (132).

5.1.3.2 THE METABOLISM OF LA

Reduction

After absorption, exogenously applied LA enters the mitochondrial matrix and is reduced by dihydrolipoamide dehydrogenase to dihydrolipoic acid (DHLA) (FIGURE 6), using electrons from NADH (129;133). Surprisingly, also human erythrocytes, which lack mitochondria, are able to reduce LA: the glutathione reductase, which resides mainly in the cytosol, is responsible for this NADPH-dependent LA-reduction (134). In the rat liver, reduction occurs equally by dihydrolipoamide dehydrogenase and glutathione reductase (135). Furthermore, also the thioredoxin reductase can catalyse LA reduction even more efficiently than dihydrolipoamide dehydrogenase.

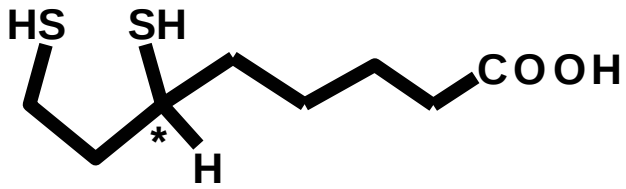


FIGURE 6: Dihydrolipoic acid

β-Oxidation

Another metabolic event of lipoic acid is the β-oxidation of its pentanoic acid side chain. In urine of rats, the following metobolites were identified after [1,6-^{14}C]-lipoate-administration: see FIGURE 7. In conclusion, DHLA, bisnorlipoic acid, 13-hydroxybisnorlipoic acid and tetranorlipoic acid may contribute to the antioxidative effect of LA *in vivo*.

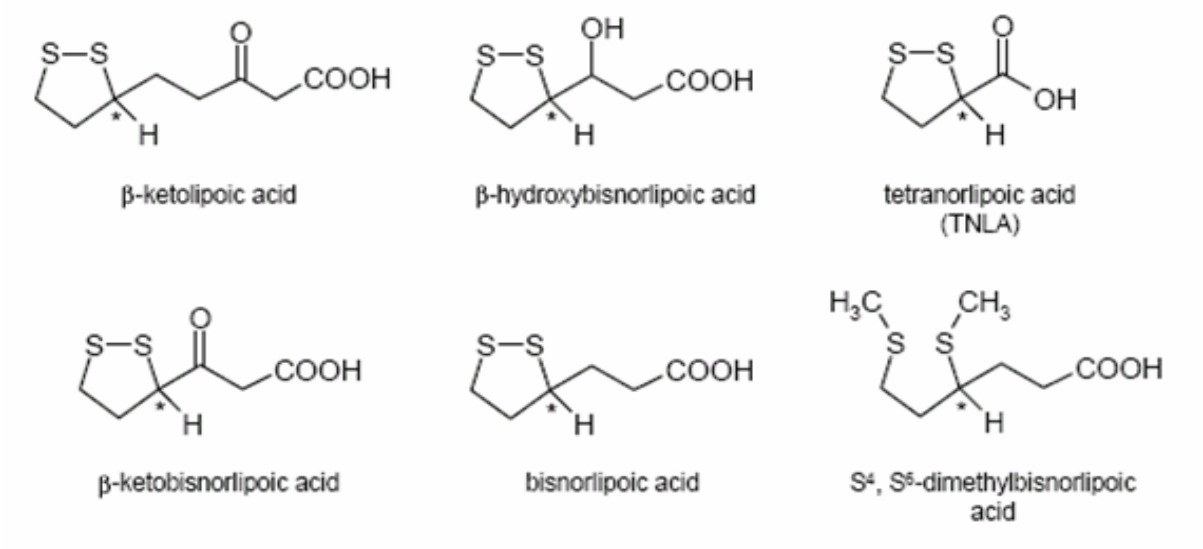


FIGURE 7: Main metabolites of LA

Toxicity of LA

Acute toxicity of LA is very low with approximately 400 mg/kg after i.v. administration of LA.

5.1.4 THERAPEUTIC POTENTIAL OF LA

In 1966, German physicians started to administer lipoic acid to patients with liver cirrhosis, mushroom poisoning, heavy metal intoxication and diabetic polyneuropathy. Originally, the reason for this treatment was the observation, that patients with liver cirrhosis, diabetes mellitus and polyneuropathy had lower levels of endogenous LA (136). Thus, it was assumed that supplementation with LA may help to overcome the shortage, thereby restoring the 2-oxo acid oxidation. Indeed, destruction of the cofactor function of LA may be involved in pathological processes.

Diabetic neuropathy represents a major health problem being responsible for substancial morbidity, increased mortality, and impaired quality of life. The therapeutic efficacy of LA to inhibit advanced glycation endproducts has been investigated in several clinical trials. Comprising all these studies, it seems that LA used in doses of at least 600 mg/day has beneficial effects: Already a short term treatment with LA (600 mg/day;

i.v.; 3 weeks) reduced the chief symptoms of diabetic polyneuropathy and decreased neuropathic complaints such as pain and paresthesia (137). Long term treatment over 4-7 months even reduced neuropathic deficits and improved cardiac autonomic neuropathy (138;139). LA (1,200 mg/day; p.o.; 4 weeks) lowered plasma glucose levels in patients with type II diabetes (140). Preliminary data indicate a possible long-term improvement in motor and sensory nerve conduction in the lower limbs (141). Further, LA improves neuropeptide deficits, nerve blood flow, and neurological symptoms (137;142;143). It has been suggested that oxidative stress has a role in the causation of noninsulin-dependent diabetes since high glucose levels produce oxidative stress (144;145). In streptozotocin diabetic rats, antioxidants and LA delayed the onset of polyneuropathy (143;146). Besides these effects, LA exhibits further protective effects in hyperglycaemia, microangiopathy, and excessive oxidative stress known to cause nerve damage in diabetes.

LA exhibits well known and efficient antioxidative, redox regulating and metal chelating properties and is therefore approved for the treatment of diabetic neuropathy, and suggested for the treatment of liver cirrhosis, heavy metal intoxication, glaucoma, mushroom poisoning, and cellular oxidative damage (147-149). Furthermore, several studies display a protective role of LA in ischemia and IRI in cerebellum, kidney, retina, and liver (150-153).

Beyond the antioxidative properties of LA, however, little is known about molecular targets of LA in liver damage. Since apoptosis plays a major role in liver diseases (see above), a possible antiapoptotic effect of LA on hepatocytes was investigated.

5.2 NATRIURETIC PEPTIDES AND ANP

The atrial natriuretic peptide (ANP) was first described by *de Bold et al.* (154). Twenty years ago, in 1984, the circular structure of ANP was elucidated: it is a 28 amino acid circular peptide containing an intrachain disulfide bond (155;156) (FIGURE 8). All natriuretic peptides (NP) exhibit this cyclic structure of 17 amino acids formed by a disulfide bond between two cysteine residues, being required for the biological activity (155;156). Eleven of the cyclic amino acids are homologous in each NP, whereas the number of amino acids differs at the C- and N-terminal end. To date, further members of

the NP family are known, namely brain natriuretic peptide (BNP), secreted by atrial and ventricular myocardial cells (157;158), C-type natriuretic peptide (CNP), expressed primarily in the central nervous system and in vascular endothelium (159), and the recently identified dendroaspis natriuretic peptide (DNP). Related peptides include guanylin and uroguanylin (for review see (158)).

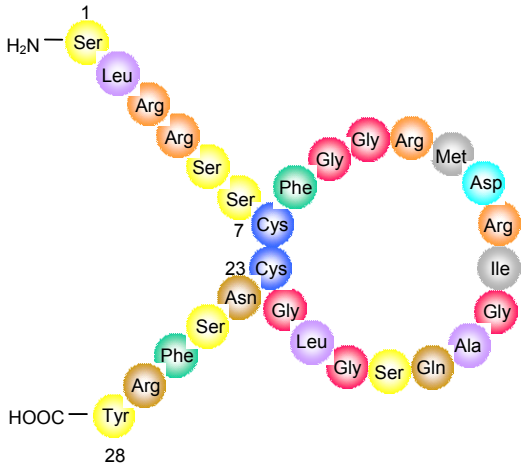


FIGURE 8: Structure and amino acid sequence of ANP

ANP is secreted primarily by cardiac atria in response to atrial stretch. It is involved in the regulation of blood pressure and volume homeostasis by compensating the renin-angiotensin-aldosterone system and exerts natriuretic as well as diuretic actions (160).

The biosynthesis of ANP is typical for peptide hormones: the ANP gene is transcribed into mRNA encoding a 152 amino acid peptide, the so called pre-pro-ANP. Pre-pro-ANP is cleaved at the N-terminus and thus released pro-ANP accumulates in granula (161). By stimulation, such as stretching of the atria, pro-ANP is further cleaved into an amino-terminal fragment and the biologically active hormone ANP. After activation, ANP is secreted into the circulation from atrial myocytes *via* exocytosis (162).

5.2.1 NATRIURETIC PEPTIDE RECEPTORS AND SIGNAL TRANSDUCTION

Three distinct natriuretic peptide receptors (NPR) have been identified for NPs (FIGURE 9): the NP receptor-A and -B bind all three NPs, with preference of NPR-A for ANP and BNP, and NPR-B for CNP (163;164). The natural ligand for NPR-B is CNP. Both receptors contain an intracellular kinase-like domain and a guanylate cyclase domain. Consequently, binding of NPs to their receptors activates the guanylate cyclase, leading to intracellular cGMP formation (cyclic guanosine monophosphate). NPs mediate most of their physiological effects through binding to the membrane-bound guanylate cyclase.

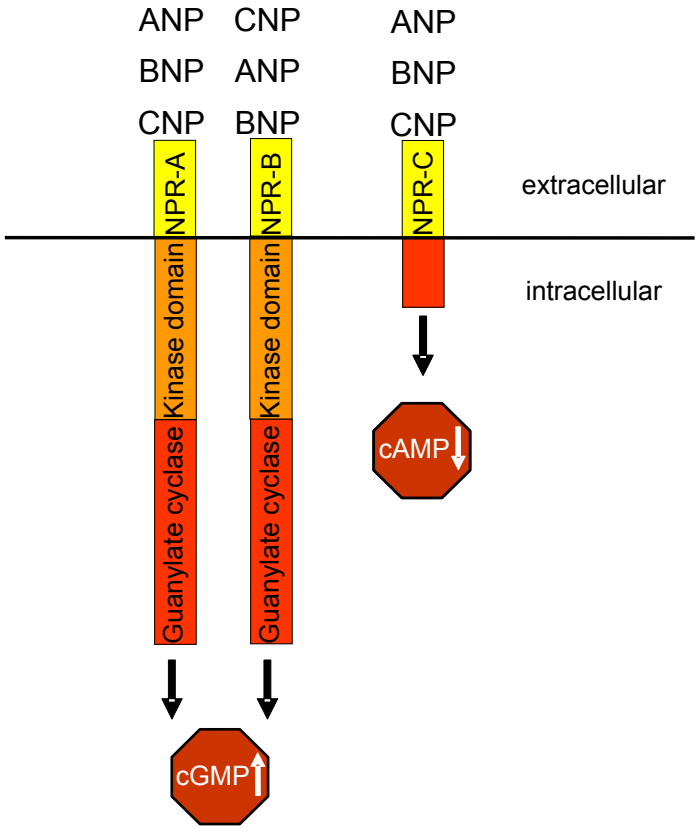


FIGURE 9: Natriuretic peptide receptors: after extracellular binding at NPR-A/-B, intracellular cGMP is elevated *via* the guanylate cyclase domain. NPR-C is able to clear NPs and exerts other biological effects. NPR-A binds ANP and BNP, NPR-B only CNP, and NPR-C all three NPs.

NPR-C does not contain an intracellular guanylate cyclase domain. All NPs bind to this so-called “clearance” receptor with equal affinity and are subsequently internalised and enzymatically degraded. Besides its clearance function, this receptor is discussed to mediate certain NP effects: ANP attenuates proliferation in astroglial cells, reduces endothelin expression in endothelial cells, and inhibits COX-2 expression in macrophages *via* NPR-C (165-167). In certain cases, NPR-C is discussed to mediate inhibition of adenylate cyclase activity and activation of phospholipase C (168).

NPRs are widely distributed in mammalian tissues, predominantly in the cardiovascular system, adrenal glands and kidney (169). In addition, all three receptors have also been identified in the liver (170).

5.2.2 cGMP-DEPENDENT SIGNALLING

After NP binding to NPR-A or NPR-B, the cellular actions of the peptides are mediated by their second messenger cGMP, which is generated upon activation of the particulate guanylate cyclases. Thus, generated cGMP can exert its regulatory functions by interacting with different cGMP-receptor proteins:

(i) the most prominent target of cGMP is the cGMP-dependent protein kinase PKG (171;172).

(ii) in high concentrations, cGMP can also activate the cAMP-dependent protein kinase PKA (173).

(iii) cGMP can also interact with the cAMP pathway *via* binding to phosphodiesterases, thus causing either a decrease or an increase of cAMP levels (174).

(iv) cGMP-gated ion channels, contributing to cGMP-mediated natriuresis (175).

ANP mediates most of its effects *via* the guanylate cyclase-coupled A-receptor (NPR-A) and the second messenger cGMP (176;177). cGMP exerts its regulatory functions *via* interaction with the cGMP-receptor proteins PKG and in high concentrations also with PKA (171;173;178). Since cGMP seems to suppress apoptotic cell death (179), aim of the present study was to identify the cGMP target molecules responsible for ANP's protective actions in IRI.

5.2.3 ANP AND IRI

The protection of rat livers against IRI by ANP has been demonstrated in both, warm and cold ischemic and reperfused rat livers (176;180;181). Liver perfusion with ANP 20 min prior to ischemia/reperfusion (pretreatment) caused:

(i) a decrease in cell damage as assessed by release of lactate dehydrogenase.

(ii) reduced hepatocyte apoptosis as well as hepatocyte and endothelial necrosis as assessed by TUNEL assay, Caspase-3 activity, and trypan blue uptake in cold ischemia.

(iii) improvement of liver function as seen by increased bile flow.

All ANP effects are cGMP-mediated, which indicates an NPR-A-mediated signalling of hepatocyte protection. Furthermore, mechanistic investigations suggested that ANP mediates its protective actions *via* a reduced activation of the redox sensitive transcription factors NF-κB and AP-1, resulting in decreased expression of TNF-α (182). Additionally, ANP protects liver cells against oxidative stress induced by activated KCs whithout affecting the superoxide formation of KCs (183). Preconditioning of the liver with ANP activates the p38 MAPK pathway (184). ANP is furthermore able to induce the heat shock response by activating the heat shock transcription factor and inducing the heat shock protein Hsp70 (177;185). In addition to confer thermotolerance (187;188), Hsp70 is discussed to protect against ROS toxicity and IRI *via* renaturation/refolding of affected proteins (186), to prevent apoptotic cell death, and to reduce hepatic IRI (177;189).

Thus, ANP appears to protect liver cells against IRI. Since it is still unknown how ANP

may protect against apoptosis, aim of the present study was to elucidate the signalling pathways involved in ANP-mediated protection aginst ischemic apoptosis and probably against secondary necrosis.

5.2.4 p38 MAPK

p38 MAPK is a member of the mitogen-activated protein kinase (MAPK) family and is activated predominantly by cellular stress and inflammatory signals. For activation, p38 MAPK requires phosphorylation at both, threonine and tyrosine residues by dual specificity MAPK kinases (MKK 3/6). These MAPKKs are in turn activated by upstream MAPKK kinases (MEKKs, ASK1, TAK1, MLK) (for review see (190-193)). Once activated, p38 MAPK phosphorylates its substrates at specific serine/threonine residues. Several observations suggest a protective role of p38 MAPK during warm ischemia and hypoxia (66;194;195). On the other hand, p38 MAPK is widely known as a proapoptotic kinase (196;197).

Therefore, aim of the present study was to elucidate the role of p38 MAPK in the ANP-induced inhibition of hepatic apoptosis.

5.2.5 cGMP-DEPENDENT PROTEIN KINASE

ANP mediates most of its effects *via* the cGMP-coupled NPR-A receptor and thus *via* the second messenger cGMP. Therefore, the most prominent target of cGMP, PKG, and its involvement in hepatocyte apoptosis was investigated.

PKG is a cGMP-activated serine/threonine kinase. Two isoenzymes of PKG have been identified in mammals, PKG I and II (172;178;198). PKG I is a predominantly cytosolic protein, whereas PKG II is bound to the plasma membrane. PKG I and II represent homodimers consisting of monomeric subunits with a molecular mass of 77 kDa and 87 kDa, respectively. PKGs are composed of a regulatory and a catalytic domain. Binding of cGMP to the regulatory domain results in conformational change. Thus, the substrate can interact with the binding domain inside the catalytic domain and a phosphate residue of ATP is transferred to either serine or threonine of the substrate.

PKG I expression was shown in a broad range of cell types, including platelets, smooth muscle cells, kidney, endothelial cells, neutrophil granulocytes, macrophages, and is highest in Purkinje cells of the cerebellum (174;199-203). In contrast, PKG II shows only limited, tissue-specific expression. Interestingly, there is so far no distinct information available concerning PKG expression in the liver.

The physiological and pathophysiological functions of PGK include modulation of secretory processes in kidney and intestine. PKG I knockout mice display impaired cGMP-dependent vasorelaxation, elevated blood pressure, and enhanced platelet activation during ischemia and reperfusion (IR) (for review see (204)). In bones, PKG II is important for the longitudinal growth (205).

In some tissues ANP-induced protective effects seem to be mediated *via* cGMP and PKG: in the kidney, PKG II inhibits secretion and expression of renin, thus affecting the regulation of blood pressure, referring to an ANP-mediated effect (206). Furthermore, ANP can increase the glomerular filtration rate and this might be a result of PKG I being located in mesangial and smooth muscle cells of the vasculature (201). In addition, also the protective effects of 17β-estradiol on cardiomyocytes seem to be mediated *via* ANP/cGMP/PKG (207).

5.2.6 cAMP-DEPENDENT PROTEIN KINASE

In higher concentrations cGMP can also activate PKA.

PKA is expressed in all mammalian tissues investigated, including the liver (208). In contrast to PKG, the regulatory and the catalytic domain are localised on different polypeptide chains. Inactive, PKA is available as tetramer consisting of two regulatory and two catalytic domains. Binding of cAMP leads to dissociation of this tetramer and subsequently to the release of the two catalytic subunits. These domains are then responsible for the transfer of γ-phosphoryl groups (209-211).

Two major regulatory subunits have been identified as products of different genes, namely PKA I (43 kDa) and II (45 kDa). These subunits together with one of the three isoforms of the catalytic subunit (40 kDa) form the complete enzyme. The PKA I complex is mainly cytosolic, whereas the PKA II complex is almost exclusively

particulate associated.

PKA is involved in a variety of metabolic processes, including glycogenesis and lipogenesis, as well as secretory processes, muscle contraction, learning, ion channel conductance, proinflammatory cytokine production, differentiation, and growth control (212). Furthermore, several studies describe a participation of PKA in signalling mechanisms mediated by NPs (213-216).

C MATERIALS AND METHODS

1 THE ISOLATED PERFUSED RAT LIVER

1.1 CHEMICALS

Racemic LA, DHLA, and R-LA were a gift from Viatris Inc. (Frankfurt/Main, Germany). ratANP was purchased from Calbiochem/Novabiochem (Bad Soden, Germany). If not stated otherwise all other chemicals were bought from Sigma (Deisenhofen, Germany) or VWR international (Munich, Germany). All solutions were prepared with double distilled H_2O and were subsequently autoclaved or sterile filtrated.

1.2 ANIMALS

For liver perfusion and hepatocyte isolation male Sprague-Dawley rats weighing 220-280 g were purchased from Charles River WIGA GmbH (Sulzfeld, Germany). The animals had free access to chow (Sniff, Soest, Germany) and water up to the time of experiments. All animals received humane care. The study was registered with the local animal welfare committee.

1.3 SOLUTIONS AND SUBSTANCES

Krebs-Henseleit buffer (KH-buffer)

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

substances	vendor	final concentration	dissolution medium	treatment period during ischemia
ratANP 99-126	Calbiochem Mw 3062.5	200 nM	0.9% NaCl solution	10.-30. min and cold ischemia
SB203580	Calbiochem Mw 377.4	2 µM	H_2O	0.-30. min and cold ischemia

TABEL 1: Substances for liver treatment

1.4 MODEL OF THE ISOLATED PERFUSED RAT LIVER

Perfusion experiments for the model of cold ischemia and reperfusion were carried out by Dr. T. Gerwig (Department of Pharmacy, University of Munich, Germany.).

For the perfusion model male rats were anaesthetised with pentobarbital sodium (Narcoren®, 50 mg/kg body weight, intraperitoneal, Merial GmbH, Hallbergmoos, Germany) and 0.1 ml heparin (250 I.U., Braun-Melsungen AG, Melsungen, Germany) was injected into the *vena femoralis* to prevent blood clotting. After incision of the abdominal wall, the portal vein was cannulated and the liver was perfused *in situ* in a non-recirculating manner at a constant flow rate of 3.0 - 3.5 ml x min^{-1} x g $liver^{-1}$ with a carbogen-oxygenated (95% CO_2 and 5% O_2, Linde, Unterschleißheim, Germany), hemoglobin- and albumin-free Krebs-Henseleit buffer (pH 7.4) at 37°C. The outflow of the perfusate was possible *via* the *vena cava inferior*.

1.4.1 COLD ISCHEMIA AND REPERFUSION

Isolated rat livers were just flushed exsanguinous (0' perfusion) with KH buffer or were perfused with KH buffer before ischemia for 30 min. Preconditioning of the organs was performed by adding ANP (200 nM) to the pre-ischemic perfusate for 20 min in the presence or absence of protein kinase inhibitors. Livers were then kept in University of Wisconsin (UW) solution (DuPont, Bad Homburg, Germany) containing ANP for 24 h at 4°C (FIGURE 10). At the indicated times livers were snap-frozen and stored at -85°C until further analysis.

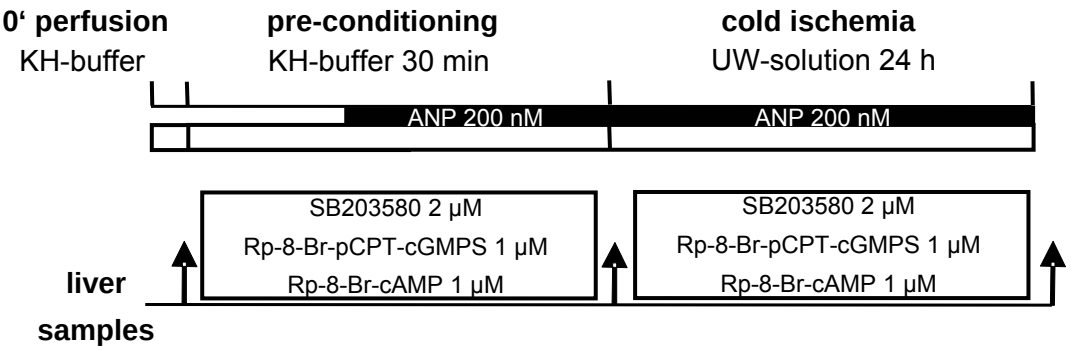


FIGURE 10: Experimental protocol. Livers were just flushed exsanguinous (0 min perfusion) or were perfused with KH buffer before ischemia for 30 min (preconditioning) either in the absence or in the presence of 200 nM ANP or 50 µM 8-Br-cGMP given 20 min prior to ischemia and to the storage solution. After preconditioning livers were stored for 24 h at 4°C (cold ischemia). In additional experiments, livers were pretreated by adding different protein kinase inhibitors (2 µM SB203580, 1 µM Rp-8-Br-pCPT-cGMPS, 1 µM Rp-8-Br-cAMP) to the pre-ischemic perfusate in the absence or presence of 200 nM ANP. Arrows indicate time points when liver samples were taken.

2 LIVER CELL CULTURE

2.1 ANIMALS

Animals were purchased and housed as described under B.1.2.

2.2 SOLUTIONS

PBS (phosphate buffered saline) (pH 7.4)

Na_2HPO_4	1.48 g
KH_2PO_4	0.43 g
NaCl	7.20 g
H_2O	ad 1000 ml

Solution A (pH 7.35)

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

Hepes	20 mM
EGTA	0.5 mM

Solution B (pH 7.35)

NaCl	115 mM
$NaHCO_3$	25 mM
KCl	5.9 mM
$MgCl_2$	1.18 mM
NaH_2PO_4	1.23 mM
Na_2SO_4	1.2 mM
$CaCl_2$	2.5 mM
Hepes	20 mM
Collagenase H	0.05%

Percoll™ gradient

NaCl	136 mM
Na_2HPO_4	10.2 mM
KCl	2.68 mM
KH_2PO_4	1.46 mM
$MgCl_2$	0.5 mM
Percoll™ solution	50% (v/v)

Hanks' balanced salt solution (HBSS) (pH 7.35)

$CaCl_2$	0.95 mM
KCl	5.3 mM
KH_2PO_4	0.44 mM
$MgCl_2$	0.49 mM
$MgSO_4$	0.41 mM
NaCl	136.75 mM
Na_2HPO_4	0.34 mM
Hepes	20 mM

Medium199 or L-15 Medium (Pan Biotech, Aidenbach, Germany), both supplemented with:

Glucose	8.3 mM
Glutamin	2 mM
Dexamethasone	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 of precultivation

Trypan blue solution

Trypan blue	0.08 g
NaCl	0.03 g
H_2O	ad 20 ml

2.3 CULTIVATION

Cell cultivation was performed in a cell incubator (Heracell, Heraeus, Hanau, Germany) at 37°C and 5% CO_2.

2.4 ISOLATION OF KUPFFER CELLS

Isolation of Kupffer cells (KC) was performed by PD Dr. Alexandra K. Kiemer, Department of Pharmacy, University of Munich, Germany (217).

Rat KC were isolated according to the method of *Knook et al.* (218) with modifications as reported by *Armbrust et al.* (219). Briefly, rats were anaesthetised as per description under 2.4. Subsequently the liver was perfused *in situ* after cannulation of the portal vein with 100 ml of Gay´s balanced salt solution (GBSS), then digested by perfusion with pronase and pronase/collagenase-solutions. The liver cells thus obtained were suspended in a pronase/collagenase-solution, shaken carefully for 30 min and passed through a Falcon cell strainer (100 µm, BD Labware, Bedford, MA, USA). The hepatocytes were segregated by differential centrifugation and the remaining nonparenchymal cells were separated by a Nycodenz density gradient (1,500 x *g*, 15 min). The cells of the interphase were collected and segregated according to size by counterflow elutriation using a Beckman-centrifuge (J 2-21, JE-6B rotor, Beckman Instruments, Munich, Germany). The sedimented KC were 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 2.25 x 10^6 cells/well in 6-well tissue plates and cultivated for 1 to 3 days. Two hours after plating, cells were washed to eliminate non-adherent cells. 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.

2.5 ISOLATION OF PRIMARY HEPATOCYTES

Hepatocytes were isolated using a modified two step collagenase digestion method (220). Male Sprague-Dawley rats weighing 200-300 g were anaesthetized by intraperitoneal injection of Narcoren© (50 mg/kg body weight). Then, 250 I.U. 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 the liver was perfused with Ca^{2+}-free solution A to dissolve desmosomes by Ca^{2+}-deprivation. Perfusate flow was adjusted by a roller pump (Watson Marlow 101U/R, Falmouth, UK) to 30 ml/min. The perfusate was oxygenated with carbogen (95% CO_2 and 5% O_2, Linde, Unterschleißheim, Germany) for 15 min and kept at 37°C. Incision of the *aorta abdominalis* ensured a sufficient drain of the perfusate. After thorakotomia, the *vena cava inferior* was cannulated *via* the right heart atrium. This vein was then ligated above the kindney and below the liver vein to prevent outflow of the perfusate. Then the liver was flushed exsanguinous for 4 to 7 min with solution A and subsequently perfused in a recirculating manner with solution B (oxygenated with carbogen for 15 min at 37°C) containing 0.05% Collagenase H (Roche Diagnostics, Mannheim, Germany). After further 6 to 8 min, the liver displayed an increasing number of small rips, indicating the digestion of the extracellular matrix by Collagenase H. Afterwards the liver was carefully excised, rinsed with Ca^{2+}-containing solution B and then placed into a solution B containing petri dish. The following procedure was performed in a laminar flow work bench (Herasafe, Heraeus, Hanau, Germany). The hepatic cells were isolated by tearing the liver capsule apart and carefully scratching out the liver cells with a tea spoon. The cell suspension thus obtained was filtered with a Falcon cell strainer (100 µm, BD Labware, Bedford, MA, USA) and sedimentation of the cells was allowed for 10 min. Afterwards the supernatant was removed and the hepatocytes were separated from the obtained cell suspension *via* Percoll™ (Amersham Pharmacia Biotech, Upsala, Sweden) density gradient centrifugation (650 x *g*, 10 min, 4°C). After centrifugation the 3 layers in the supernatant were removed and the remaining pellet, containing the hepatocytes, was resuspended in L-15 Medium. Then, after staining the cells with trypan blue (500 µl trypan blue solution, 450 µl solution B, 50 µl cell suspension), the number and viability of the cells was checked in a Neubauer counting chamber (vitality was always over 80%). Subsequently,

hepatocytes were seeded into collagen R (0.2 mg/ml, Serva, Heidelberg, Germany) coated 12-/6-well culture plates (Peske, Aindling-Pichl, Germany) at a concentration of 8 x 10^5/ 10^6 cells/ml in L-15 Medium (Pan Biotech, Aidenbach, Germany) containing 5% (v/v) FCS (Gibco^{TM} Invitrogen Corporation, Karlsruhe, Germany). After 4 h, cells were washed and made quiescent with FCS free medium. To remove cell debris, plates were washed twice before each medium change with HBSS. Hepatocytes were used for experiments after 24 and 48 h of culture. After the indicated treatment time, medium was removed, plates were washed twice with HBSS and after addition of the appropriate lysis buffer cells were stored at -85°C until further investigations.

2.5.1 cGMP Measurement

Assay procedure

For cGMP measurement isolated hepatocytes were cultured in 6-well plates (10^6 cells/ml). Cells were left untreated or treated for 20 min with ANP (20 nM, 200 nM and 1 µM) or with SNP (10 µg/ml). Hepatocytes were lysed on ice by addition of buffer A (Amersham Pharmacia, Freiburg, Germany). After shaking the culture plates for 10 min, cells were frozen over night at -85°C. For determination of cGMP content, cells were defrosted on ice, centrifuged (14,000 *g*, 10 min, 4°C) and 200 µl of the clear supernatants were transferred to a 96-well plate. Then the cGMP measurement was performed with a commercially available kit (Amersham Pharmacia, Freiburg, Germany), based on a competitive enzyme immunoassay system. Assay performance was done as indicated by the manufacturer (protocol IV).

3 KUPFFER CELL DEPLETION

3.1 ANIMALS

Animals were purchased and housed as described under 1.2.

3.2 GENERAL ASPECTS/THEORY

The following experiments were carried out to create animals with Kupffer cell (KC) depleted livers. With this approach we wanted to study cell-cell-interactions possibly involved in ANP-mediated p38 MAPK activation in whole organs.

Kupffer cells were depleted using clodronate (FIGURE 11) containing liposomes (FIGURE 12), kindly provided by Dr. Nico van Rooijen (Vrije University, Amsterdam, The Netherlands). Clodronate (Cl_2MBP) as well as liposomes prepared of phosphatidylcholine and cholesterol are nontoxic drugs in it. Since liposomes are not able to cross vascular barriers (capillary walls), only macrophages in the liver (Kupffer cells), spleen (different macrophage subpopulations), lung (alveolar but not interstitial macrophages), peritoneal cavity, lymph nodes, joints (phagocytic synovial lining cells), and testis can be depleted with this method (221;222). *Schiedner et al.* described that a single intravenous clodronate injection induced complete KC depletion, whereas the amount of splenic macrophages was reduced by only approximately 20% (223). Thus, organ-specific macrophage depletion is almost possible and depends on the administration route of the liposomes.

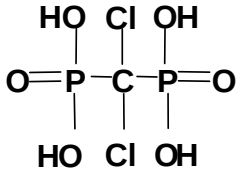


FIGURE 11: Clodronate (dichloromethylene-bisphosphonate, Cl_2MBP): chemical structure.

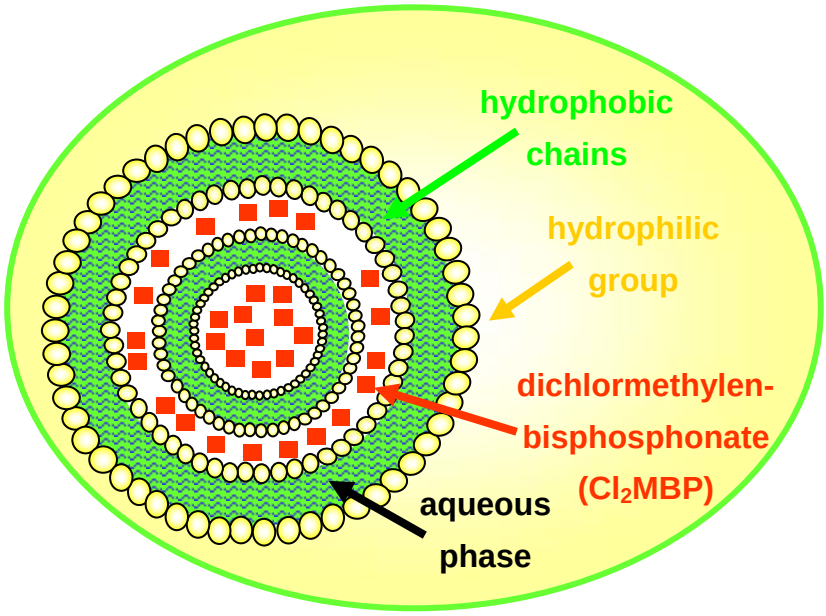


FIGURE 12: Assembly of clodronate liposomes: Liposomes are synthesised spheres, consisting of concentric phospholipid bilayers (yellow and green) separated by aqueous compartments (white). These compartments are formed, when phospholipid molecules are dispersed in water. During the formation of the liposomes part of the aqueous solution together with hydrophilic molecules such as clodronate (red squares) are encapsulated (according to (221)).

3.3 PROCEDURE

For KC depletion male Sprague-Dawley rats weighing 200-300 g were anaesthetised with diethyl ether. Then 900 µl of a solution containing liposome-encapsulated Cl_2MBP were administered 48 h before perfusion experiments *via* a single intravenous injection into the tail vein. Animals of the control group received 900 µl NaCl instead. After injection, liposomes are ingested by KCs and once inside the macrophages, the phospholipid bilayers of the liposomes are disrupted under the influence of lysosomal phospholipases. Thus, Cl_2MBP which is dissolved in the aqueous compartments between the liposomal bilayers, is released into the cell and accumulates intracellularly. After Cl_2MBP exceeds a threshold concentration, KC apoptosis is induced (224) (FIGURE 13). Free Cl_2MBP released from dead macrophages has an extremely short half life in the circulation and is removed by the renal system.

After 2 days the livers of Cl_2MBP- and NaCl-pretreated rats were perfused for 30 min ± ANP (200 nM; 20 min), snap-frozen, and stored at -85°C until further analysis (TABEL 2,

perfusion protocol see B.1.4 and B.1.4.1).

Group (n=5)	Injection 48 h prior to perfusion with:	Liver perfusion with:
Co	NaCl	NaCl
Cl_2MBP [900µl / ± 200 g body weight]	Cl_2MBP	NaCl
ANP [200 nM]	NaCl	ANP
Cl_2MBP+ANP	Cl_2MBP	ANP

TABEL 2: Schema of liver treatment.

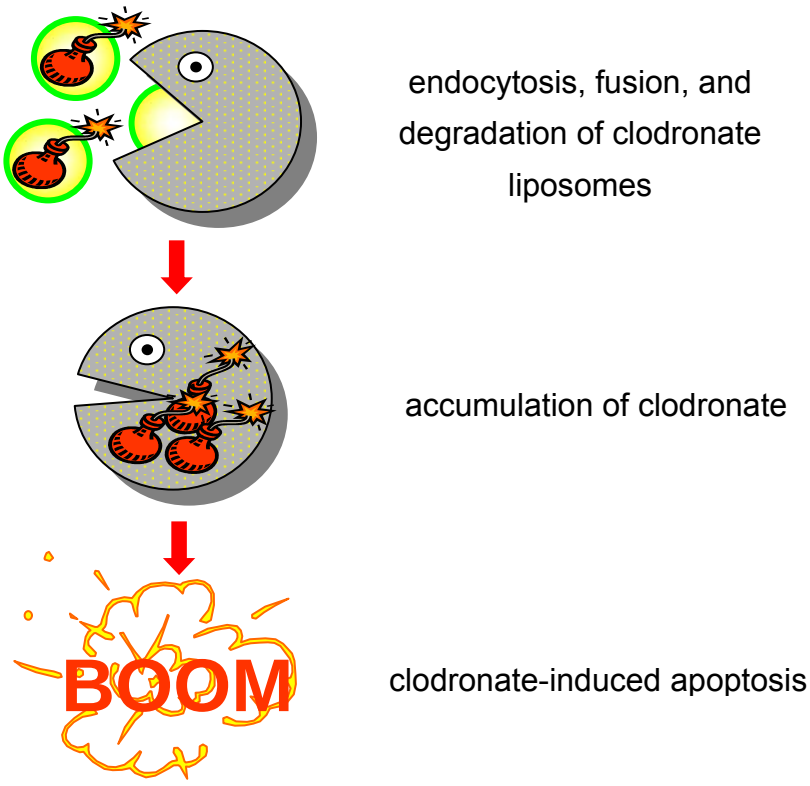


FIGURE 13: Schematic representation of the Kupffer cell depletion (according to (224)).

3.4 IMMUNHISTOLOGICAL ANALYSIS

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

Liver slices were stained with antibodies against the KC-specific surface marker ED2 to verify the Cl_2MBP-dependent depletion of the KCs. For staining, organ 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. To verify KC-depletion, ED2 as KC marker (antibody from Serotec, Oxford, England) was used. Blue staining of ED2-positive cells was realized with the ChemMate™ APAAP Kit (Dako, Hamburg, Germany) based on the alkaline phosphatase-anti alkaline phosphatase method. The Alkaline Phosphatase Substrate Kit III (Linaris, Wertheim, Germany) served as substrate for the alkaline phosphatase. Samples were counterstained in hematoxylin solution.

4 LACTATE DEHYDROGENASE EFFLUX

4.1 SOLUTIONS

Phosphate buffer (pH 7.5)

K_2HPO_4	50 mM
KH_2PO_4	50 mM

Both components are mixed until pH 7.5 is reached.
Then add:

Pyruvate	60 mM

NADH solution

NADH-Na_2	10 mg/ml

Dissolved in 0.5% $NaHCO_3$ solution.

4.2 LACTATE DEHYDROGENASE EFFLUX

Lactate dehydrogenase (LDH) release into the cell medium is a sensitive indicator of cell damage (225). The assay is based on the LDH-catalysed conversion of pyruvate to lactate. Thereby, NADH is oxidized to NAD^+, resulting in a decrease in NADH extinction.

4.3 ASSAY PROCEDURE

For measurement, the following solutions were pipetted into a volume-reduced cuvette (Peske, Aindling-Pichl, Germany):

	Reference	Sample
Phosphate buffer	1000 µl	500 µl
Perfusate or cell supernatant		500 µl
NADH solution		500 µl

The extinction was continuously monitored for 1 min at room temperature (RT) by a plotter. The enzymatic activity was calculated based on the decrease in NADH extinction (ε_{365}=3.34 $mM^{-1}cm^{-1}$).

5 WESTERN BLOT ANALYSIS

p38 MAPK protein and apoptosis related proteins (Akt, BAD) were investigated by Western blot analysis. With this specific method proteins are separated by electrophoresis and subsequently transferred to an immobilising membrane.

5.1 SOLUTIONS

Lysis buffer (pH 7.5)

Hepes	50 mM
NaCl	50 mM
EDTA	5 mM
$Na_4P_2O_7$	10 mM
NaF	50 mM
Na_2VO_4	1 mM
Add just before use:	
PMSF	1 mM
Triton® X-100	1%
Complete™ (25x)	

SDS sample buffer (stock solution)

Tris-HCl (pH 6.8)	37.5 ml
SDS	6 g
Glycerol	30 ml
Bromphenol blue	15 mg

SDS sample buffer (3 x)

Stock solution	850 µl
β-mercaptoethanol	150 µl

Separation gel 10%		**Stacking gel**	
PAA solution 30%	5.0 ml	PAA solution 30%	
1.5 M Tris, pH 8.8	37.5 ml	1.25 M Tris, pH 6.8	850 µl
SDS 10%	6 g	SDS 10%	150 µl
H_2O	30 ml	H_2O	
TEMED	15 mg	TEMED	
APS		APS	

Electrophoresis buffer (5x)		**TBS-T (pH 8.0)**	
Tris-Base	3 g	Tris-base	3 g
Glycine	14.4 g	NaCl	11.1 g
SDS	1 g	Tween 20	2 ml
ad 1,000 ml H_2O		ad 1,000 ml H_2O	

Tris-CAPS (5x) (pH 9.6)	
Tris-base	36.34 g
CAPS	44.26 g
ad 1,000 ml H_2O	

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

Coomassie staining solution		**Coomassie destaining solution**	
Coomassie brilliant blue G	1.5 g	Acetic acid (100%)	100 ml
Acetic acid (100%)	50 ml	Ethanol (96%)	335 ml
Ethanol (96%)	225 ml	ad 1,000 ml H_2O	
ad 500 ml H_2O			

5.2 ANTIBODIES

All primary antibodies were purchased from Cell Signaling (Frankfurt/M, Germany).

antibody	**isotype**	**dilution**
phospho p38 MAPK (Thr180/Thy182)	Rabbit IgG	1:1,000, BSA
totp38 MAPK	Rabbit IgG	1:1,000, Blotto
phospho Akt (Ser473)	Rabbit IgG	1:1000, BSA
totAkt	Rabbit IgG	1:1,000, BSA
phospho BAD (Ser136)	Rabbit IgG	1:1,000, BSA
phospho BAD (Ser112)	Rabbit IgG	1:1,000, BSA
totBAD	Rabbit IgG	1:1,000, BSA

5.3 PREPARATION OF PROTEIN EXTRACTS FROM RAT LIVER TISSUE

All work was performed in a 4°C cooling chamber. Livers were stored on dry ice during the lysate preparation. 1 ml ice-cold lysis buffer was added to 100 mg liver tissue. After homogenisation with a Potter S device (Braun Biotech, Melsungen, Germany), lysates were cleared by centrifugation (10 min, 4°C, 14,000 *g*). The obtained supernatants were mixed with 3x SDS sample buffer, boiled for 5 min at 95°C, aliquoted and then stored at -85°C. Protein concentrations were determined by the method of Lowry.

5.4 PREPARATION OF CELLULAR PROTEIN EXTRACTS FROM ISOLATED RAT HEPATOCYTES

Untreated or treated hepatocytes (1 x 10^6 cells/ml) grown in 6-well tissue culture plates were harvested on ice by washing twice with ice-cold HBSS (see 2.2) and addition of 100 µl lysis buffer. After 30 min incubation on ice, cells were scraped off and the lysates were transferred to Eppendorf tubes. Then the homogenates were centrifuged and treated as described above (5.3). Protein concentrations were determined by the Bradford Assay (BioRad Laboratories, Munich, Germany) (226).

5.5 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Separation of proteins was performed by denaturating SDS-polyacrylamide gel electrophoresis, allowing the separation of proteins according to their size. Before electrophoresis, all samples were denaturated by boiling and charged negatively by addition of 3x SDS sample buffer. Hence, SDS-polypeptide complexes migrate through the polyacrylamide gel towards the anode simply according to their molecular weight.

For gel preparation an acrylamide 30%/ bis-acrylamide 0.8% stock solution (37.5:1 v/v; Rotiphorese™ Gel 30 from Roth, Karlsruhe, Germany) was applied.

Procedure

Equal amounts of protein (100 µg) were loaded and separated by SDS-PAGE (Mini PROTEAN 3, BioRad Laboratories, Munich, Germany). Proteins were stacked at 100 V for 21 min and resolved at 200 V for 40 min.

5.6 PROTEIN TRANSFER BY SEMIDRY BLOTTING AND DETECTION OF PROTEINS

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.

Procedure

The blotting membrane was cut to the size of the resolving gel and placed for 5 min in methanol and subsequently at least for 30 min in anode buffer. On top of the graphite plate (anode), forming an air bubble free stack, were placed in turn: one thick blotting paper (BioRad, Munich, Germany) moisturised in anode buffer, the membrane, the gel, and subsequently one thick blotting paper (BioRad, Munich, Germany) moisturised in cathode buffer.

Blotting was performed at a current of 1.5 mA per cm^2 of blotting surface for 55 min. After transfer, unspecific binding sites were masked by blocking the membrane for 1 h at room temperature in a 5% (m/v) solution of low fat milk powder (Blotto, BioRad, Munich, Germany) in TBS-T. Then the blocked membrane was incubated with diluted antibody solution of the adequate primary antibody (5.2) at 4°C overnight on a shaking platform. Four washing steps for 5 min in TBS-T preceded the incubation with the secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:20,000 in Blotto, Dianova, Hamburg, Germany). After additional washing steps (2 x 10 min, 2 x 5 min), the immunoreactive bands were visualized with a chemiluminescent detection kit (ECL Plus™, Amersham Pharmacia, Braunschweig, Germany) and subsequent exposure to a medical X-ray film (Fuji, Duesseldorf, Germany). To exclude loading differences the blots were reprobed with antibody against the unphosphorylated form of the respective protein (Cell Signalling, New England Biolabs, Frankfurt/Main, Germany).

Coomassie blue staining

In order to control equal protein concentrations in each sample and equal transfer of proteins, gels were stained with Coomassie blue staining solution (5.1) after blotting. Briefly, gels were incubated for 30 min in staining solution and subsequently washed for 10 min in destaining solution (5.1).

6 IMMUNOPRECIPITATION

Cell or tissue lysates were prepared as described in the Western blot section (see "Materials and Methods" 5). After centrifugation, protein concentration in the supernatant was determined and equal amounts of protein were adjusted by addition of lysis buffer. Total BAD was immunoprecipitated from the lysate after 12 h incubation and gentle shaking with a phosphorylation state independent anti-BAD antibody (1:200) followed by addition of 50 µl protein A agarose beads (Sigma, Deisenhofen, Germany) to 500 µl lysate for 2 h at 4°C. After centrifugation the supernatant was discarded, the beads were washed three times with 500 µl ice-cold lysis buffer and were resuspended in 50 µl 2.5x SDS sample buffer. Immune complexes were cleared from the agarose beads by heating to 95°C for 5 min and subsequent microcentrifugation. 45 µl of the supernatant were used for SDS-PAGE and Western blotting ("Materials and Methods" 5).

7 CASPASE-3-LIKE ACTIVITY

The activation of the downstream caspase Caspase-3, considered as one of the major effector caspases (227), can be measured by applying the synthetic peptide substrate DEVD (asp-glu-val-asp). To make detection possible, the DEVD substrate is labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin (AFC). DEVD-AFC is cleaved after the aspartate residue by activated caspases (228). The liberation of this fluorophore (FIGURE 14) shows a blue to green shift in fluorescence at an extinction wavelength of 390 nm and an emission wavelength of 535 nm. AFC as fluorophore is highly sensitive and sufficiently stable to handle.

Since besides Caspase-3 other downstream caspases (e.g. 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.

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

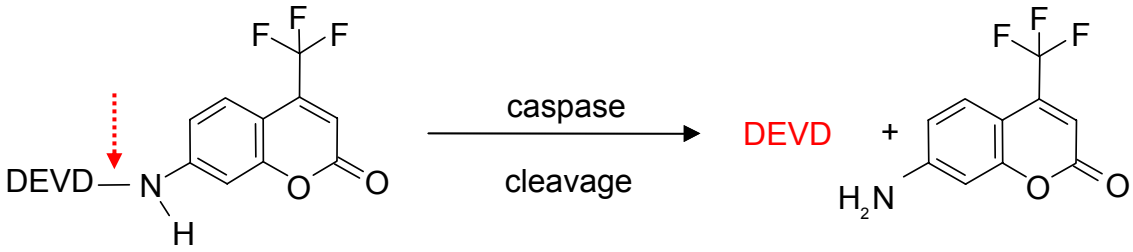


FIGURE 14: Liberation of AFC from DEVD-AFC by caspase cleavage (see text).

7.1 SOLUTIONS

Lysis buffer

$MgCl_2$	5 mM
EGTA	1 mM
Triton® X-100	400 µl
Hepes pH 7.5	25 mM

Substrate buffer

Hepes pH 7.5	50 mM
Sucrose	1% (m/v)
CHAPS	0.1% (m/v)
Ac-DEVD-AFC	50 µM
DTT	10 mM

7.2 MEASUREMENT

Measurement of Caspase-3-like activity in liver tissue

Caspase-3-like activity in liver tissue was measured by Dr. T. Gerwig (Department of Pharmacy, University of Munich, Germany) (217).

Measurement of Caspase-3-like activity in primary rat hepatocytes

Caspase-3-like activity was determined according to *Thornberry et al.* (228) with modifications as reported by *Hentze et al.* (229).

Untreated or treated hepatocytes (8 x 10^5 cells/ml; 12-well tissue culture plates) were washed twice with ice-cold HBSS (see 2.2), incubated with 50 µl ice cold lysis buffer and frozen at -85°C over night. After thawing on ice, cells were scratched off the culture plates, collected by centrifugation (14,000 rpm, 10 min, 4°C), and 10 µl supernatants (blank: 10 µl lysis buffer) were transferred to a flat-bottom 96-well microtiter plate (Greiner, Frickenhausen, Germany). After addition of 90 µl substrate buffer, the

generation of free AFC was kinetically determined by fluorescence measurement (excitation: 390 nm, emission: 535 nm; 37°C) using a fluorometer microplate reader (SpectraFluorPlus, Tecan, Hannover). Control experiments revealed the linear character of activity regarding duration and protein concentration. Further control experiments demonstrated that LA does not interfere with the assay (153). Protein concentrations of the corresponding samples were again quantified with the Pierce Assay using a BSA-standard (Pierce, Rockford, USA).

8 RT-PCR

Since there is no distinct information available concerning the PKG expression in the liver, we investigated the expression of the protein in the rat liver by RT-PCR.

PCR experiments were performed by PD Dr. Alexandra K. Kiemer and Dr. T. Gerwig, both Department of Pharmacy, University of Munich, Germany.

RNA isolation

RNA was isolated using the guanidine thiocyanate/caesium chloride method according to *Chirgwin et al.* (230) and mRNA was separated from totRNA (total RNA) with the PolyATract® mRNA isolation system (Promega, Heidelberg, Germany).

Reverse transcription

Reverse transcription was performed for 20 min at 42°C with 1 µg mRNA using viral reverse transcriptase from avian myeloblastosis virus (all substances from Promega, Heidelberg, Germany). The reaction was terminated by boiling for 5 min and the samples were stored at –20°C until further investigation. All oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany).

Polymerase chain reaction

The RT-PCR was set up using the respective primers according to TABLE 3. Reaction was performed employing a hot start method followed by 30-35 cycles for both isoforms. The amplified products were separated by gel electrophoresis followed by staining with ethidium bromide.

Gene	Orientation	Sequence
PKG I rat	sense	5'-CTT ACC TGC TTC TGC CTT GC-3'
	antisense	5'-CAG GAC CAC CAT GTC AAC TG-3'
PKG II rat	sense	5'-GTG GCC AGA TTC TCC TCA ACC
	antisense	5'-ACC TCG GGG GCC ACA TAC TCT-
GAPDH rat	sense	5'-AGA TCC ACA ACG GAT ACA TT-3'
	antisense	5'-TCC CTC AAG ATT GTC AGC AA-3'

TABLE 3: Primer sequences for RT-PCR.

9 *IN VITRO* PHOSPHORYLATION BY p38 MAPK

Activitiy of p38 MAPK was determined by an *in vitro* phosphorylation assay using MBP (Myelin basic protein) as kinase substrate. After SDS-polyacrylamide gel electrophoresis, gels were quantified by phosphorimaging.

9.1 SOLUTIONS

Lysis buffer

EDAT	2 mM
NaCl	137 mM
Glycerol	10%
$Na_4P_2O_7$	2 mM
Tris	20 mM
Triton®X-100	1%
$C_3H_7Na_2O_6P$	20 mM
NaF	10 mM
Add just before use:	
Na_3VO_4	2 mM
PMSF	1 mM
Complete™ (25x)	

Kinase buffer

Hepes	20 mM
$MgCl_2$	20 mM
$C_3H_7Na_2O_6P$	25 mM
Add just before use:	
Na_3VO_4	100 µM
DTT	2 mM

ATP mix

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

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

Laemmli buffer (1 ml)

Tris-HCl, pH 6.8	3.125 M
Glycerol	500 µl
SDS	20%
DTT	16%
5% Pyronin Y	5 µl
ad 20 µl H_2O	

9.2 ASSAY PROCEDURE

Immunoprecipitation (FIGURE 15)

Cell lysates were prepared from primary isolated hepatocytes, tissue lysates from frozen liver sections. Hepatocytes (1 x 10^6 cells/ml) grown in 6-well tissue culture plates or 100 µg liver tissue were homogenized in ice-cold lysis buffer with a dounce homogenizer and centrifuged (10,000 rpm, 10 min, 4°C). Aliquots of the supernatant were taken for determination of protein concentrations and the tissue extract was frozen at -85°C. The protein concentrations were estimated after the method of Pierce. Equal amounts of protein were incubated with the respective antibody (1.5 µl of anti-p38 polyclonal rabbit antibody, Cell Signaling, New England Biolabs, Frankfurt/Main, Germany) shaking for 2 h. Afterwards, immunoprecipitation was performed with protein A agarose (5 µl) shaking overnight at 4°C. After centrifugation (10,000 rpm, 4 min, 4°C) the precipitates were washed three times with lysis buffer and once with kinase buffer.

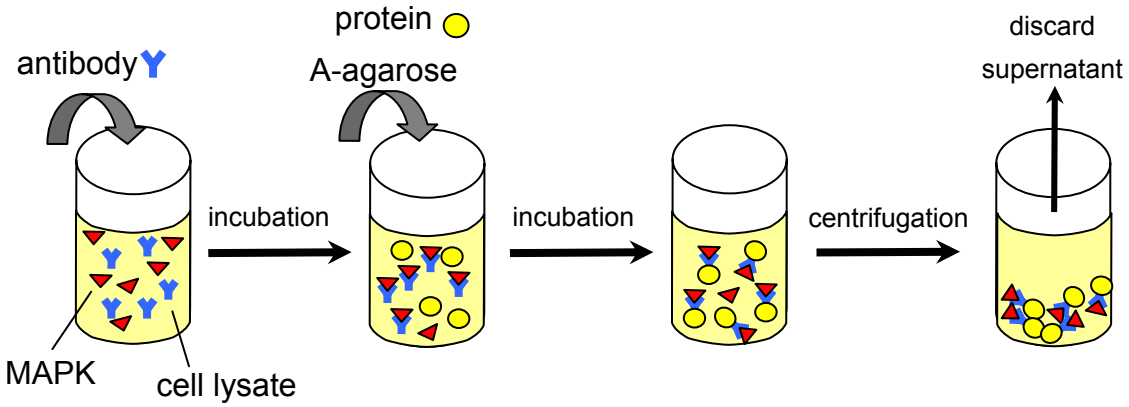


FIGURE 15: schematic diagram of the immunprecipitation.

In vitro phosphorylation assay (FIGURE 16)

Immunoprecipitates were resuspended in 20 µl of kinase buffer. Then 3 µl of substrate solution (1 mg MBP dissolved in 300 µl kinase buffer) and 10 µl ATP-mix were added. The reaction mixture was incubated at 30°C for 20 min shaking. Phosphorylation was stopped by the addition of 6 µl Laemmli buffer and heating for 3 min at 90°C. 30 µl of the reaction mixture were resolved in a 12 % SDS polyacrylamide gel in a Laemmli system at 200 V. Band intensities were quantified by phosphorimaging (Packard, Meriden, USA). Ratio of digital light units (DLU) of respective values vs. controls was determined.

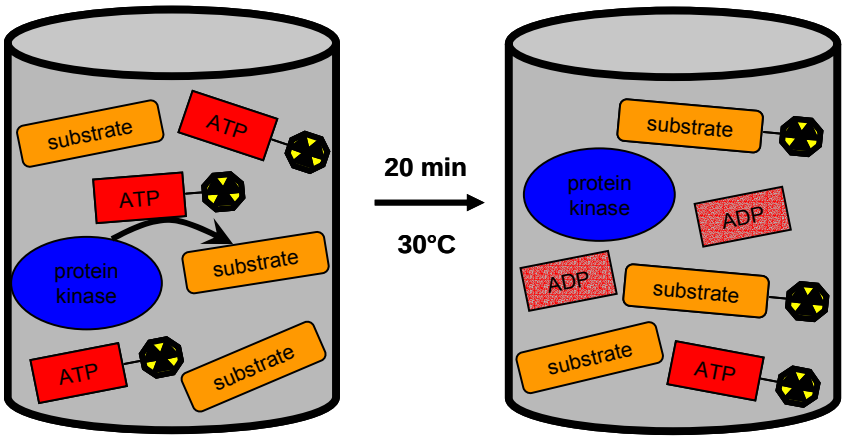


FIGURE 16: schematic diagram of the kinase assay.

10 STATISTICAL ANALYSIS

All data are expressed as the mean ± SEM (standard error) (n=number of organs or number of independent cell preparations). Unless stated otherwise, all experiments were performed with five organs/triplicates per treatment group. Statistical significance between groups was determined by one sample t-test or student's t-test. A p value < 0.05 was considered to be statistically significant. Statistical analyses were performed with GraphPad Prism® (Version 3.02, GraphPad Software Inc., San Diego, USA).

D RESULTS

1 R-LIPOIC ACID PROTECTS HEPATOCYTES AGAINST TNF-α-/ ACTINOMYCIND-INDUCED APOPTOSIS

Several studies have demonstrated hepatoprotective effects of LA (150-153). Since apoptosis plays an important role in several liver diseases, aim of the present study was to investigate possible protective effects of LA in hepatocyte apoptosis.

For these purposes, we used a common model of hepatocyte apoptosis: programmed cell death was induced *via* treatment of cells with the inflammatory cytokine **TNF-α** (**T**, 28 ng/ml) and the inhibitor of transcription **ActinomycinD** (**ActD**/**A**, 0.2 µg/ml). *Leist et al.* showed that not stimulation with this cytokine alone, but a co-stimulation with TNF-α and an inhibitor of transcription induces apoptosis in isolated hepatocytes (231).

1.1 LA AND R-LA REDUCE TNF-α-/ACTD-INDUCED APOPTOSIS

α-lipoic acid (LA) contains a chiral carbon and therefore exists in two isoforms. LA consists as a 1:1 racemic mixture of the R- and S-enantiomers (R-/S-LA), with R-LA being the naturally occurring form.

Since caspase activation is a characteristic feature of the apoptotic process, the activity of this enzyme was used as a measure to determine the effect of racemic LA, R-LA, and DHLA on T/A-induced apoptosis.

To test the effects of R-/S-LA, R-LA and DHLA on the Caspase-3-like activity, hepatocytes were incubated for 2 h with different concentrations of the indicated substance followed by 15 min incubation with A and then 12 h incubation with T. As seen in FIGURE 17 and 18, pretreatment with both, R-/S-LA and R-LA reduces T/A-induced Caspase-3-like activity and thus apoptosis. Whereas R-/S-LA even seems to reduce the basal apoptosis rate occurring in untreated cells (FIGURE 18).

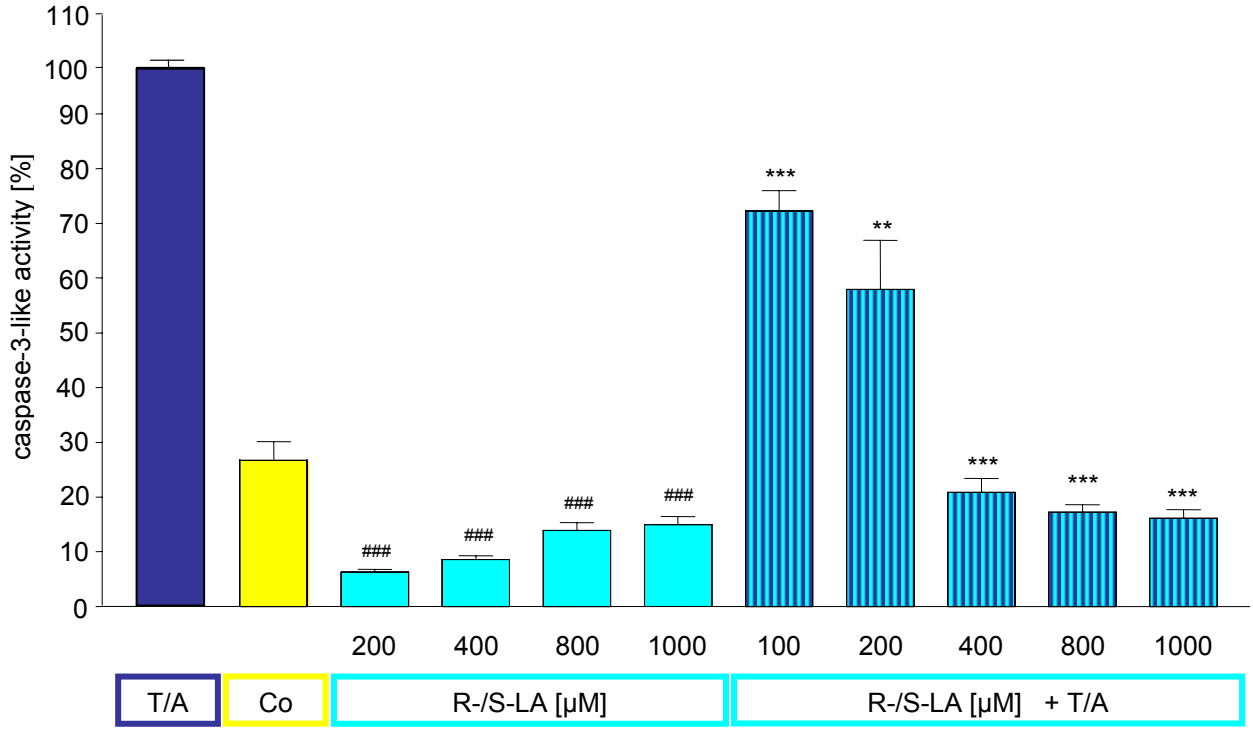


FIGURE 17: R-/S-LA-preincubation significantly reduces T/A-induced Caspase-3-like-activity in isolated hepatocytes.

24 h after isolation hepatocytes were left untreated (Co), or were treated for 2 h with different concentrations of R-/S-LA prior to 15 min incubation with A followed by 12 h T. After stimulation, hepatocytes were lysed and Caspase-3-like activity was measured as described under "Materials and Methods". Data are expressed as percent of enzyme activity of T/A-treated cells set equal 100%. Columns show mean ± SEM of 5 independent experiments performed as triplicates. ###p<0.001 significantly different from Co group, ***p<0.001 and **p<0.01 significantly different from T/A-treated hepatocytes.

Since R-LA is the naturally occurring form and described in the literature to be more potent than the racemate (232), we decided to use the R-isomer for the following investigations.

FIGURE 18 also displays the effects of various stimulation orders: hepatocytes were first incubated for 15 min with A, followed by 15 min, 1 h or 2 h T treatment, and then for 12 h with R-LA. Also the effect of 15 min incubation with A alone followed by co-stimulation with R-AL and T for 12 h on caspase-activity was tested. Further, cells were incubated for 1 h and 2 h with R-LA, followed by 15 min A and subsequent 12 h T stimulation.

As a result of these experiments, we found the most efficient protection after 2 h

pretreatment with 200 µM R-LA, followed by 15 min A and 12 h T incubation. Thus, we further investigated the signal transduction pathway involved in protection against T/A-induced apoptosis with this stimulation pattern.

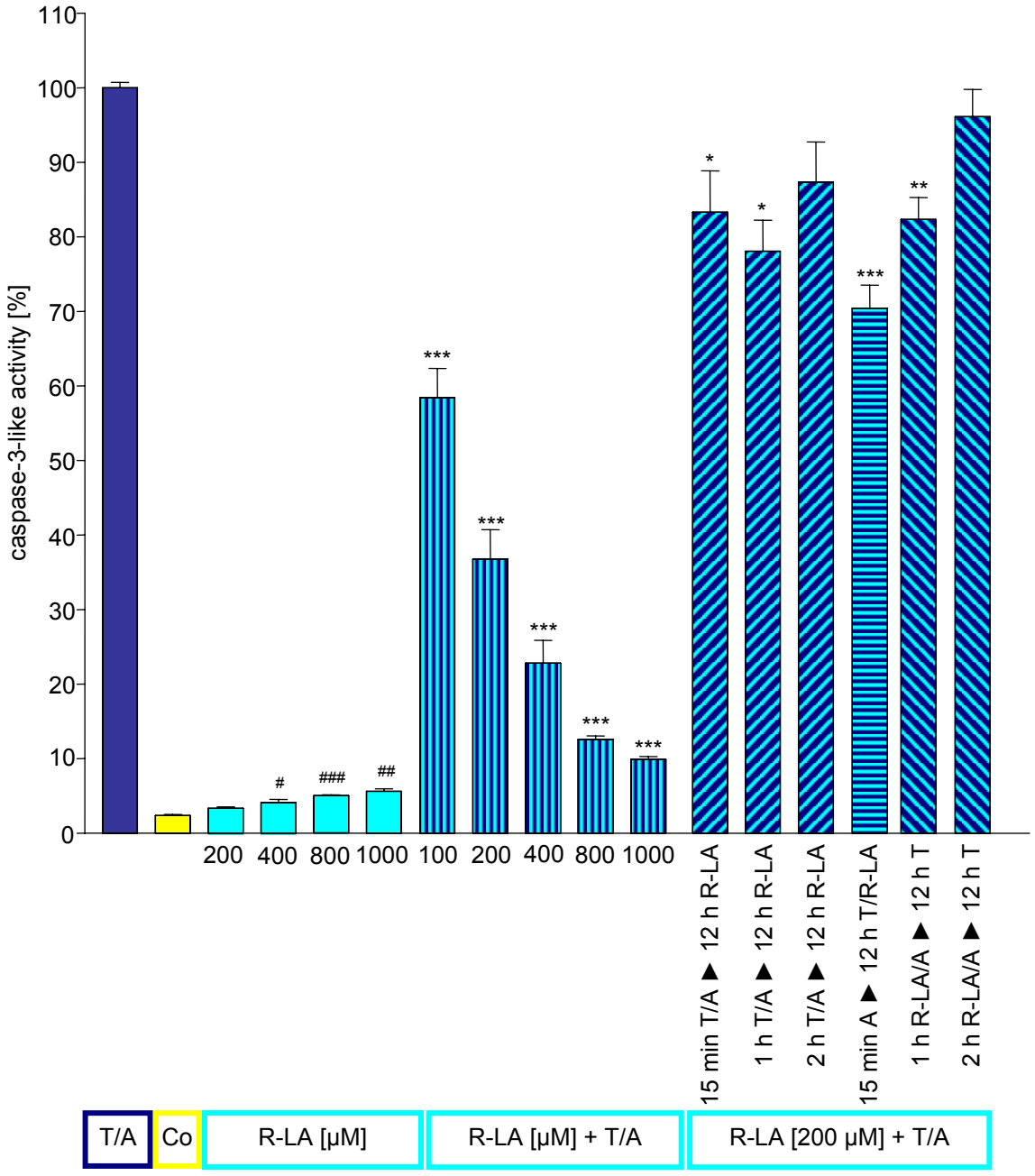


FIGURE 18: R-LA-preincubation significantly reduces T/A-induced Caspase-3-like-activity in isolated hepatocytes.

24 h after isolation hepatocytes were left untreated (Co), or were treated for 2 h with different concentrations of R-LA prior to 15 min incubation with A followed by 12 h T. After stimulation, cells were lysed and Caspase-3-like

activity was measured (see “Materials and Methods”). Data are expressed as percent of enzyme activity of T/A treated cells set equal 100%. Columns show mean ± SEM of 3 independent experiments performed as triplicates. ***p<0.001, **p<0.01 and *p<0.05 significantly different from T/A-treated cells and ###p<0.001, ##p<0.01 and #p<0.05 significantly different from untreated cells.

FIGURE 19 shows that DHLA reduces T/A-induced Caspase-3-like activity, but at the same time it induces caspase activity in untreated cells.

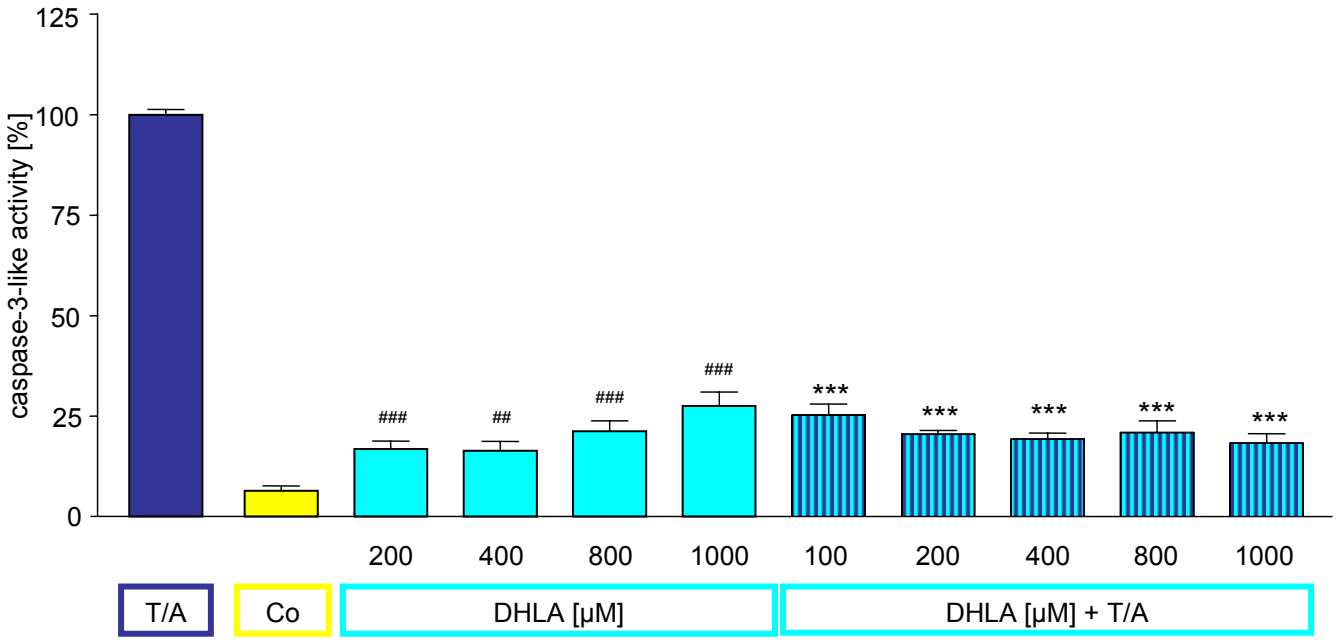


FIGURE 19: DHLA-preincubation reduces T/A-induced Caspase-3-like activity in isolated hepatocytes.

24 h after isolation hepatocytes were left untreated (Co) or were treated for 2 h with different concentrations of DHLA prior to 15 min incubation with A followed by 12 h T. After stimulation, hepatocytes were lysed and Caspase-3-like activity was measured as described under “Materials and Methods”. Data are expressed as percent of enzyme activity of T/A treated cells set equal 100%. Columns show mean ± SEM of 2 independent experiments performed as triplicates. With ***p<0.001 being significantly different from T/A-treated cells, and ###p<0.001, ##p<0.01 significantly different from untreated hepatocytes.

Since R-LA incubation alone increased caspase activity in concentrations over 400 µM, but only very weakly, and DHLA significantly increased caspase activity in all tested

concentrations compared to untreated cells, we further investigate whether DHLA and R-LA alone induce apoptotic or necrotic cell death. LDH efflux was used as measure for severe cell damage. FIGURE 20 and 21 display LDH release in DHLA and R-LA stimulated cells. Considering the LDH-efflux and the morphology of the treated hepatocytes it seems that DHLA alone induces cell death (FIGURE 21). Additionally, the LDH-data display that R-LA protects T/A-treated cells not only against apoptotic cell death, as seen in FIGURE 19, but also prevents cells from a secondary necrotic cell death (FIGURE 20).

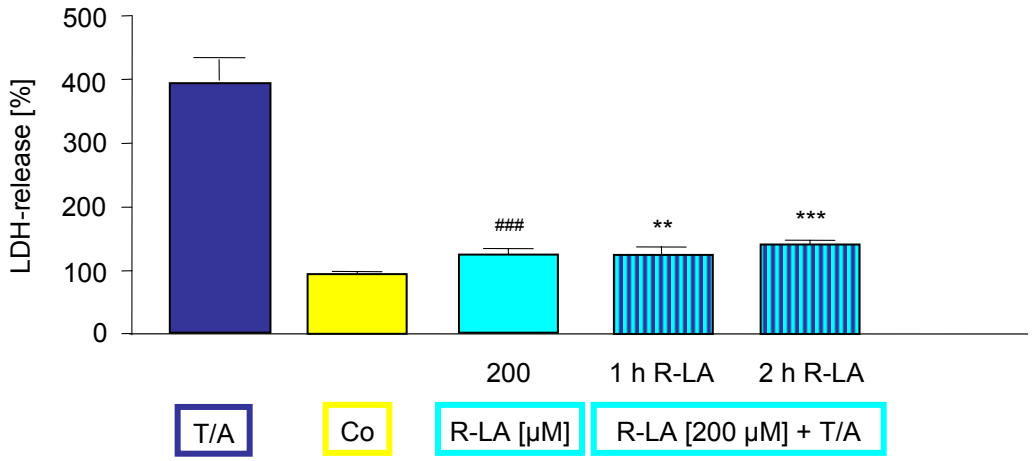


FIGURE 20: R-LA-preincubation reduces LDH-efflux in apoptotic cells.

Hepatocytes were left untreated (Co), or were treated with R-LA (2 h) ± T/A to different time points. After stimulation, the LDH-efflux was measured in the supernatant of the isolated cells (see “Materials and Methods”). Data are expressed as percent of enzyme activity of untreated cells set equal 100%. Columns show mean ± SEM of 2 independent experiments. With ***p<0.001, **p<0.01 being significantly different from T/A-treated and $^{###}$p<0.001 being significantly different from untreated hepatocytes.

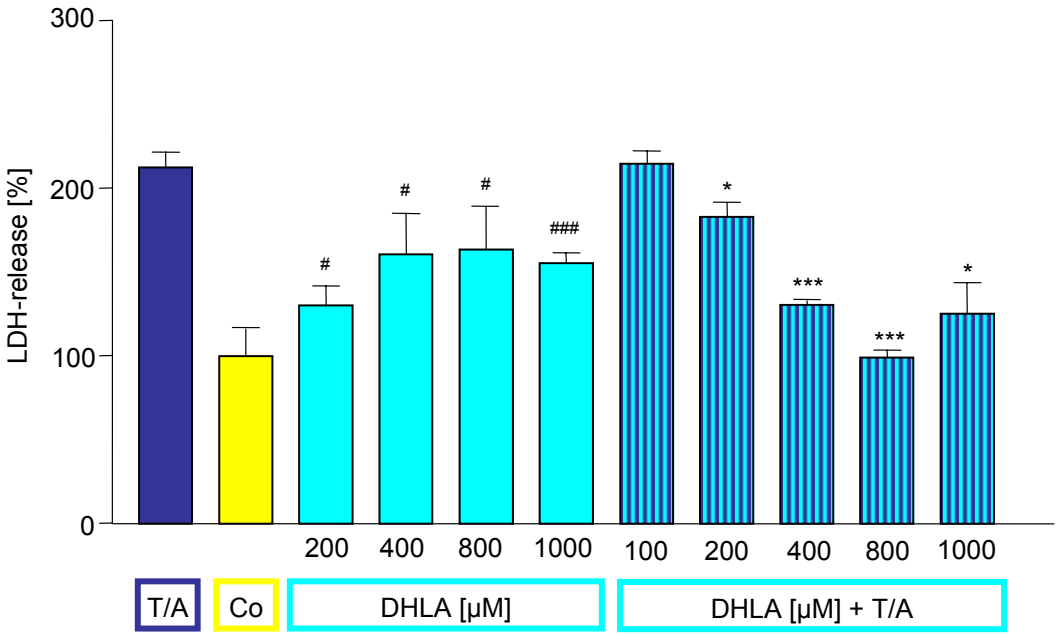


FIGURE 21: DHLA-preincubation increases LDH-release compared to unstimulated cells.

Measurement of the LDH-efflux in the supernatant of the cells (see "Materials and Methods"): data are expressed as percent of enzyme activity of untreated cells (Co) set equal 100%. Columns show mean ± SEM of 2 independent experiments. ***p<0.001 and *p<0.05 significantly different from T/A-treated cells. $^{\#\#\#}$p<0.001 and $^{\#}$p<0.05 significantly different from untreated cells.

1.2 EFFECTS OF ANTIOXIDANT- AND FE-CHELATOR-TREATMENT ON TNF-α-/ACTD-INDUCED APOPTOSIS

Since lipoic acid is known to be a potent antioxidant and also is able to chelate a variety of transition metals such as $Fe^{2+/3+}$ (149), we aimed to determine whether antioxidative or Fe-chealting treatment affects T/A-induced apoptosis. To investigate these effects, hepatocytes were preincubated with different antioxidants (2,6-Di-tert-butyl-4-methylphenol (BHT), α-Tocopherol (Vitamin E), N-acetyl cysteine (NAC)), Fe-chelating agents (2.2-Dipyridyl, Deferoxamine mesylate), and the non-chelating agent 4.4-Dipyridyl.

Reduction of Caspase-3-like activity is not due to antioxidative properties of R-LA

FIGURE 22 shows that treatment of non-apoptotic hepatocytes with different antioxidants alone displayed no activation of Caspase-3-like activity. Only the antioxidant BHT significantly reduced the caspase activity in apoptotic hepatocytes (FIGURE 22). Whereas neither Vitamin E nor NAC showed a significant effect on T/A-induced apoptosis. These results suggest that antioxidant properties do not exert prominent action on T/A-induced hepatocyte apoptosis.

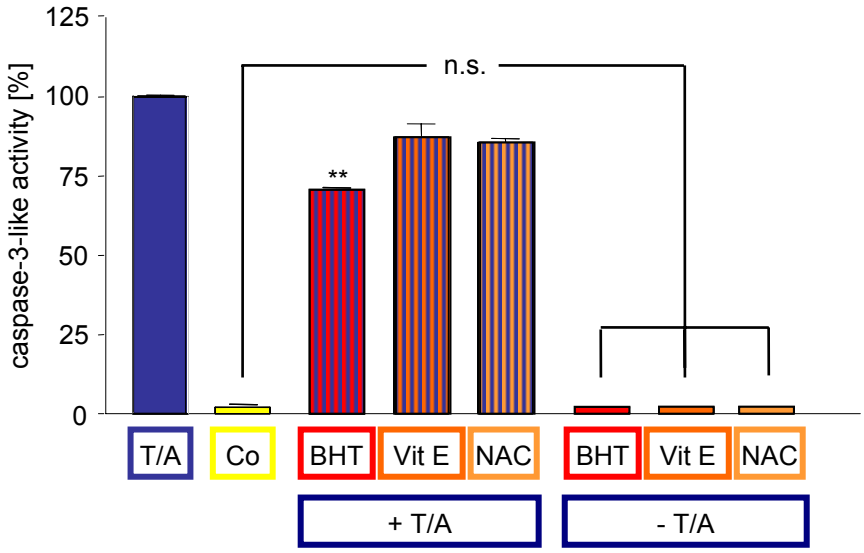


FIGURE 22: R-LA reduces Caspase-3-like activity not depending on its antioxidative properties.

24 h after isolation hepatocytes were left untreated (Co), or were treated for 2 h with different antioxidants (BHT [20 µM], Vitamin E [60 µM], N-acetyl-cysteine [10 mM]) prior to 15 min incubation with A followed by 12 h T. After stimulation, hepatocytes were lysed and Caspase-3-like activity was measured as described under "Materials and Methods". Data are expressed as percent of enzyme activity of T/A treated cells set equal 100%. Columns show mean ± SEM of 3 independent experiments performed as triplicates. **p<0.01 significantly different from T/A-treated hepatocytes.

Reduction of Caspase-3-like activity is also not due to R-LA's chelating properties

Isolated hepatocytes were incubated with different Fe-chelating and non-chelating agents alone and this displayed no significant effect on caspase activity compared to untreated cells (FIGURE 23). Incubation of the cells with different chelating agents prior to induction of apoptosis *via* TNF-α/ActD exhibited different effects on the caspase activity: Deferoxamine reduced the enzyme activity in apoptotic cells. **Both,** the chelating (2-2) and the non-chelating (4-4) form of dipyridyl displayed no reducing effect on the caspase-activity (FIGURE 23). Interestingly, the chelating (2,2) dipyridyl even slightly but significantly elevated T/A-induced apoptosis. Taken together, these data support a minor role of iron complexation in the regulation of T/A-induced apoptosis.

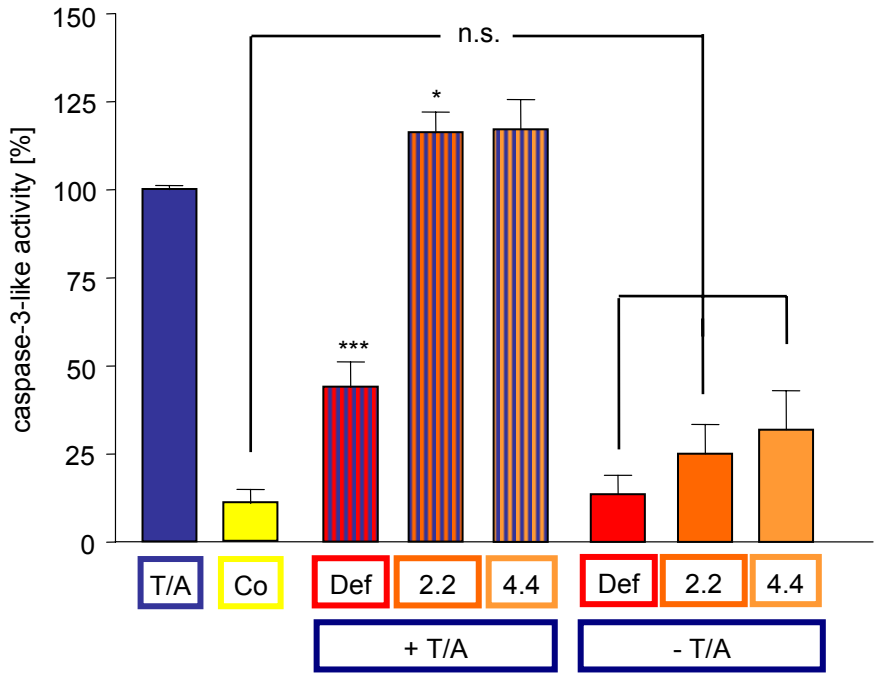


FIGURE 23: R-LA reduces Caspase-3-like activity not depending on its Fe-chelating properties.

24 h after isolation, hepatocytes were left untreated (Co) or were treated for 2 h with different Fe-chelating (Deferoxamine [10 mM]; 2.2 Dipyridyl [100 µM]) agents and with the non-chealting agents 4.4 Dipyridyl [100 µM] prior to 15 min incubation with A followed by 12 h T. After stimulation, cells were lysed and Caspase-3-like activity was measured (see "Materials and Methods"). Data are expressed as percent of enzyme activity of T/A treated cells set equal 100%. Columns show mean ± SEM of 3 independent experiments performed as triplicates. ***p<0.001 and *p<0.05 significantly different from T/A-treated hepatocytes.

1.3 R-LA REDUCES TNF-α-/ACTD-INDUCED CASPASE-3-LIKE ACTIVITY VIA THE PI3-K-/AKT-PATHWAY AND SUBSEQUENT BAD-PHOSPHORYLATION

The above presented data suggest that R-LA mediates its antiapoptotic effects rather independently of its well known antioxidative or chelating properties. Thus, other targets possibly involved in this hepatoprotective signalling pathway were investigated.

Activation of the PI3-K/Akt pathway and subsequent Akt phosphorylation are involved in the R-LA-induced antiapoptotic signalling cascade

Since the PI3-K/Akt pathway is known to participate in the antiapoptotic signalling cascade of different cells, e.g. hepatocytes (233;234) (for review see:(27;235)), it was first investigated whether incubation of hepatocytes with R-LA induces Akt phosphorylation. In fact, FIGURE 24 shows that R-LA is able to phosphorylate Akt at Ser473 in a time-dependent manner.

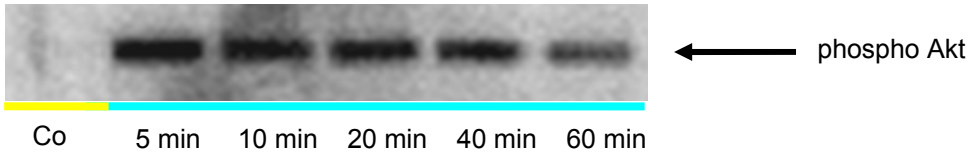


Figure 24: Time course of R-LA-induced Akt-phosphorylation in hepatocytes.

24 h after isolation hepatocytes were treated with 200 µM R-LA for different time points. After incubation, Western blot analysis (see “Materials and Methods”) was performed detecting phosphorylated Akt (phospho Akt). The figure shows one representative blot out of three independent experiments.

Preincubation of the cells with the specific PI3-K-inhibitor Wortmannin (WM) demonstrated that the observed Akt-phosphorylation after R-LA stimulation depends on the activation of the PI3-K/Akt pathway (FIGURE 25).

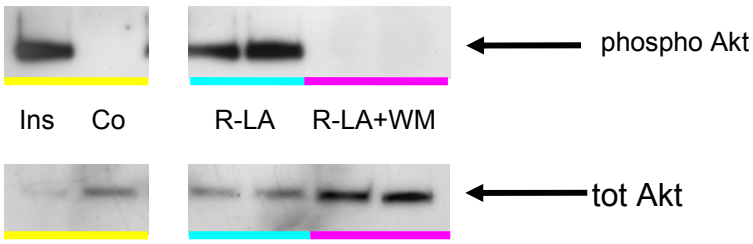


FIGURE 25: R-LA induces Akt-phosphorylation *via* the PI3-K-pathway.

24 h after isolation hepatocytes were treated with or without Insulin (Ins, 20 min, 50 ng/ml) as positive control, ± R-LA (200 µM, 20 min) or were preincubated ± Wortmannin (WM, 1 h, 800 nM). Equal amounts of DMSO, which was used to dissolve WM, were added to each well. The subsequent Western blot analysis (see "Materials and Methods") was performed detecting phosphorylated and total Akt (phospho/tot Akt). The figure shows one representative blot out of three independent experiments.

Activation of the PI3-K/Akt pathway leads to BAD phosphorylation

Akt is able to phosphorylate BAD, a proapoptotic member of the Bcl-2-family *in vitro* and *in vivo*, and thus is able to inhibit BAD-induced apoptotic cell death in different cell types (46;47). Since FIGURE 26 shows that R-LA-incubation results in Akt phosphorylation, it was further investigated if this kinase activation leads to a subsequent BAD phosphorylation at Ser136.

FIGURE 26 shows, that R-LA phosphorylates BAD at Ser136 in a time-dependent manner.

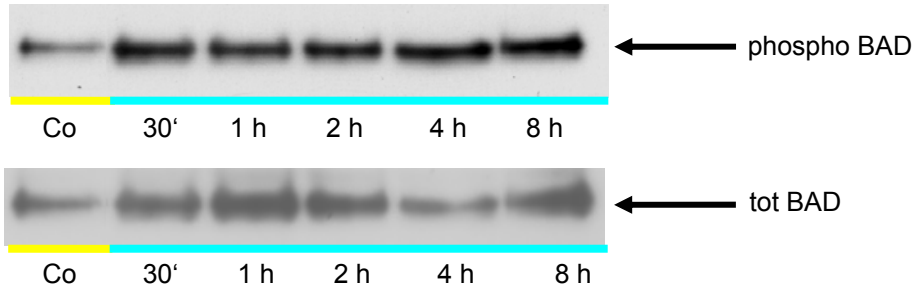


FIGURE 26: Time-dependent BAD-phosphorylation in hepatocytes after R-LA-treatment.

24 h after isolation hepatocytes were treated at different time points with 200 µM R-LA. After immunoprecipitation of tot BAD, Western blot analysis (see "Materials and Methods") was performed detecting phosphorylated and total BAD (phospho/tot BAD). The figure shows one representative blot out of three independent experiments.

We demonstrated that the R-LA-mediated decrease in caspase activity depends on activation of the PI3-K-pathway (FIGURE 25). In order to determine whether the observed R-LA-induced BAD phosphorylation (FIGURE 26) is regulated through the PI3-K/Akt signallig pathway, primary hepatocytes were incubated with increasing concentrations of the specific PI3-K inhibitor Wortmannin. FIGURE 27 displays that WM inhibits R-LA-induced BAD phosphorylation in a concentration-depended manner.

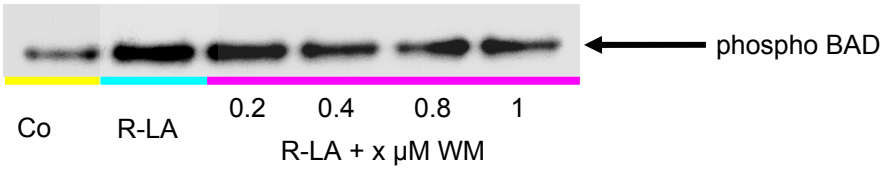


FIGURE 27: R-LA-induced BAD-phosphorylation depends on the PI3-K/Akt-pathway.

24 h after isolation hepatocytes were treated ± WM (1 h preincubation, 800 nM) before stimulation with 200 µM R-LA (8 h). Equal amounts of DMSO, which was used to dissolve WM, were added to each well. After immunoprecipitation of tot BAD, the subsequent Western blot analysis (see "Materials and Methods") was performed detecting phosphorylated (phospho BAD). The figure shows one representative blot out of three independent experiments.

Activation of the PI3-K/Akt pathway is involved in the R-LA-induced antiapoptotic signalling cascade

The previous data showed a PI3-K-dependent activation of Akt, a pathway having been described to mediate antiapoptotic action. We therefore aimed to determine a causal relationship between R-LA-induced PI3-K/Akt-activation and caspase-inhibition. To investigate this, Caspase-3-like activity in hepatocytes pretreated with or without Wortmannin was measured.

The PI3-K-inhibitor WM applied in concentrations able to largely prevent R-LA-induced BAD phosphorylation significantly abrogated the protective action of R-LA on T/A-induced apoptosis (FIGURE 28). This shows a causal contribution of the PI3-K/Akt-pathway in R-LA-mediated protection against hepatocyte apoptosis.

It has to be noted that WM did not completely abrogate R-LA's protective effect. We would like to point out, however, that WM+T/A slightly but significantly reduced caspase activity (FIGURE 28).

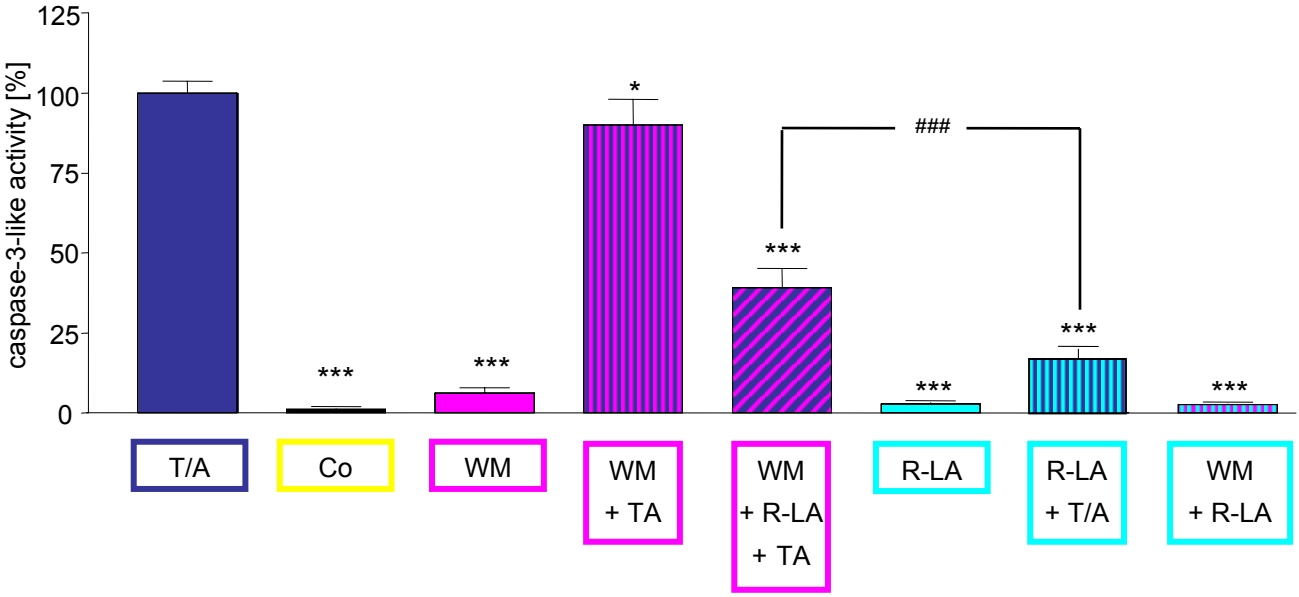


FIGURE 28: Wortmannin abrogates the reducing effect of R-LA on Caspase-3-like activity.

24 h after isolation hepatocytes were left untreated (Co), or were treated for 2 h with 200 µM R-LA prior to 15 min incubation with A followed by 12 h Incubation with T. Some cells were preincubated with the PI3-K-inhibitor WM [800 nM] prior to R-LA stimulation, the others with 0.08% DMSO instead. After stimulation, hepatocytes were lysed and Caspase-3-like activity was measured as described under "Materials and Methods". Data are expressed as percent of enzyme activity of T/A treated cells set equal 100%. Columns show mean ± SEM of 3 independent experiments performed as triplicates. ***$p<0.001$, *$p<0.05$: significantly different from T/A-treated hepatocytes; ###$p<0.001$: WM+R-LA+T/A significantly different from R-LA+T/A.

Activation of the Akt is causally involved in the R-LA-induced antiapoptotic signalling cascade

The previous data showed causal contribution of the PI3-K/Akt-pathway in R-LA-mediated protection against hepatocyte apoptosis. Thus, we additionally examined whether treatment of apoptotic cells with R-LA induces Akt phosphorylation, and whether WM again is able to abrogate this R-LA induced Akt-activation.

The PI3-K-inhibitor WM applied in concentrations able to largely prevent R-LA-induced BAD phosphorylation and T/A-induced caspase activation completely inhibited Akt phosphorylation (FIGURE 29).

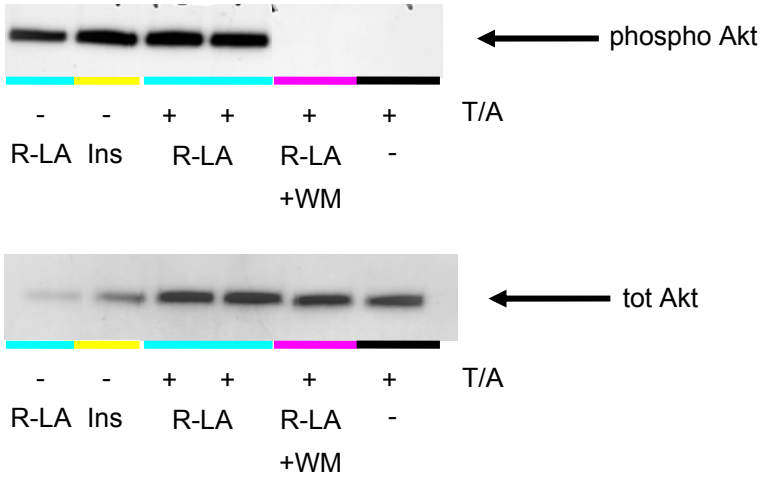


FIGURE 29: R-LA-induced Akt-phosphorylation can be inhibited by WM.

24 h after isolation hepatocytes were treated ± WM (1 h preincubation, 800 nM) before stimulation with 200 µM R-LA (20 min) ± T/A (A: 15 min; T: 30 min). After incubation, Western blot analysis (see “Materials and Methods”) was performed detecting phosphorylated and total Akt (phospho-/tot-Akt). The figure shows one representative blot out of three independent experiments.

Comprising, our data indicate that a protection from T/A-induced hepatocyte apoptosis is rather independent from its antioxidative effects, but mediated by activation of the PI3-K/Akt pathway and involves BAD phosphorylation.

2 PROTEIN KINASE A DEPENDENT SIGNALLING MEDIATES ANTIAPOPTOTIC EFFECTS OF ANP IN POST-ISCHEMIC LIVERS

Preconditioning of rat livers with Atrial Natriuretic Peptide (ANP) reduces hepatic ischemia reperfusion injury (IRI) (176;180) comprising attenuation of apoptosis in post-ischemic organs (181). The role of stress-activated signalling pathways in hepatic IRI and their modulation by protective interventions such as ANP are widely unknown. However, we have previously shown that ANP-preconditioning markedly increases p38 MAPK activity (184;236). In addition, several groups suggest a protective role for p38 MAPK during warm ischemia and hypoxia (66;194;195). Thus, we wanted to investigate whether the ANP-induced p38 MAPK activation contributes to the observed antiapoptotic effects of ANP-preconditioning after cold ischemia (181).

2.1 ANP-PRECONDITIONING INDUCES NEITHER IN ISOLATED HEPATOCYTES NOR IN ISOLATED KUPFFER CELLS A MARKED p38 MAPK ACTIVATION

ANP-preconditioning in the whole liver leads to a strong p38 MAPK activation (184;236). Since ANP protects against IRI, we wanted to investigate the possible involvement of p38 MAPK in this signal transduction pathway of hepatoprotection. Therefore, we aimed to employ isolated hepatocytes for detection of possible downstream targets of this kinase pathway participating in ANP-mediated liver protection.

ANP is known to mediate its effects primarily *via* binding to type A natriuretic peptide receptors. Therefore, cGMP levels were measured as a parameter of guanylate-cyclase-coupled A-receptor (NPR-A) function on isolated hepatocytes. This kind of assay is required, as it is known, that the isolation procedure may destroy surface receptors (237). To test whether the measured cGMP levels are induced by binding of ANP to NRP-A and not by activation of soluble guanylate cyclase, cGMP levels were also measured after incubation with the NO-donor S-nitroso-N-acetylpenicillamine (SNP). Thus, if ANP- but not SNP-treatment of hepatocytes results in a significant elevation of intracellular cGMP levels, this indicates functional NPR-As. We measured cGMP-levels in isolated hepatocytes 24 h, 48 h, and 72 h after isolation as a parameter of guanylate-

cyclase-coupled A-receptor (NPR-A) function. Due to the obtained results, we used hepatocytes only 48 h after isolation (FIGURE 30).

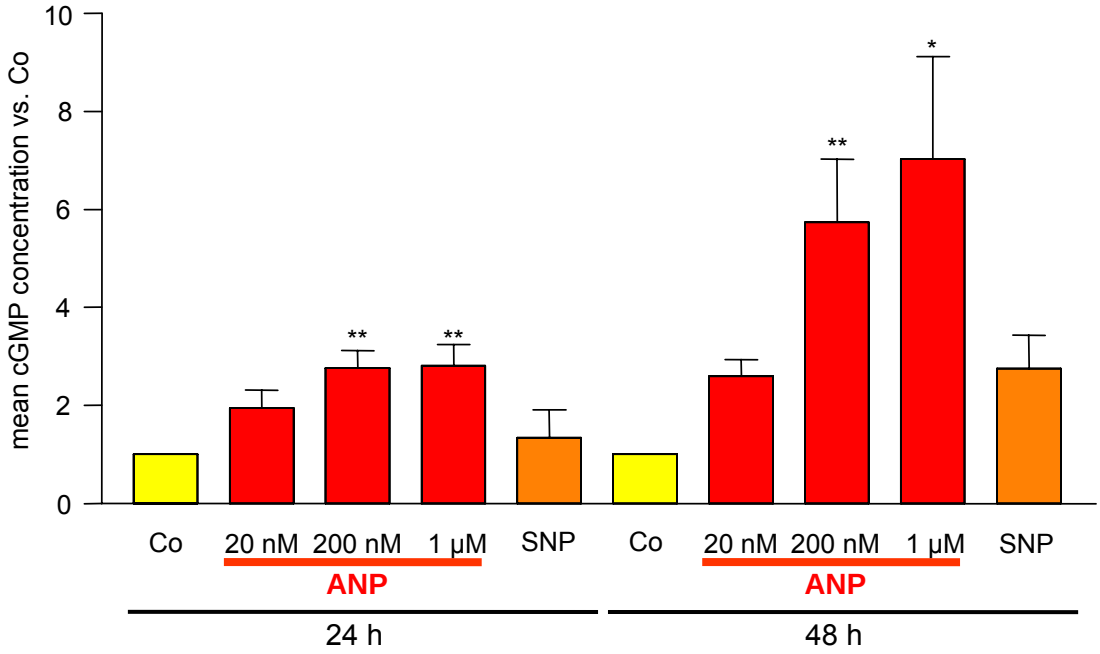


FIGURE 30: ANP-treatment of hepatocytes induces intracellular cGMP formation.

Cells were isolated as described under “Materials and Methods”. Then, hepatocytes were incubated with different ANP concentrations 24h and 48 h after isolation. cGMP measurement was performed with a commercially available kit. Columns show mean ± SEM of 2 independent experiments performed in triplicates with **p<0.01 and *p<0.05 being statistically different from the control group.

Then, we examined ANP-mediated p38 MAPK activation in isolated hepatocytes by Western blot (FIGURE 31A) and by *in vitro* phosphorylation of MBP (myelin basic protein) (FIGURE 31B).

Both methods showed only a slight increase (WB: about 1.5-fold; KA: 1.8-fold) in the kinase activity compared to the strong p38 MAPK activation in the isolated perfused liver (FIGURE x; almost 3.5-fold). This weak activation in the hepatocytes might be explained by the culture conditions and the lack of hepatic stellate cells (Ito cells), endothelial cells, and KCs, possibly additionally involved in the signal mediation.

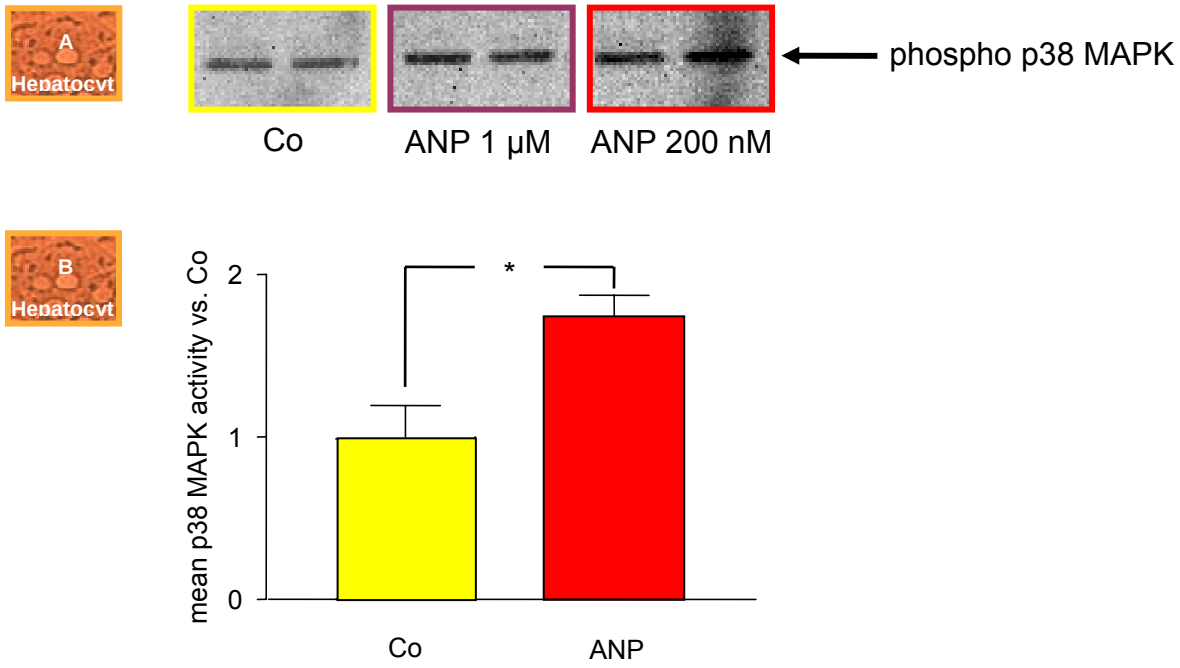


FIGURE 31: ANP-treatment of hepatocytes leads to a modest increase in p38 MAPK phosphorylation.

Cells were isolated as described under “Materials and Methods”. **A**: Western blot analysis was performed detecting phosphorylated p38 MAPK (phospho p38 MAPK) after 20 min incubation of hepatocytes ± ANP (200 nM, 1 µM). **B**: hepatocytes were incubated for 20 min ± ANP (200 nM) with subsequent determination of kinase activity by *in vitro* phosphorylation of MBP. Determination of digital light units was performed by phosphorimaging and values of ANP-treated cells were divided by mean values of untreated cells. Columns show mean ± SEM of 2 independent experiments performed as triplicates with *$p<0.05$ being statistically different from the control group.

Since p38 MAPK is only slightly activated in isolated hepatocytes compared to the whole liver, and ANP is described to be a regulator of KC function, we investigated whether ANP mediates the kinase activation *via* KC interaction (183;238-240). Therefore, we determine ANP’s action on p38 MAPK in isolated liver macrophages. FIGURE 32 shows no increased p38 MAPK phosphorylation in isolated KCs. Thus we suggest no direct involvement of KCs in ANP-mediated p38 MAPK activation.

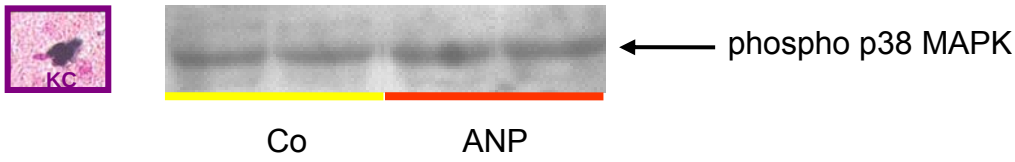
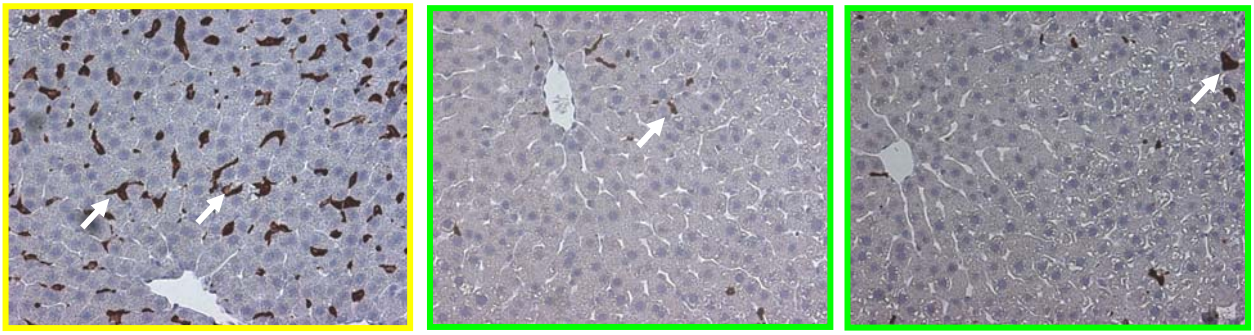


FIGURE 32: Treatment of KC with ANP does not increase p38 MAPK phosphorylation.

KC were isolated as described under Materials and Methods 2.4. Western blot analysis was performed detecting phosphorylated p38 MAPK (phospho p38 MAPK) after 20 min incubation of the KC ± ANP (1 µM).

2.2 ANP-INDUCED p38 MAPK ACTIVATION DOES NOT DEPEND ON A HEPATOCYTE-KUPFFER CELL-INTERACTION

Neither in isolated hepatocytes nor in KCs does ANP induce such strong p38 MAPK activation as in the whole liver (FIGURE 34). Since ANP represents a regulator of KC function, we hypothetised that ANP-mediated kinase activation needs a cell-cell-interaction between KCs and hepatocytes: thus, ANP-induced KC activation could lead to a p38 MAPK activation in hepatocytes. To verify this potential cell interaction, we investigated the ANP-mediated effect on the kinase in KC-depleted livers. For this purpose, we first audited the Cl_2MBP-dependent KC depletion *via* immunhistological staining of the macrophages with ED2 antibody (FIGURE 33). In fact, Cl_2MBP led to a depletion of the KCs compared to NaCl-treated livers (FIGURE 33). Furthermore, LDH efflux into the perfusate was determined to exclude Cl_2MBP-induced liver damage. The measurement indicated no increase in LDH-activity and thus no liver toxicity of Cl_2MBP (data not shown).



Co livers **KC-depleted livers**

FIGURE 33: Immunhistological staining of KCs in untreated (Co) and KC-depleted livers.

Rats received an intravenous injection of 900 µl NaCl (Co) or 900 µl liposomes (12 mM Cl_2MBP) into the tail vein. After 48 h rat livers were perfused, snap-frozen and liver slices were stained with an antibody against the KC-specific surface marker ED2 (see “Materials and Methods”) to verify the Cl_2MBP-dependent depletion of the KCs. Exemplarily KCs are hallmarked with white arrows.

Subsequently, ANP-induced p38 MAPK activity was measured by determination of the radioactive labelled kinase substrate MBP. Surprisingly, liver perfusion with ANP exhibited no differences in kinase activity in KC-depleted or non-depleted livers (FIGURE 34). Due to this observation we suggested, that ANP-mediates p38 MAPK activation not *via* hepatocyte-KC-interaction.

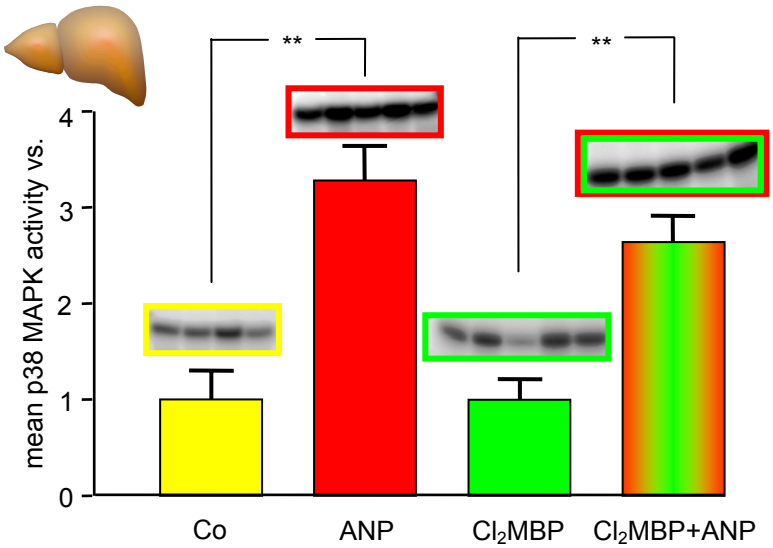


FIGURE 34: KC depletion does not diminish ANP-induced p38 MAPK activation.

KCs were depleted with Cl_2MBP as described under “Materials and Methods”. After 48 h control organs (Co) or KC-depleted livers (12 mM Cl_2MBP) were perfused ± ANP (200 nM) for 20 min. Then p38 MAPK activity was investigated by *in vitro* phosphorylation assay (see “Materials and Methods). Determination of density light units was performed by phosphorimaging and values of ANP-pretreated cells were divided by mean values of the respective control group. Columns show mean ± SEM of 4 to 5 independent perfusion experiments with **p<0.01 being statistically different from the respective control group.

2.3 p38 MAPK ACTIVATION IS NOT INVOLVED IN ANP-INDUCED INHIBITION OF APOPTOSIS

Since Caspase-3-like activity is significantly elevated at the end of ischemia compared to control livers (FIGURE 34) and we recently showed that ANP-preconditioning increases p38 MAPK activity (184;236), the possible involvement of p38 MAPK in ANP-mediated hepatoprotection was investigated. In order to explore the effect of p38 MAPK activity on post-ischemic apoptotic cell death, the specific p38 MAPK inhibitor SB203580 was employed in the presence and absence of ANP 30 min prior to the ischemic period. FIGURE 35 confirms the protective effect of ANP against ischemia-induced apoptotic cell death (181). Compared to control organs, ANP-preconditioning significantly reduced post-ischemic Caspase-3-like activity, whereas the p38 MAPK inhibitor SB203580 did not abrogate this protective effect (FIGURE 35). Surprisingly, pretreatment with SB203580 alone was protective.

This results suggested no effect of p38 MAPK activation on protection from ischemic apoptosis, which was further supported by our observation that the cell permeable cGMP analogue 8-Br-cGMP exerted no significant increase of p38 MAPK activity (236). Nevertheless, ANP does mediate its protective effects *via* cGMP (176;181) and preconditioning with 8-Br-cGMP decreases apoptotic cell death in the ischemic liver even more potently than ANP (181;241).

Therefore, our data suggest that the two mechanisms, the cGMP-mediated hepatoprotection conveyed by ANP and the ANP-induced activation of p38 MAPK, are not causally linked to each other.

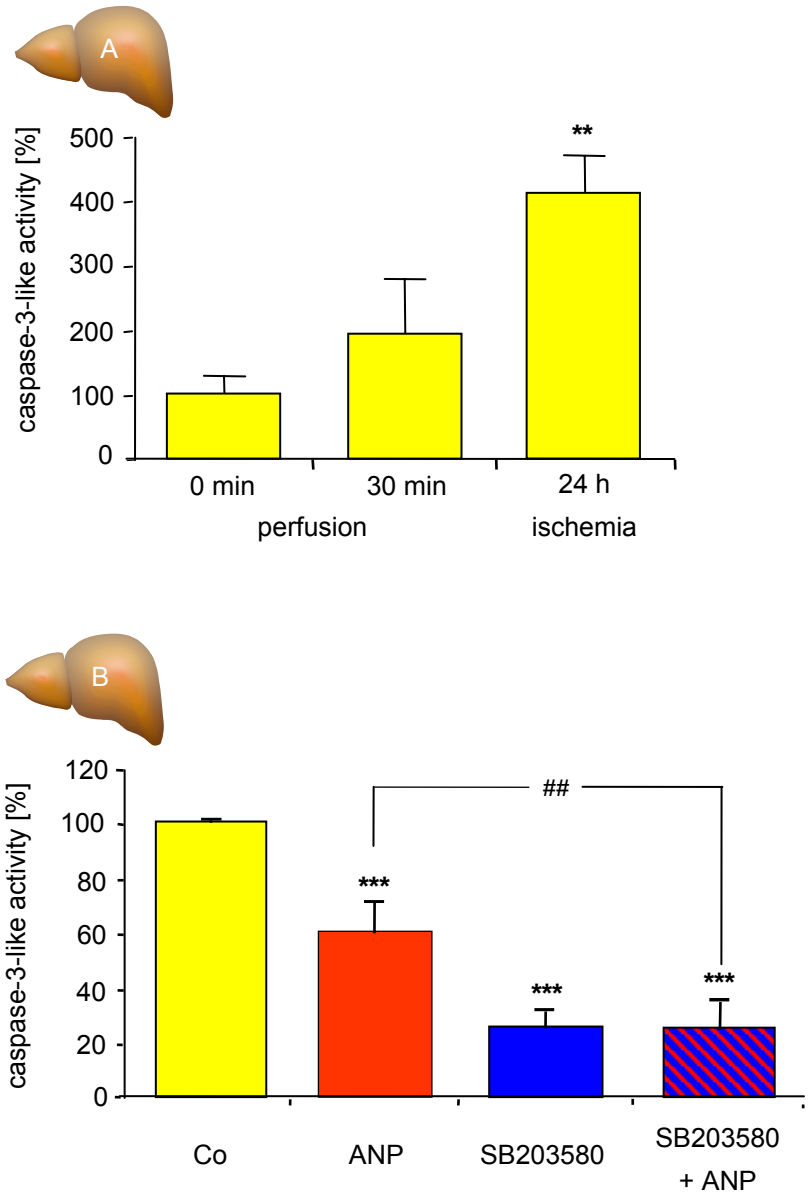


FIGURE 35: p38 MAPK activation is not involved in ANP-mediated antiapoptotic effects.

Either isolated rat livers were flushed exsanguinous (0' perfusion) with KH buffer or were perfused with KH buffer before ischemia for 30 min in the absence (Co) or presence of 200 nM ANP ± 2 µM SB203580, which was added 20 min prior to ischemia. **A**: The figure shows the Caspase-3-like activity induced by ischemia. Livers were briefly perfused blood-free (0' perfusion) and Caspase-3-like activity was measured as described under "Materials and Methods". Activities in sham treated livers (0' perfusion) were set equal 100% and rose up to 350 mU/mg at the end of 24 h ischemia. Columns show mean ± SEM of two independent experiments with four to five rat livers. **p<0.01 vs. 0' perfusion. **B**: After 24 h of cold ischemia livers were snap frozen and Caspase-3-like activity was measured as described under "Materials and Methods". Data are expressed as percent of enzyme activity of untreated control livers (Co) set equal 100%. Columns show mean ± SEM of three independent experiments with four to five rat livers. ***p<0.001 vs. control, $^{\#\#}$p<0.01 vs. ANP.

2.4 PKG ACTIVATION IS NOT INVOLVED IN THE ANTIAPOPTOTIC ACTION OF ANP

The second messenger cGMP protects against apoptosis in different cell types (181;242-244). Since cGMP mediates its effects in various cell types *via* PKG (171;172), also the involvement of PKG in ANP-mediated hepatoprotection was investigated. However, when rat livers were preconditioned by adding ANP ± Rp-8-Br-pCPT-cGMPS to the perfusion buffer 20 min prior to the ischemic period, the PKG inhibitor did not abolish the antiapoptotic effect of ANP (217). Perfusion with the inhibitor alone did not significantly affect Caspase-3-like activity (217).

So far, there is no distinct information available concerning PKG expression in the liver. Furthermore, it was shown that a PKG-inhibitor could not abrogate ANP-mediated effects (217). Therefore, we investigated PKG expression in the liver by RT-PCR. We found that neither the predominantly cytosolic isoenzyme PKG I nor the plasma membrane bound isoform PKG II is expressed in the liver, supporting that the ANP-induced protection is not mediated *via* PKG (FIGURE 36).

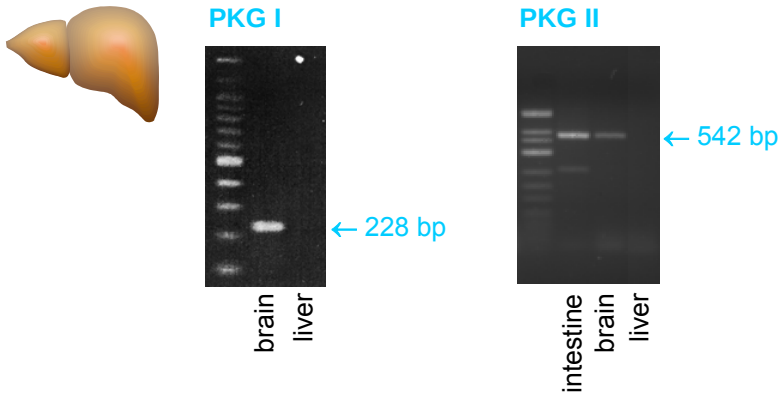


FIGURE 36: PKG activation is not involved in the antiapoptotic action of ANP.

Livers were flushed blood-free and cDNA was prepared as described under “Materials and Methods”. Brain and intestine as control organs were instantly removed, rinsed in PBS and snap frozen in liquid nitrogen. RT-PCR experiments were performed with primers detecting rat PKG isoforms I/II. Data show one representative of one/three independent experiments.

2.5 PKA ACTIVATION IS CRUCIAL FOR INHIBITION OF APOPTOSIS BY ANP

Having excluded the most prominent target molecules of cGMP signalling, PKGs, we tried to figure out other potential signalling options. In this context it is interesting to note that PKA can also act as target molecule for cGMP (173;216). Furthermore, liver perfusion with ANP in the presence of the PKA inhibitor Rp-8-Br-cAMPS abrogated the antiapoptotic effect of ANP (217). Additionally, ANP significantly increased hepatocyte PKA activity as did 8-Br-cGMP and the well described activator of PKA, Bt_2-cAMP (217). Therefore, we investigated possible downstream targets involved in the ANP-mediated protection against apoptosis.

2.6 ANP-MEDIATED PKA ACTIVATION LEADS TO BAD PHOSPHORYLATION

PKA *in vitro* phosphorylates the proapoptotic molecule BAD at Ser112, thereby inactivating BAD and reducing apoptotic cell death in isolated cells. Therefore, we investigated BAD as a possible target of ANP-induced PKA phosphorylation (245). FIGURE 37 shows that pretreatment of livers with ANP significantly increased the phosphorylation of BAD.

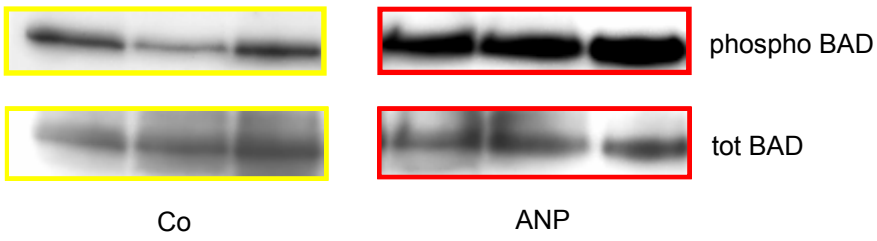


FIGURE 37: ANP-preconditioning leads to BAD phosphorylation.

Livers were perfused for 30 min in the absence (Co) or presence of 200 nM ANP, which was added 20 min prior to cold ischemia (24 h, 4°C). Western blot analysis (see “Materials and Methods”) was performed detecting phosphorylated BAD (phospho BAD) and total BAD (tot BAD) after 30 min reperfusion.

E DISCUSSION

1 R-LA-INDUCED PROTECTION AGAINST TNF-α-/ACTINOMYCIND-INDUCED LIVER CELL APOPTOSIS

R-LA reduces TNF-α-/ActD-induced apoptosis

Due to its potent antioxidant and redox-regulating properties, α-lipoic acid (LA) was originally proposed for the treatment of diseases mediated by free radicals, such as liver diseases, diabetes, heavy metal poisoning, and radiation damage (147-149). Further LA has been suggested to reverse oxidative stress and redox disturbances associated with HIV infection or IRI (129).

The present work shows for the first time an apoptosis-reducing effect of both, the racemic LA and R-LA. Further, it was demonstrated that the reduced form, DHLA, seems to induce cell death in hepatocytes.

Several publications examine the protective effects of α-lipoic acid, a racemate consisting of both, the naturally occurring R- and the unnatural S-isomer. Divers studies report differences in the efficiency of the racemate, the R- and the S-isomer contributing to protection. The S-enantiomer (S-LA) is a less effective antioxidative and chelating agent compared to R-LA, which might explain different regulatory actions in redox sensitive processes (246). Besides, differences in the antioxidative potential between the two enantiomers, several protective effects of R-LA can not be mimicked by S-LA: R-LA increased ATP synthesis and aortic blood flow during reoxygenation after hypoxia, whereas the S-enantiomere had no effect on ATP synthesis and improved blood flow (247). Administration of both, R-/S-LA and R-LA increased the GSH and GSSG levels, as well as the levels of other antioxidants in lens tissue and protected against cataract formation. The S-enantiomere being completely ineffective was explained by preferential uptake of R-LA (248;249). Additionally, R-, but not S-LA can stimulate deficient brain pyruvate dehydrogenase complexes in dementia of the vascular type, but not in Alzheimer dementia (250). R-/S-LA and R-LA are described to increase insulin-stimulated glycogen synthesis and to enhance insulin-mediated glucose oxidation,

whereas S-LA had no significant effect on both parameters (251). Since the ratio of S- and R-enantiomers in the racemate is 1:1, with S-LA being less effective, the presented results reflecting that the racemic LA inhibited T/A-induced apoptosis to a minor degree compared to pure R-LA, are not surprising. This observation is constistent with a study showing that the anti-inflammatory activities of R-LA are 10-fold stronger than those of the racemate (232).

Interestingly, our data showed that DHLA significantly increases apoptotic cell death, R-LA only slightly but not significantly. Since DHLA is a more potent antioxidant than R-LA, and several studies describe apoptosis-inducing properties of LA due to its antioxidative properties (252), DHLA might induce cell death in our model. And the fact that R-LA is reduced to DHLA explains the slight increase in apoptosis induced by R-LA.

Antioxidative properties play a minor role in R-LA-mediated reduction of TNF-α-/ActinomycinD-induced apoptosis

LA is described to be a potent antioxidant (149). Since the role of ROS in hepatocyte apoptosis is yet unclear, our results suggesting R-LA-mediated protection being rather independent of antioxidant actions are astonishing.

Several studies ascribe ROS as inducers or at least mediators of apoptosis (253;254): depletion of mitochondrial GSH sensitises hepatocytes to TNF-α- and Fas-induced apoptosis (255). Others report an inhibition of hepatocyte apoptosis after cytoplasmic GSH depletion (229;256). *Fernandez-Checa et al.* suggest a higher sensitivity towards apoptosis in alcoholic liver diseases, with ethanol being known to preferentially deplete mitochondrial GSH (257). In contrast, oxidative stress induced by increased intracellular superoxide anion concentrations can abrogate apoptosis in tumor cells (258). *Rauen et al.* describe iron-mediated ROS-formation as key mediator of cold-induced hepatocyte apoptosis (259). Thus, it seems that ROS-mediated effects being pro- or antiapoptotic depending on the intensity and localisation of the generated ROS and the apoptotic inducers looked at. The antioxidant LA has been reported to protect against IRI in the kidney, heart, retina, and brain (150-152;260).

In contrast, our data indicate that T/A-induced hepatocyte apoptosis is rather

independent of redox homeostasis. In fact, our data show an R-LA-induced inhibition of hepatocyte apoptosis that could not be mimicked by two out of three tested antioxidants. The antioxidants were the same and used in the same concentrations as in a work of *Rauen et al*., showing cold-induced ROS-release being the mediator of hepatocyte injury (261). *Van de Mark et al.* described LA-induced effects that could not be mimicked by antioxidants (262). Furthermore, *Persson et al.* observed an LA-induced protection against oxidant-mediated apoptosis, whereby the protective actions of LA arise from intralysosomal iron chelating and not from radical-scavenging actions (263). This results suggest that the observed antiapoptotic actions of R-LA seem to be widely independent of its radical scavenging properties.

This raises the possibility that LA works as metal-chelator, thereby suppressing harmful metal-catalysed redox reactions, enzyme inactivation or lipid peroxidation, thus inhibiting apoptosis (246;264-266). *Persson et al.* describe antiapoptotic effects of LA acting as iron-chelator in a murine macrophage cell line (J774), even though they observed additive cytoprotective effects by combination of an Fe-chelator and LA indicating other mechanisms than chelating actions to contribute to protection (263). Cold-induced apoptosis of hepatocytes appears to be mediated by a rapid increase in the cellular chelatable iron pool and subsequent formation of ROS (267). *Kerkweg et al.* observed decreased cold-induced hepatocyte apoptosis after incubation with different iron-chelating agents, the same Fe-chelators in the same concentrations as used for our investigations (268). Interestingly, only one of these Fe-chelators was able to inhibit hepatocyte apoptosis in our model of T/A-induced cell death, and the administration of the non-chelating dipyridyl induced caspase activity. Surprisingly, the chelating dipyridyl even slightly but significantly induced caspase activity compared to T/A-treated cells. Thus, our data support the assumption that iron complexation plays only a minor role in LA-mediated protection against T/A-induced apoptosis.

Comprising this, our data indicate that R-LA mediates hepatoprotective effects largely independent of its antioxidative and Fe-chelating properties.

Role of R-LA in the signalling of apoptotic cell death

Our data demonstrate R-LA as a potent inhibitor of T/A-induced apoptosis. However,

α-lipoic acid has also been described to promote apoptotic cell death: LA but no other antioxidant promotes apoptosis in retinal cell layers (269). Likewise, *van de Mark et al.* demonstrated LA-mediated apoptosis in transformed but not in normal cells, independent of the Fas signalling pathway (262). Furthermore, exposure to LA has been suggested to induce apoptosis by Fas in leukemic Jurkat cells but not in peripheral blood lymphocytes, with LA potentiating all effects following Fas activation, such as a rapid loss of cell thiols, an increase of intracellular Ca^{2+} concentrations, and an activation of PKC and Caspase-3 (270). Besides, DHLA is also described to induce apoptosis in Jurkat T-cells (270). In addition, *Casciari et al.* determined that LA was not only toxic to different tumor cell lines, but also enhanced the cytotoxicity of ascorbate in tumors in a synergistic fashion (271). A possible reason for this synergy might be the ability of LA to modify the cellular redox status or to recycle ascorbate. Moreover, LA induced apoptosis in both, proliferating and non-proliferating cells, whereas non-proliferating cells are relatively resistant to ascorbate toxicity.

Summarising these data, it seems that LA mediates its proapoptotic effects *via* both, the Fas/Fas ligand signalling pathway and antioxidative properties. Since our model of apoptotic cell death is described to be independent of the Fas/Fas ligand system (85), it is not surprising that R-LA mediates apoptotic cell death only to a minor extend. As described above, the proapoptotic effect of R-LA probably depends on the intracellular reduction of R-LA to DHLA.

In addition, some protective effects of LA are also described to occur in different cell types, most of them mediated *via* antioxidative properties: *Weitsman et al.* reported concordantly with the results presented here a decrease in caspase activity due to LA-stimulation in TNF-α-induced apoptosis of human breast cancer cells (272). Furthermore, *Myzak et al.* determined the effects of the two antioxidants LA and ascorbate on oxidant-mediated cell death in HL-60 cells (273). Again the authors observed an LA-induced inhibition of the Caspase-3 activation and suggest that this antiapoptotic effect is due to oxidant scavenging. *Honma et al.* as well describe an apoptosis reducing effect of LA in a cell model of acute diabetic neuropathy (274). In addition to these results, *Persson et al.* presume that the observed protective effects of LA on oxidant-induced J774 cell apoptosis arise from indirect antioxidative effects mediated by intralysosomal iron-chelation (263). Consequently, most of the

investigations exhibit the antiapoptotic effects of LA depending on its antioxidative properties, even though in these studies mostly ROS-induced apoptosis was investigated (263;273;274).

Other studies indicate antiapoptotic effects by chelating activities of LA to contribute to protection (263). Furthermore, in most of the examinations LA is just used as an antioxidant and thus LA-mediated effects are only compared to those of other oxidant-scavenging agents. To our knowledge, besides the here presented data no other studies exist examining the antiapoptotic effects of LA mediated through signalling pathways different from the antioxidative or the chelating ones.

Alongside the data reported here, there is only little known about LA-mediated effects on liver cell apoptosis. *Pierce et al.* described LA-mediated protection against T/A-induced apoptosis in a murine hepatocyte cell line to be mediated by antioxidative properties of LA (275). Such a conclusion is contrary to the results presented here indicating no major effect of antioxidant treatment on T/A-induced apoptosis, since other antioxidants demonstrated no antiapoptotic actions. As the authors did not test the effect of any other antioxidant on hepatocyte apoptosis, we conclude that oxidative stress is essential for the development of apoptosis is not evidenced.

Zhang et al. reported that LA mediates neuroprotective effects at least partially through activation of the PI3K/Akt pathway (276) and activation of this pathway has been shown to inhibit apoptosis in a variety of cell types (277;278). Therefore, the involvement of Akt in hepatocyte apoptosis was further investigated.

Role of Akt in apoptosis

Our data exhibit that activation of the PI3-K/Akt pathway participates in the antiapoptotic signalling induced by R-LA.

Concordantly with this, *Zhang et al.* report an Akt-mediated inhibition of cell death in rat cortical neurons after LA treatment (276). Several reports show antiapoptotic effects of Akt activation in the liver: multiple studies examined that IL-6 or Epidermal growth factor protect against TGF (Transforming growth factor)-β-induced apoptosis through activation of the PI3-K/Akt pathway and subsequent inhibition of Caspaspe-3 activation in the

human hepatoma cell line Hep3B and in rat fetal hepatocytes (279-281). Consistent with these achievements, *Hong et al.* reported a rapid Akt activation being the key mediator of the antiapoptotic signalling involved in TGF-β-induced cell death and liver regeneration (49).

In line with our results, several studies on human hepatocytes also describe a PI3-K/Akt-mediated protection against TNF-α-induced apoptosis (100;282;283). In addition, it was demonstrated that the PI3-K/Akt pathway plays a protective role in TNF-α- and Fas-induced apoptosis in mouse hepatocytes, including the activation of NF-κB and inducible Bcl-2 (284;285). Moreover, insulin is described to exert antiapoptotic effects in HepG2 by activation of the PI3K/Akt- and Erk-signalling pathways, by suppression of the Caspase-3 activation, and *via* the reduction of ROS generation (286;287).

Thus, besides the here presented data showing for the first time a LA-mediated protection against T/A-induced apoptosis by Akt-activation, some antiapoptotic effects in the liver are described to be mediated *via* activation of the PI3-K/Akt signalling pathway. Nevertheless, possible downstream targets of the PI3-K/Akt signalling involved in the antiapoptotic mechanisms are only insufficiently investigated in the literature.

Role of one possible downstream target of Akt in apoptosis: BAD

Unphosphorylated BAD, a proapoptotic member of the Bcl-2 gene family, binds Bcl-2 and Bcl-X_L, thus inhibiting their antiapoptotic potential (13). Once phosphorylated at Ser136, BAD dissociates from Bcl-2/Bcl-X_L and interacts with the 14-3-3 proteins instead. Bcl-2/Bcl-X_L are then free to exert their activity to prevent apoptosis (33).

Consistent with our data presented here, showing for the first time an LA-induced protection against hepatocyte apoptosis *via* Akt and subsequent BAD phosphorylation, several reports indicate that Akt mediates its antiapoptotic effects through phosphorylation and thus inactivation of the possible downstream target BAD: the estrogen 17-β estradiol (E_2) decreases apoptosis in TNF-α-treated breast cancer cells *via* phosphorylation of BAD at Ser112 and Ser136 by Akt (48). Furthermore, *del Peso et al.* identified the death agonist BAD to be a substrate of Akt in IL-3-treated FL5.12 cells.

Since BAD phosphorylation was prevented by incubating IL-3-dependent FL5.12 lymphoid progenitor cells with specific PI3-K inhibitors, the authors claimed that BAD phosphorylation depends on the activation of the PI3-K/Akt signalling pathway (47). Additionally, growth factor activation of the PI3-K/Akt signalling pathway culminates in the phosphorylation of BAD *in vitro* and *in vivo*, thereby suppressing apoptosis and promoting cell survival in primary neurons (46). Taken together and in addition to our data, these studies defined a mechanism directly inactivating critical components of the cell-intrinsic death machinery, namely BAD, and thus protecting against apoptosis.

In contrast to these findings, *Webster et al.* presented a study indicating that hepatocyte growth factor inhibits bile acid-induced apoptosis in rat hepatocytes through activation of PI3-K, Erk, and PKA, but indepedent of BAD phosphorylation on Ser112 (288). Thus, the authors suggested this antiapoptotic protection to be independent of BAD phosphorylation (288). As opposed to these achievements, our data demonstrate that the observed BAD phosphorylation depends on the activation of the PI3-K/Akt pathway, and is thus inhibited by incubation with Wortmannin.

Apart from the results of this work, only *Danial et al.* determined the role of BAD in hepatocyte apoptosis: the authors demonstrated that BAD phosphorylation elevates ATP-levels, thus protecting against hepatocyte apoptosis (289). Conforming with this, our data show that R-LA also protects against apoptosis through activation of Akt and phosphorylation of BAD. Consistently, LA is described to influence ATP levels in different systems: in neurodegenerations by attenuating metabolic changes such as a decrease in cellular ATP levels (290). In the liver, LA protects against IRI, increases the ATP levels, and activates the survival kinase Akt (153).

Concluding, the here presented data do indicate for the first time cytoprotective effects of R-LA in hepatocytes:

(I) R-LA protects against T/A-induced apoptosis.

(II) this antiapoptotic effects depend on the activation of the cytoprotective kinase Akt.

(III) activation of the PI3-K/Akt pathway leads to phosphorylation of the proapoptotic protein BAD.

Worth to note, these cytoprotective effects of R-LA seem to be rather independent of antioxidative or chelating properties.

2 ANP-INDUCED PROTECTION AGAINST LIVER CELL APOPTOSIS

ANP-induced liver protection does not depend on p38 MAPK activation

The cardiovascular hormone ANP is known to protect livers against IRI (ischemia reperfusion injury) *via* the particulate guanylate cyclase (176;180;181) comprising attenuation of apoptosis in ischemic organs. This fact combined with the result of ANP-preconditioning increasing p38 MAPK activity (184;236) led to the suggestion that p38 MAPK activation participates in a potential signal transduction pathway of ANP-mediated hepatoprotection (184). Mainly the observation that the kinase activity peaks at the end of the preconditioning period and thus before ischemia argued for a crucial role of p38 MAPK in ANP-induced liver protection.

So we hypothesised that ANP induces the markedly p38 MAPK activation in hepatocytes. Astonishingly, neither isolated hepatocytes, nor isolated KCs displayed such a strong p38 MAPK activation as the whole ANP-preconditioned liver did. Since we verified *via* cGMP-measurement that the hepatocytes used possess functional NPR-A receptors, we presume that other hepatic cells or mediators missing in an isolated hepatocyte or KC culture are additionally involved in the ANP-induced signalling pathway leading to p38 MAPK activation. Furthermore, KC depletion also did not alter ANP-mediated p38 MAPK activation, suggesting that an interaction between hepatocytes and KCs is not involved in ANP-mediated p38 MAPK activation.

Prior to further investigations of the signalling pathway, we intended to examine whether the ANP-induced kinase activation is crucial for the ANP-mediated protection against ischemic injury. Reports of the potency of ANP to strongly attenuate post-ischemic apoptotic cell death and p38 MAPK as an antiapoptotic mediator prompted us to determine the function of p38 MAPK particularly in ANP-mediated inhibition of apoptosis (195). Astonishingly, inhibition of this kinase did not abrogate the hepatoprotective effects of the cardiovascular hormone and thus the present data suggest that p38 MAPK is not causally involved in the decrease of ischemic injury. Therefore, the hypothesis suggesting that the ANP-mediated p38 MAPK activation contributes to protection (184) could not be confirmed. The present data provide evidence against a protective effect of p38 MAPK and rather indicate a detrimental role of this kinase during ischemic injury.

Interestingly, employment of the p38 MAPK inhibitor SB203580 (SB) itself protected against ischemia-induced apoptosis. These findings comply with the results of *Mackay et al.*, describing reduced apoptosis in SB-treated rat cardiac myocytes after ischemia (196). In line with the present results, inhibition of p38 MAPK by FR167653 (FR) attenuates hepatic IRI *via* reduced production of inflammatory cytokines (291;292). Consequently, not only the pyridinyl imidazole inhibitor SB, but also FR, described to resemble the pharmacological characteristics and chemical structure of SB (FIGURE 38) (293), is able to inhibit p38 MAPK and thus diminish IRI. Furthermore, *Martin et al.* demonstrated that the ability of SB to attenuate IRI is abolished when cells express a mutant p38α MAPK isoform insensitive to inhibition by SB (294). Concordantly with this, *Kaiser et al.* recently described that transgenic mice expressing a dominant negative MKK6 or p38α MAPK mutant are protected against cardiac IRI (295).

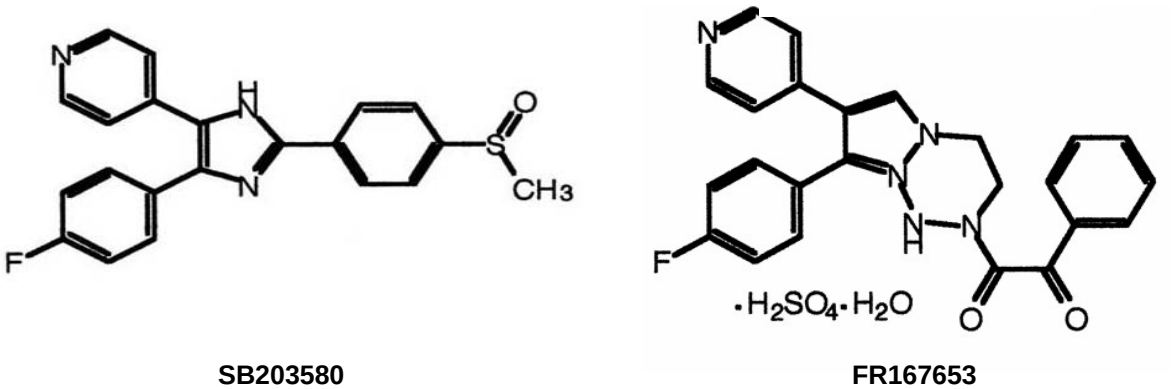


FIGURE 38: Chemical structure of the p38 MAPK inhibitors SB203580 and FR167653.

In summary, these data provide evidence against a protective effect of p38 MAPK activation in IRI. In fact, they rather indicate a detrimental role of this kinase during IRI. Yet, ANP has been shown to be a strong activator of p38 MAPK and *Carini et al.* suggested ANP-induced p38 MAPK activation to protect against hypoxic injury in isolated hepatocytes *via* cGMP (195). These controversial data might be explained by differences in the models employed: isolated hepatocytes *versus* organ perfusion. Data recorded in the whole organ model used here indicate that 8-Br-cGMP does activate p38 MAPK only to a minor extent in the whole liver although cGMP is known to be responsible for ANP-induced liver protection. Therefore, these findings further support

the notion that the strong activation of p38 MAPK after hormonal preconditioning is not involved in ANP-mediated protection against apoptotic cell death (FIGURE 39).

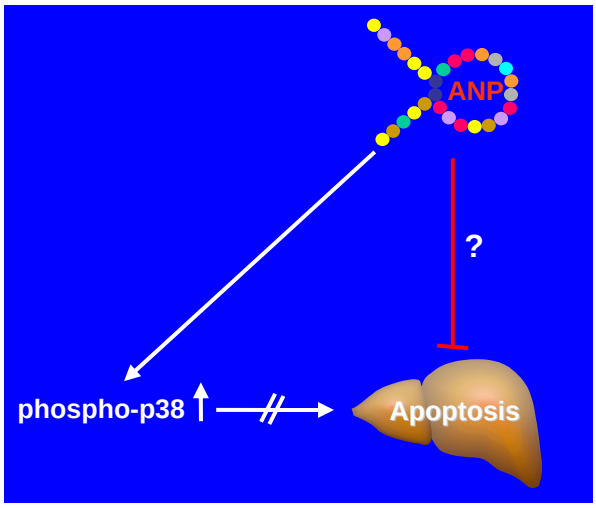


FIGURE 39: ANP-induced p38 MAPK activation does not contribute to protection against liver cell apoptosis.

ANP-mediates its protective effects against liver cell apoptosis *via* PKA

Since the present data exhibit that the ANP-induced p38 MAPK activation is not essential for ANP-mediated protection against hepatic apoptosis, other downstream targets of ANP possibly involved in liver protection were investigated.

ANP protects against IRI *via* intracellular formation of the second messenger cGMP (176;181). Thus, the role of the second messenger cGMP in hepatoprotection was explored. The signalling pathways downstream of the second messenger are still unclear in the liver. Since cGMP-dependent protein kinases (PKG) are central target proteins of cGMP (178), PKGs were hypothesised to be involved in the ANP-mediated protection. PKGs are responsible for a multitude of actions conveyed by ANP and nitric oxide (NO). In this context PKGs were shown to influence intracellular ion homeostasis, inhibit platelet aggregation, modulate multiple renal functions, and lower intracellular calcium concentrations in smooth muscle cells (for review see: (198)). In contrast to other tissues (199-201;203;296-301), the expression of PKGs in the liver is still questionable. *Ecker et al.* did in fact demonstrate PKG expression in the liver by Western blot analysis and ELISA (302). However, taking into account that platelets contain large amounts of PKG protein, it is likely that the positive data gained by

Western blot and ELISA were due to thrombocytes or other PKG-containing blood cells. Other groups reported low to no PKG mRNA expression in the liver, and therefore the presence and significance of these kinases in the liver has to be questioned (300;303). In addition, *Kosmopoulou et al.* studied the presence of different protein kinases such as PKAs and PKGs in microsomal membranes and displayed no PKG activity in membrane preparations of rat liver endoplasmatic reticulum, further supporting the absence of PKGs in this tissue (208). The present data endorse these findings, since the RT-PCR experiments revealed no amplification of either PKG isoforms in the liver, whereas control organs like brain and intestine were positive. Thus, an involvement of PKGs in ANP-mediated hepatoprotection seems to be unlikely. Furthermore the present data show no functional relationship between ANP and PKG which can be explained by the lack of detrimental effects after perfusion with ANP and the competitive PKG inhibitor Rp-8-pCPT-cGMPS. Taken together, the data presented here suggest that PKG is not the main target of ANP and cGMP in the liver. Still, cGMP is the second messenger exerting ANP's protective action (176;181). Thus, the interest is now focussed on alternative pathways.

Under certain conditions cAMP-dependent protein kinase (protein kinase A, PKA) can act as a target molecule for cGMP (173). For example, *Schumacher et al.* displayed that the stimulation of testosterone production by ANP in isolated mouse Leyding cells results from activation of PKA in a cGMP-dependent manner (304). Furthermore it has been shown in mice that in the absence of PKGI NO can relax vessels *via* cGMP-dependent PKA activation (305). Recently *Aizawa et al.* described that the ANP homologue CNP (C-type natriuretic peptide) mediates transcriptional inhibition controlled by NF-κB *via* a cGMP-dependent activation of PKA (216). Data presented by *Gerwig T* support further evidence for a crosstalk of cGMP with the cAMP/PKA pathway: liver perfusion with the PKA inhibitor Rp-8-Br-cAMPS abrogated the antiapoptotic effects of ANP and treatment of hepatocytes with the hormone enhanced PKA activity (217).

Harada et al. showed that *in vitro* PKA is able to mediate antiapoptotic effects *via* phosphorylation and inactivation of the proapoptotic protein BAD at Ser112 (245). Consistent with these findings the recorded data demonstrate that ANP-preconditioning phosphorylates BAD in the perfused rat liver, assuming that ANP-mediated PKA activation induces BAD inactivation. Thus, the data presented here suggest that the

natriuretic peptide protects against apoptosis occuring during ischemic injury *via* PKA-mediated phosphorylation of BAD.

In summary, the present data show that ANP-preconditioning protects against apoptosis triggered by ischemic injury *via* activation of the protein kinase A and subsequent BAD phosphorylation, whereas p38 MAPK activation seems irrelevant. Therefore, this study shows for the first time the causal involvement of PKA activation as early signalling event in ANP-mediated inhibition of liver cell apoptosis (FIGURE 40).

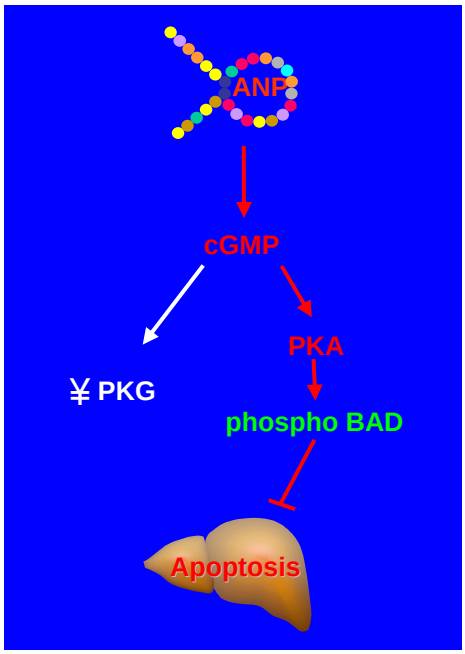


FIGURE 40: ANP protects against liver cell apoptosis *via* PKA-activation and subsequent BAD phosphorylation. ¥: PKGs are absent in the liver.

F SUMMARY

Both, the R-enantiomer of the antioxidant α-lipoic acid (R-LA) and the hormone atrial natriuretic peptide (ANP) are known to exert potent hepatoprotective action. The present work characterises α-lipoic acid- and ANP-mediated signal transduction pathways involved in the regulation of apoptotic cell death in two different models: primary hepatocytes and ischemic isolated perfused rat livers.

α-lipoic acid was shown to protect isolated hepatocytes from TNF-α-/ActinomycinD-induced apoptosis. Astonishingly, this effect did not seem to be governed neither by its well described antioxidative nor its Fe-chelating properties. In fact, the LA-mediated activation of the PI3-K/Akt survival pathway seemed to be responsible for the antiapoptotic properties of α-lipoic acid. Consequently, incubation with a specific PI3-K-inhibitor significantly reduced both, R-LA-mediated decrease in caspase activity and R-LA-induced BAD phosphorylation. Thus, PI3-K-mediated Akt activation and subsequent phosphorylation of the proapoptotic protein BAD at Ser136 are causally involved in the antiapoptotic signalling mediated by R-LA.

Perfusion with ANP 20 min prior to the ischemic period is known to reduce apoptotic cell death occurring at the end of the ischemic period. We could previously show that this preconditioning of rat livers leads to a marked activation of p38 MAPK. Since ANP reduces apoptotic cell death, the potential connection between this ANP-induced p38 MAPK activation and apoptosis reduction was investigated. Astonishingly, liver perfusion with an p38 MAPK inhibitor even decreased apoptotic cell death, supporting a detrimental role of this kinase. PKA-specific inhibitors demonstrated the involvement of PKA in this ANP-mediated protection. Interestingly, it also turned out that PKA phosphorylates the proapoptotic protein BAD at Ser112, an effect known to contribute to the inhibition of apoptosis.

In summary, the present data show for the first time that phosphorylation of BAD at either Ser136 or Ser112 turns out to be a central protective mechanism to defend from hepatocyte apoptosis.

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G APPENDIX

1 ABBREVIATIONS

A	Ampère
ANP	Atrial natriuretic peptide
ATP	Adenosine-5'-triphosphate
BNP	Brain natriuretic peptide
Bp	Basepair
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)
Cl_2MBP	Clodronate = Dichloromethylene-bisphosphonate
CNP	C-type natriuretic peptide
Co	Control
Da	Dalton
DHLA	Dihydrolipoic acid
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(aminoethylether)-tetraacetic acid

EtOH	Ethanol
FCS	Fetal calf serum
h	Hour
HRP	Horseradish peroxidase
IRI	Ischemia reperfusion injury
kDA	Kilo Dalton
L	Liter
LA	α-Lipoic Acid
LDH	Lactate dehydrogenase
m	Milli
M	Molar
MeOH	Methanol
min	Minute
mRNA	Messenger ribonucleic acid
n	Nano
NADH	Nicotinamide adenine dinucleotid
NPR	Natriuretic peptide receotor
PAA	Polyacrylamide
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3-K	Phosphatidylinositol-3 kinases
PIP_3	Phosphatidylinositol-3,4,5-triphosphate

PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PMSF	Phenylmethylsulfonylfluoride
R-LA	R-enantiomer of LA
R-/S-LA	Racemic LA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of mean
TBS-T	Phosphate buffered saline solution with Tween
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor alpha
totRNA	Total RNA
Tris	Tris-hydroxymethyl-aminomethan
U	Unit
V	Volt
W	Watt
% (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
BioRad Laboratories	Munich, Germany
Biosource	Nivelle, Belgium
Biozym Diagnostics	Oldendorf, 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
Eppendorf	Maintal, Germany
Gibco/Invitrogen	Karlsruhe, Germany
Greiner	Frickenhausen, Germany
Heraeus	Hanau, Germany
Linde	Unterschleißheim, Germany
Merck-Eurolab	Munich, Germany
Millipore	Eschborn, Germany

MWG-biotech	Ebersberg, Germany
NEN	Cologne, Germany
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
Roche Diagnostics	Mannheim, Germany
Roth	Karlsruhe, Germany
Santa Cruz	Heidelberg, Germany
Serva	Heidelberg, Germany
Sigma	Taufkirchen, Germany
Ssniff	Soest, Germany
Stratagene	Heidelberg, Germany
Stressgen	San Diego, USA

3 PUBLICATIONS

3.1 ABSTRACTS

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Kiemer AK, Kulhanek S, Gerwig T, Gerbes AL, Vollmar AM. Aktivierung der p38 MAP-Kinase und Verringerung apoptotischer Zellschäden durch das Atriale Natriuretische Peptide bei hypothermer Konservierung am Modell der isoliert perfundierten Rattenleber. Zeitschrift für Gastroenterologie, 2001, 1: 78.

Kiemer AK, Kulhanek-Heinze S, Gerwig T, Vollmar AM, Gerbes AL. MAP Kinase Activities during ischemia and reperfusion of the rat liver: influence of ANP preconditioning. Liver Transplantation, 2001, 7: 214.

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