Dissertation zur Erlangung des Doktorgrades

der Fakultät für Chemie und Pharmazie der

Ludwig-Maximilians-Universität München

α-Lipoic Acid Attenuates Ischemia Reperfusion Injury of the Rat Liver: Mechanisms of Protection

von

Christian Müller

aus

Ingolstadt

2002

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29.01.1998 von Prof. Dr. A. M. Vollmar betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am 13.06.2002

Dissertation eingereicht am	17.06.2002
1. Gutachter	Prof. Dr. A. M. Vollmar
2. Gutachter	Prof. Dr. E. Wagner
Mündliche Prüfung am	10.07.2002

Für meine Eltern

A. Contents

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B. Introduction

1. Overview and aim of this study

Ischemia reperfusion injury (IRI) is a serious clinical problem during liver resections, liver transplantation, and haemorrhagic shock. The pathomechanisms of this injury can be divided into incidents during ischemia and events occuring during reperfusion. Some main pathophysiologic features of ischemic liver cell injury comprise depletion of ATP, disturbance of natrium-calcium homeostasis, and activation of phospholipase A_2 (Bilzer and Gerbes, 2000;Bilzer *et al.*, 1994;Jaeschke *et al.*, 1988). Reperfusion of livers leads to an aggravation of ischemic liver cell damage: reactive oxygen species (ROS) derived from activated Kupffer cells as well as the activation of proinflammatory, redox-sensitive transcription factors, such as NF-κB and AP-1, are discussed to contribute to hepatic reperfusion injury (Banafsche *et al.*, 2001;Zwacka *et al.*, 1998;Fan *et al.*, 1999). Numerous studies show beneficial effects of antioxidants like superoxide dismutase, katalase, glutathione, and N-acetylcysteine on IRI (Bilzer *et al.*, 1999;Serracino-Inglott *et al.*, 2001;Mizoe *et al.*, 1997b;Nagel *et al.*, 1997).

Among substances with known antioxidative properties, α-lipoic acid (LA), a compound established in the therapy of diabetic polyneuropathy (Coleman Michael D., 2001), is especially interesting: LA exhibits distinct regulatory action on signal transduction processes playing a central role in tissue damage and protection. In this context the potential of LA to regulate stress-related signalling pathways, such as NF-κB and AP-1 on the one hand (Saliou *et al.*, 1999), and to activate cytoprotective protein kinases on the other hand, has recently been reported (Yaworsky *et al.*, 2000;Maddux *et al.*, 2001).

α-lipoic acid is found naturally occuring as a prosthetic group in α-keto acid dehydrogenase complexes of mitochondria, and therefore plays a fundamental role in metabolism. Administration of LA to cells leads to a rapid uptake and reduction to dihydrolipoic acid (DHLA) (Han *et al.*, 1997). LA and DHLA are both potent scavengers of ROS, such as hydroxyl radical, hypochlorous acid or singlet oxygen, as well as effective metal chelators forming complexes with e.g. Fe^{2+} (Biewenga *et al.*, 1997;Packer *et al.*, 1995). The low redox potential of the

LA/DHLA redox couple allows an increased *de novo* synthesis of GSH (Packer *et al.*, 1995;Packer *et al.*, 2001).

Aim of this study

The present study examined whether the administration of α-lipoic acid has protective potential in hepatic IRI. Special interest focussed on the characterization of mechanisms in LA-mediated hepatoprotection. Thereby, potential influence of LA on necrotic and apoptotic processes, hepatic redox state, energy metabolism, activation of proinflammatory transcription factors, and cytoprotective kinases was investigated.

2. Hepatic ischemia reperfusion injury

Hepatic ischemia reperfusion injury is a typical incident occuring during liver transplantation, liver resection, and hemorrhagic shock. It is characterized by reduced or completely missing blood supply accompanied by limited oxygen availability of the liver within a certain time period, called ischemia. This *ischemic injury* induces pathomechanisms which are aggravated by recovering blood flow during reperfusion, called *reperfusion injury*. Generally two forms of ischemic periods are distinguished: cold and warm ischemia. Cold ischemia occurs, when organs are prepared for transplantation. Thereby, organs are flushed with cooled storage solutions and subsequently stored at 4°C until transplantation. Warm ischemia especially occurs during liver resections, when blood supply is interrupted for surgery and the organ rests at body temperature. In transplantations warm ischemia happens during implantation of the organ.

The following chapters describe the clinical impact of hepatic IRI and show main pathomechanisms of ischemia and reperfusion.

2.1 Clinical relevance

When Starzl and colleagues in 1963 (Starzl T.E., 1996) performed the first human liver transplantation they could not expect that the number of transplantations from former 12 per year would arise in the year 2000 to approximately 4500 only in the United States (Keeffe, 2001).

Advances in immunosuppression and surgical technique made it possible that liver transplantation became a widely accepted therapy for children and adults

with end-stage liver disease (e.g. chronic hepatitis C, alcoholic liver disease, etc.). Nowadays, long-term survival rates (7 years) reach approximately 70%. Currently, a major problem is the limited availability of donor livers. Although the number of donors and liver transplants increased 2.4-fold from 1988 to 1997, the number of patients on the liver transplant list increased 15.6-fold and the number of waiting list deaths increased 5.8-fold over the same time period (Keeffe, 2001).

Of those receiving livers, primary graft nonfunction leading to graft failure and retransplantation still occurs in 5-15% of patients. This represents the most common reason for retransplantation after immunological rejection (Lemasters and Thurman, 1997). Initial poor function of transplanted liver grafts occurs in 10-25% of patients. The incidence of primary graft failure and initial poor function is strongly dependent on duration of warm ischemia and time of cold storage. Thus, the initial performance of the donor organ is strongly related to IRI associated with graft harvest, storage, and transplantation (Neuberger, 2000;Lemasters and Thurman, 1997).

This work especially concentrated on *warm* hepatic ischemia reperfusion injury, which appears during transplantation at the end of the surgery procedure implanting the organ, with the liver still lacking blood supply (Jaeschke, 1996).

Warm ischemia and reperfusion injury also occurs during surgical liver resections, where parts of the liver are removed, while blood flow is interrupted by clamping supplying vessels, followed by resumption of normal blood flow. The complications arising from liver resection due to IRI are comparable in quantity and severity to that described for transplantation. Data from the “Klinikum der Universität München” Großhadern in 2000 (724 patients) show dysfunction of the liver in 14% and liver failure in 5% of all patients (Schauer R. 2001, unpublished data).

Due to the fact that the only effective treatment of primary liver failure is transplantation of the organ, it is of greatest clinical interest to reduce or even to prevent IRI.

2.1.1 Ischemic injury

A hallmark of ischemic liver injury represents ATP depletion due to lacking oxygen in the mitochondrial respiratory chain during anoxia. Physiological

consequence of this alteration in ATP availability is deterioration of energy dependent metabolic pathways and transport processes, leading to a massive imbalance of the intracellular ion status (Jaeschke, 1996). Thereby, cellular concentration of Na^+ increases because of an inhibition of the Na^+/K^+-ATPase, directly connected to a rise in intracellular Ca^{2+}-concentrations (Bilzer and Gerbes, 2000). The tremendous increase of intracellular Na^+ is of greatest relevance for the development of cell swelling during ischemia, leading to cellular burst in the worst case. A central role in the pathogenesis of ischemic injury is also attributed to the increase of intracellular Ca^{2+} concentrations. As an essential consequence, activation of Ca^{2+} dependent proteases, phospholipases, and endonucleases were described leading to cell degradation processes and cell damage (Bilzer and Gerbes, 2000). Inflammatory incidents during ischemia are mediated *via* activation of phospholipase A_2 (Bilzer *et al.*, 1994) resulting in prostaglandin and leukotriene formation.

2.1.2 Reperfusion injury

While reperfusion injury is not harmful following short periods of ischemia, it brings about the full expression of injuries induced by long periods of ischemia, e.g. warm ischemia > 30-45 min (Henderson, 1999). Reperfusion injury is a consequence and amplification of cell activation and damage developed during ischemia.

Kupffer cell activation and reactive oxygen species

Interest in mechanisms of reperfusion injury increased dramatically with the findings that xanthine oxidase- and mitochondria-derived reactive oxygen species (ROS) might be responsible for its pathophysiology. The initial, simplistic view was that these oxygen radicals generated during reperfusion cause cell damage by lipid peroxidation. However, more mechanistic studies characterizing the role of reactive oxygen-mediated liver injury showed that no relevant intracellular oxidant stress could be detected in the reperfused liver either *in vitro* or *in vivo* (Kobayashi *et al.*, 1992;Jaeschke *et al.*, 1988;Jaeschke and Farhood, 1991;Jaeschke, 1998). The extent of lipid peroxidation necessary to cause significant liver cell damage is by far higher than that ever measured during reperfusion (Jaeschke, 1998). The lack of intrahepatocellular reactive oxygen formation under pathophysiologically relevant conditions has directed interest towards oxidant stress in the hepatic vasculature. Especially Kupffer

cells (KC), but also neutrophils were identified as the critical sources of ROS during the initial phase of reperfusion injury (Rauen *et al.*, 1994;Bilzer and Gerbes, 2000;Jaeschke, 1998;Lichtman and Lemasters, 1999;Serracino-Inglott *et al.*, 2001). KC can be activated by subjecting them to hypoxia with subsequent reoxygenation. Activated KC also induce a network of cytokines (e.g. TNF-α, interleukin-1, etc.), participating in sinusoidal accumulation of granulocytes and microcirculatory failure. Reducing the capacity of KC to produce ROS by gadolinium chloride or methyl palmitate effectively protected against reperfusion injury, suggesting an important role of vascular oxidant stress in its pathophysiology (Jaeschke and Farhood, 1991;Lichtman and Lemasters, 1999). Furthermore, a lot of antioxidant strategies including treatment with superoxide dismutase (Mizoe *et al.*, 1997), catalase (Okuda *et al.*, 1992), N-acetylcysteine (Dunne *et al.*, 1994), vitamin E (Nagel *et al.*, 1997), and glutathione (GSH) (Bilzer *et al.*, 1999) have been shown to possess protective potential in hepatic IRI and therefore generate a causal link between ROS formation and IRI.

Besides KC activation, sinusoidal endothelial cell death during early reperfusion represents an important pathophysiological feature of IRI (Lemasters and Thurman, 1997). Thereby, denudation of sinusoids occurs, leading to an enhanced susceptibility of hepatocytes to ROS and other inflammatory mediators aggravating tissue injury.

In addition to directly cause cell death and tissue injury, ROS generated during IRI can affect these processes indirectly by activating redox-sensitive signalling pathways, such as the transcription factors nuclear factor (NF)-κB and activator protein (AP)-1, which in turn enhance proinflammatory gene expression (Bradham *et al.*, 1997;Jaeschke, 1998;Zwacka *et al.*, 1998;Bradham *et al.*, 1999;Yamada *et al.*, 2000;Kiemer *et al.*, 2000;Tsoulfas and Geller, 2001;Ricciardi *et al.*, 2000).

The **NF-κB** transcription factor family consists of five different members, termed p50, p52, p65 (RelA), c-Rel, and RelB, which can form various homo-and heterodimers. NF-κB is normally sequestered in the cytoplasm by proteins of the IκB family including IκB-α, IκB-β, IκB-γ, and IκB-ε. The induced form of NF-κB is predominantly a p50 and p65 heterodimer, which translocates to the nucleus upon activation. The most commonly studied pathway of NF-κB activation involves phosphorylation of IκB-α on serine residues 32 and 36 by

the IκB-kinase (IKK) complex (Arrigo, 1999). This leads to ubiquitination and degradation of IκB, which thereby unmasks a nuclear target sequence on the NF-κB molecule and results in the translocation of NF-κB from the cytoplasm to the nucleus as an active transcription factor. In the early phase of IRI, activation of the proinflammatory transcription factor NF-κB may trigger upregulation of cytokines, including TNF-α, interleukin-1, and adhesion molecules, such as ICAM-1, that can mediate the subsequent subacute, inflammatory response. Expression of inducible nitric oxide synthase (iNOS)-mRNA during IRI is initiated by NF-κB activation after 1 h of reperfusion (Fan *et al.*, 1999;Tsoulfas and Geller, 2001).

The **AP-1** family is another group of redox-regulated transcription factors that has been shown to be involved in IRI. AP-1 is a collective term referring to numerous combinations of dimeric transcription factors composed of *jun*, *fos* or ATF (activating transcription factor) subunits that bind to the common DNA site, the AP-1-binding site. The AP-1 family consists of homo- and heterodimers of *jun* (v-*jun*, c-*jun*, *jun*B, *jun*D), *fos* (v-*fos*, c-*fos*, *fos*B, Fra1, Fra2) or activating transcription factor (ATF-2, ATF-3, B-ATF) proteins (Fan *et al.*, 1999;Karin *et al.*, 1997). Regulation of AP-1 activity occurs at two major levels: extracellular stimuli modulate both the expression and the activity of AP-1 proteins. The abundance of AP-1 proteins is most commonly regulated by controlling the transcription of their genes. c-*jun* and c-*fos* can also be regulated by modulation of their stability. In the case of c-*jun*, phosphorylation by c-*jun* N-terminal kinase (JNK) reduces its ubiquitination and hence its degradation. Phosphorylation of serines 63 and 73 of c-*jun* by JNK is moreover known to increase its transcriptional activity (Karin *et al.*, 1997).

The activation of both NF-κB and AP-1 during IRI has been described to lead to inflammatory liver cell damage (Ricciardi *et al.*, 2000;Jaeschke, 2000;Fan *et al.*, 1999). Recent studies even showed the direct relation between reduction of IRI in a rat liver model and blockade of NF-κB activation by the use of NF-κB antisense oligonucleotides (Banafsche *et al.*, 2001).

2.1.3 Mechanism of cell death: Apoptosis or Necrosis?

Irreversible cell damage during IRI leads to cell death. The question, whether apoptosis or necrosis prevails in IRI is controversially discussed (Kohli *et al.*, 1999;Miyoshi and Gores, 1998;Clavien *et al.*, 2001;Yadav *et al.*, 1999;Gujral *et*

al., 2001;Patel *et al.*, 1999). Although recent reports suggest that necrosis might be predominent (Gujral *et al.*, 2001;Redaelli *et al.*, 2002), both types of cell death have to be considered.

Liver cell death during IRI is triggered by a number of insults arising from the external environment or from within the cell. These insults may engage cell surface receptors with death domaines leading to a proteolytic cascade involving initiator and executioner caspases and an apoptotic demise. Alternatively, the insults may profoundly disrupt mitochondrial function and result in loss of homeostasis accompanied by activation of hydrolases and a necrotic cell death (Kaplowitz, 2000). The distinction between apoptosis and necrosis has become fluent recently by the recognition that the same stimuli can induce either form of cell death. Thereby, mitochondria play a key role: selective release of mediators, such as cytochrome c amplifies the apoptosis program and profound loss of mitochondrial function leads to necrosis (Leist and Jäättelä, 2001). ROS and NO thereby participate as initiating factors and modulators (Kaplowitz, 2000;Gabbita *et al.*, 2000;Finkel, 2001). Cell death can be described based upon morphological features as apoptotic or necrotic.

Apoptosis, a controlled unobstructive mechanism of ridding the organism of damaged or unneeded cells, appears as shrinkage, cytoplasmic and nuclear condensation and fragmentation without loss of plasma membrane integrity (Rust and Gores, 2000).

During apoptosis, a final execution phase can be distinguished from an initiation phase. In the execution phase, caspases, a family of cysteine proteases (**c**ysteinyl **asp**artate-specific prote**ases**), degrade the cell by cleavage of key proteins specifically after aspartate residues (Miller, 1997). Caspases are present in the cytosol of most cells as zymogens and need to be activated by cleavage of the proenzyme by proteolytic steps.

It is generally known that caspases are activated by other caspases. Certain caspases are called effector or downstream caspases (e.g. 3, 6, 7), because they cleave key substrates, leading to apoptotic cell death (Cohen, 1997;Sartorius *et al.*, 2001;Rust and Gores, 2000).

When initial liver cell damage is too severe, cells are no longer able to control and perform the tightly regulated and also energy-dependent apoptotic processes. This means that necrotic cell death appears. **Necrosis** involves

swelling and loss of plasma membrane integrity leading to lysis. The rapid phagocytic removal of apoptotic cells and fragments minimizes inflammation (Jaeschke, 2002), whereas the release of cellular content in necrosis promotes secondary inflammation. The most important feature of necrosis is that numerous mitochondria within a cell collapse and ATP production therefore tremendously declines. In consequence, cells swell, losing their ability to maintain ion gradients and calcium-dependent, nonspecific hydrolases start the disintegration process, ending in cell lysis (Kaplowitz, 2000).

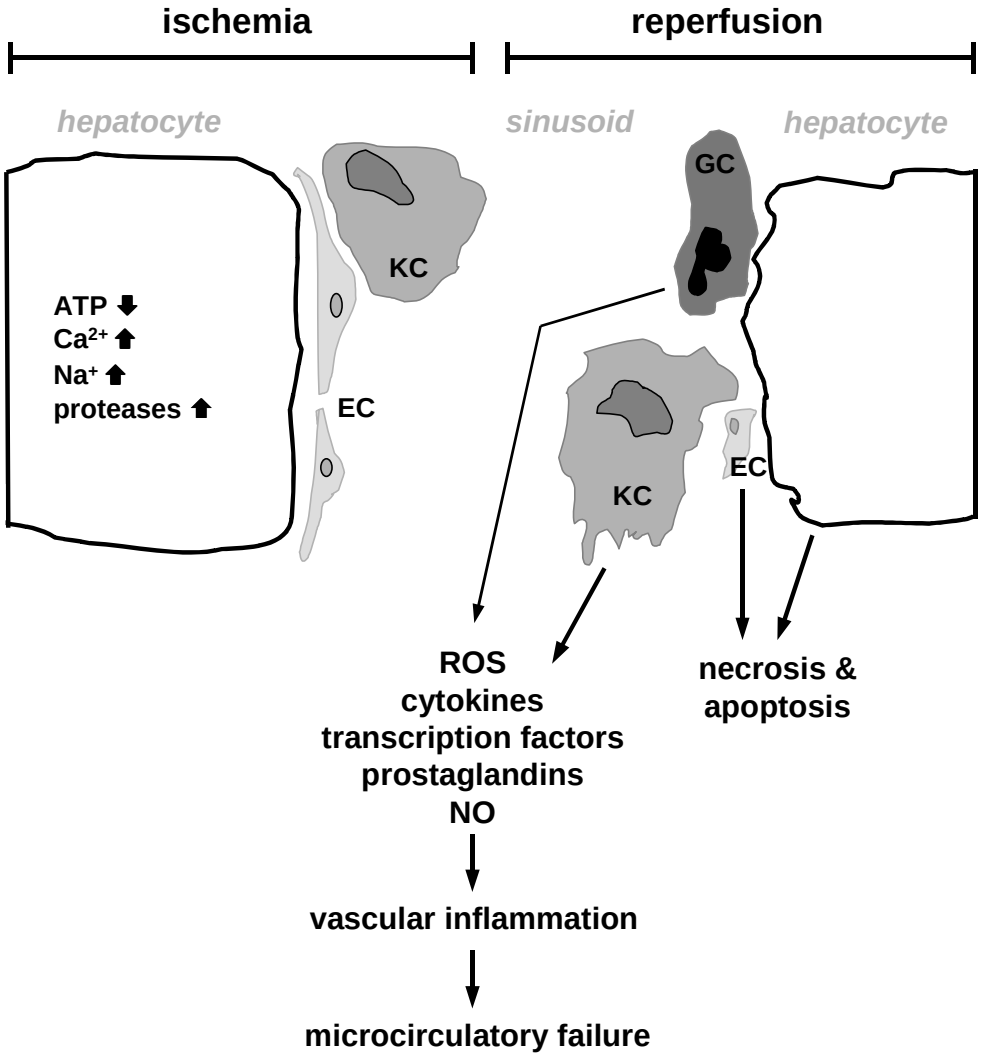


Figure 1: schematic illustration of the main pathomechanisms of hepatic IRI. *Endothelial cell (EC), Kupffer cell (KC), granulocyte (GC), reactive oxygen species (ROS), nitric oxide (NO).*

2.1.4 Therapeutic approaches

Many strategies have been developed to reduce IRI. Established proceedings thereby represent the use of University of Wisconsin (UW)- and other solutions for cold storage of livers prepared for transplantation. As protective ingredients in the UW-solution, lactobionate and glutathione were identified. Lactobionate was shown to reduce cell swelling during organ storage, due to marked osmotic properties (Bilzer M., 1997). The underlying mechanism of protective GSH effects remain unclear, but an antioxidative action was suggested (Belzer *et al.*, 1990). Beyond that, the xanthine oxidase inhibitor allopurinol, the Ca^{2+}-antagonist nicardipine, and the protease-inhibitor glycine were shown to exert protective effects when added to storage solutions.

Besides interventions during organ storage, pre- and postischemic pharmacological therapies and also surgical interventions have been investigated to reduce IRI. Among them are: treatment with antioxidants, e.g. GSH, superoxide dismutase, katalase, N-acetylcysteine, etc. (Dunne *et al.*, 1994;Jaeschke and Farhood, 1991;Okuda *et al.*, 1992;Mizoe *et al.*, 1997;Nagel *et al.*, 1997;Clavien *et al.*, 1992;Bilzer *et al.*, 1999), use of the cardiovascular hormone atrial natriuretic peptide (ANP) (Bilzer *et al.*, 1994;Gerbes *et al.*, 1998;Kiemer *et al.*, 2000;Kiemer *et al.*, 2002), and ischemic preconditioning.

The term ischemic preconditioning was introduced in 1986 by Murry and co-workers (Murry *et al.*, 1986). In this classical study the authors referred to ischemic preconditioning as an adaption of the myocardium to ischemic stress induced by repetitive short periods of ischemia and reperfusion. Meanwhile, the finding of an intrinsic protective property of the myocardium has been confirmed for other organs. Several years after characterization of ischemic preconditioning in the heart its beneficial effects were recognized in hepatic IRI after both warm (Peralta *et al.*, 1997) and cold ischemia (Arai *et al.*, 1999;Yin *et al.*, 1998). Ischemic preconditioning attenuated sinusoidal endothelial cell death and decreased ROS formation by Kupffer cells (Arai *et al.*, 1999). The very promising results reducing IRI by ischemic preconditioning of organs lead to numerous studies about the underlying mechanisms. Besides affecting adenosine and early NO formation during reperfusion, especially kinases, such as p38 mitogen activated protein kinase (p38 MAPK) were described to mediate ischemic preconditioning (Iesalnieks *et al.*, 2001;Abe *et al.*, 2000;Maulik *et al.*, 1998;Ping and Murphy, 2000;Nakano *et al.*, 2000;Ono and Han, 2000).

In this context, it has been reported that ischemic preconditioning activates p38 MAPK and blocking its activation by simultaneous administration of specific p38 MAPK inhibitors abrogated the effect of hepatic and cardiac ischemic preconditioning (Amersi *et al.*, 2002;Cohen *et al.*, 2000;Weinbrenner *et al.*, 1997;Ping and Murphy, 2000;Maulik *et al.*, 1998;Abe *et al.*, 2000) and hypoxic preconditioning of hepatocytes (Carini *et al.*, 2001).

Mitogen activated protein kinases (MAPK) are highly conserved serine/threonine kinases, which are activated in response to a wide variety of stimuli including growth factors and environmental stresses (e.g. ischemia and reperfusion). Three major MAPK family members have been extensively studied: extracellular signal-regulated kinases (ERK1 and ERK2), c-*jun* N-terminal kinase (JNK1 and JNK2), and **p38 MAPK**. MAPK are activated by protein kinase cascades that contain at least two upstream kinases. Dual phosphorylation is necessary for each MAPK to become fully activated (Chen *et al.*, 2001).

Various stressors, such as ischemia and reperfusion, hypo-osmolarity, etc. activate the p38 MAPK cascade. MEK3 and MEK6 (MEK=MAP/ERK kinase, also known as MAP kinase kinases or MKKs) are thought to be the major upstream kinases responsible for p38 activation, whereas MEK3/6 themselves are phosphorylated by MEKK (MAP kinase kinase kinases). Downstream events of p38 MAPK comprise regulation of AP-1 activity by induction of c-*fos* and c-*jun* and the activation MAPKAP kinase-2, which in turn is able to phosphorylate heat shock protein 27 (Chen *et al.*, 2001).

Another kinase, **Akt** (protein kinase B), has been reported to possess cytoprotective potential and being involved in ischemic preconditioning of the rat heart (Tong *et al.*, 2000). Thereby, a causal relation between Akt activation and reduction of IRI by ischemic preconditioning was described.

The serine/threonine kinase Akt is regarded as a key mediator of the physiological effects of insulin, of several growth factors (e.g. platelet-derived growth factor), and plays a crucial role in protecting cells from apoptosis (Leslie *et al.*, 2001). The main downstream targets of Akt represent glycogen synthase kinase, the pro-apoptotic BAD, and caspase-9. Furthermore, Akt has been found to stimulate glucose uptake, glucose transporter (GLUT)1, and GLUT4 translocation. GLUT1/4 represent insulin responsive glucose transporter isoforms, which are translocated from intracellular compartments to the plasma

membrane in response to insulin. After different growth stimuli, such as insulin, epidermal growth factor, or nerve growth factor, etc. Akt becomes activated as follows: activated phosphatidylinositol-3-kinase (PI3-K) produces phosphatidylinositol (3, 4, 5) triphosphate (PIP_3), which is necessary for Akt translocation from the cytosol to the plasma membrane. There, Akt becomes phosphorylated twice, at Thr308 by PIP_3-dependent kinase (PDK)-1 and at Ser 473 by PDK-2 (Downward, 1998;Chan *et al.*, 1999).

2.1.5 Experimental model

To study hepatic ischemia reperfusion injury (IRI), several approaches and techniques have been established. In this work the isolated perfused rat liver system was applied.

This system represents a commonly used tool for exploring the physiology and pathophysiology of the liver (Gores *et al.*, 1986) and is widely used in transplantation research (Dahl S., 1997) due to the following advantages:

The method allows without much special equipment to set up a competent and versatile system of liver perfusion, maintaining its metabolic capacity and sensitivity towards different stimuli, e.g. hormones, for experimental periods of 2-5 hours (Ahmed *et al.*, 2001;Dahl S., 1997). Its popularity is due to the fact that, in contrast to *in vivo* models, the isolated perfused rat liver allows repeated sampling of perfusate, permits easy exposure of the liver to different concentrations of test substances and is amenable to alterations in temperature that would not be workable *in vivo*. Furthermore and most importantly, examining molecular mechanisms of substances, experiments can be done independently from the influence of other organ systems, plasma constituents and neural-hormonal effects (Bilzer M., 1997) focussing on liver-specific mechanisms.

In contrast to other *in vitro* models, such as primary hepatocytes or cell organelles, hepatic architecture, communication between different liver cells, and bile flow are preserved in the isolated perfused rat liver (Gores *et al.*, 1986;Ahmed *et al.*, 2001;Dahl S., 1997).

Therefore, the isolated perfused rat liver system represents a suitable model to evaluate possible modulatory or hepatoprotective properties of pharmacological and surgical interventions in IRI.

3. α-Lipoic acid

3.1 History

In 1937, Snell and collegues reported about a nutritional factor extracted from potatoes, which was required for the growth of Lactobacillus, this factor was dubbed the ”potatoe growth factor” (Snell E.E. *et al.*, 1937). Later research described an “acetate replacing factor” and a “pyruvate oxidation factor” in yeast extracts. Snell and Broquist proposed in 1949 that all of these factors were probably the same compound. In 1951 Reed and co-workers purified pale yellow crystals of a compound called “α-lipoic acid” (Reed L.J. *et al.*, 1951). 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 2) and represents an eight-carbon disulfide containing a chiral carbon, therefore existing in two enantiomeric forms. The R-enantiomer is the naturally occuring form, whereas synthetic α-lipoic acid is a 1:1 racemic mixture of the S- and R-enantiomers.

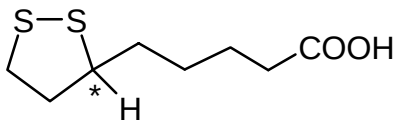


Figure 2: α-lipoic acid.

LA was initially classified as a vitamin after its isolation, but it was later found to be synthesized by plants, animals and humans (Carreau, 1979;Gueguen *et al.*, 2000). In a recent study a mouse cDNA was cloned, characterized and demonstrated that it encodes a lipoic acid synthase located in mitochondria (Morikawa *et al.*, 2001). Although it is known that octanoic acid and a sulfur source are the precursors of LA, the complete way of its biosynthesis has not yet been elucidated, (Gueguen *et al.*, 2000;White, 1980;Dupre *et al.*, 1980).

3.2 Physiological functions

3.2.1 α-Keto acid dehydrogenases

As lipoamide, LA is covalently bound to the amino group of lysine residues and functions as a prosthetic group in the α-keto dehydrogenase complexes. This multienzyme complex is composed of multiple copies of three enzymes: the α-keto acid (pyruvate, branched chain α-keto acid, or α-ketoglutarate) dehydrogenase or E_1, the dihydrolipoyl acyltransferase or E_2, and the dihydrolipoyl dehydrogenase or E_3. These three enzymes catalyze five reactions that oxidatively decarboxylate their substrates. Lipoamide is involved as a moiety of E_2 to which an acyl group is attached, transferring it from thiamine pyrophosphate (TPP) on E_1 to coenzyme A (CoA) to produce acyl-CoA. In the process lipoamide is reduced, with its disulfide linkage broken. E_3 is the enzyme that reoxidizes the lipoamide for another round of catalysis using NAD^+, which is converted to NADH (Figure 3) (Packer *et al.*, 1997;Packer, 1998). Lipoamide therefore plays a major role in energy delivering processes of carbon hydrate and protein catabolism by importing their degradation product acetyl-CoA in the citrate cycle.

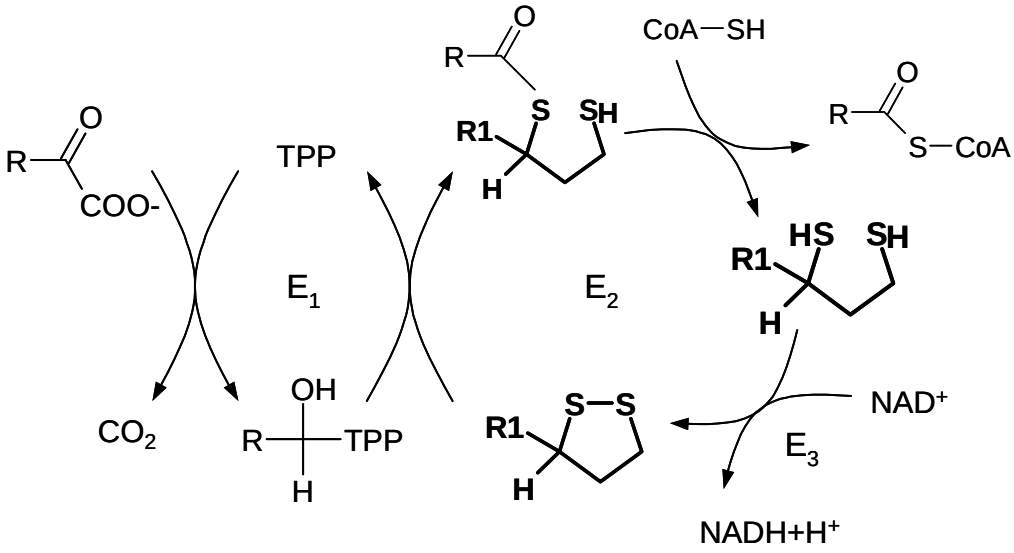


Figure 3: role of LA in oxidative decarboxylation of α-keto acids. E_1: α-keto acid dehydrogenase; E_2: dihydrolipoyl acyltransferase; E_3: dihydrolipoyl dehydrogenase.

3.2.2 Glycine cleavage system

The glycine cleavage system is a multienzyme complex that is only located in the hepatic mitochondrial matrix, catalyzing the oxidation of glycine to CO_2, and ammonia, forming NADH and 5,10-methylenetetrahydrofolate. Thereby, α-lipoate is attached to a lysine in the so called H-protein and is involved in the transfer of the methylamine moiety formed after oxidative decarboxylation of glycine (Packer *et al.*, 1997). Bound LA is again reduced during the transfer (see Figure 3) of the one-carbon group from the lipoyl residue of H-protein to tetrahydrofolate (Bustamante *et al.*, 1998).

3.3 Pharmacokinetiks of LA

3.3.1 LA absorption

After oral administration, LA is rapidly absorbed, reaching maximum plasma concentrations of approximately 2.4 µM (200 mg p.o.) and 7.3 µM (600 mg p.o.) after approximately 0.5-1 h in fasted persons (Breithaupt-Grogler *et al.*, 1999;Menke G., 1995;Preiß R. *et al.*, 1996;Hermann R. *et al.*, 1996;Gleiter *et al.*, 1996). Bioavailability of LA is indicated as ca. 29%, but is also dependent on food uptake, i.e. ingestion results in decreased plasma values (Teichert *et al.*, 1998b;Gleiter *et al.*, 1996). LA possesses a half-life period ($t_{1/2}$) of 30 min (Teichert *et al.*, 1998).

Only one report exists in the literature about the particular kinetic of LA uptake in rat liver. This work describes that LA in lower concentrations (< 75 µM) is absorbed by a saturable and in higher LA concentrations by a nonsaturable kinetic. Therefore, it is concluded that LA uptake at low concentrations is carrier-mediated. At higher concentrations diffusion becomes the major pathway (Peinado *et al.*, 1989).

3.3.2 LA metabolism

3.3.2.1 Reduction of LA

After being absorbed, exogenously applied LA enters the mitochondrial matrix, and dihydrolipoamide dehydrogenase (E_3, see B.3.2) reduces LA to DHLA (Figure 4), using electrons from NADH (Haramaki *et al.*, 1997;Handelman *et al.*, 1994).

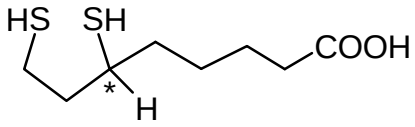


Figure 4: dihydrolipoic acid.

Surprisingly, reduction of LA was also observed in human erythrocytes, which lack mitochondria. In the erythrocyte system, it was found that glutathione reductase, which exists mainly in the cytosol, is responsible for this NADPH dependent LA reduction (Constantinescu *et al.*, 1995). In rat liver, reduction occurs equally by glutathione reductase and by dihydrolipoamide dehydrogenase (Packer, 1998). In addition, it has recently been found that thioredoxin reductase, which catalyzes the NADPH-dependent reduction of thioredoxin, reduces LA more efficiently than dihydrolipoamide dehydrogenase. The biological significance of this pathway is as yet unknown, but it represents another route of LA reduction in tissues.

3.3.2.2 β-Oxidation

Another metabolic event of lipoic acid is the β-oxidation of its pentanoic acid side chain. After administration of [1,6-^{14}C]-lipoate to rats, the following LA metabolites were identified in urine: bisnorlipoic acid, tetranorlipoic acid, β-hydroxybisnorlipoic acid, β-ketolipoic acid, and β-ketobisnorlipoic acid (Figure 5) (Spence and McCormick, 1976). In human beings, the metabolism is only poorly documented. β-Oxidation products, especially bisnorlipoic acid, were also detected in human plasma. In urine, the main metabolite was S^4,S^6-dimethylbisnorlipoic acid (Figure 5), indicating a further metabolizing step before metabolites are excreted into urine (Locher M. *et al.*, 1998;Biewenga *et al.*, 1997).

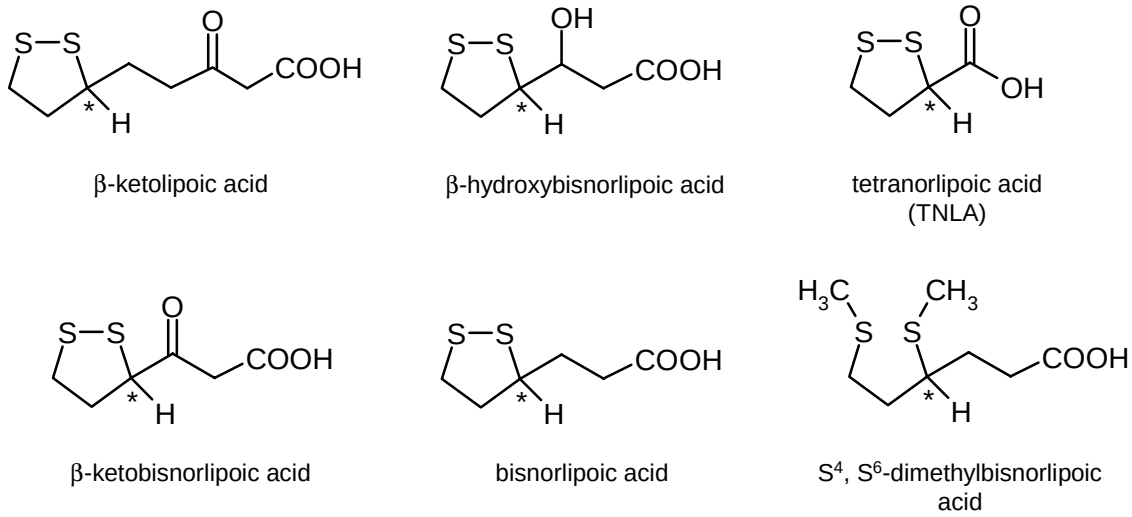


Figure 5: main metabolites of LA.

Very recent findings confirmed the mentioned S-methylation adducts of LA and even presented a new metabolite, the disulfoxide of S^2,S^4-bismethylmercapto-butanoic acid, which was identified as the major metabolite in dogs (Figure 6) (Schupke *et al.*, 2001).

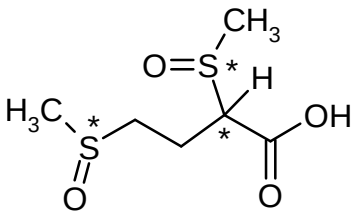


Figure 6: major LA metabolite in dogs, the disulfoxide of S^2,S^4-bismethylmercapto-butanoic acid.

3.3.3 LA toxicity

Acute toxicity of LA is very low and represents approx. 400 mg/kg after intravenous administration to rats (Hexal AG, 1998).

3.4 Therapeutic use in diabetic polyneuropathy

Diabetic polyneuropathy represents a major health problem, as it is responsible for substancial morbidity, increased mortality, and impaired quality of life. Near-normoglycaemia is now generally accepted as the primary approach to prevent diabetic polyneuropathy, but is not achievable in a considerable number of patients. The therapeutic efficacy and safety of LA in diabetic polyneuropathy has been intensely investigated. Thus far, 15 clinical trials have been completed using different study designs, durations of treatment, doses, sample sizes, and patient populations (Ziegler *et al.*, 1999). Within this variety of clinical trials, those with beneficial effects of LA used doses of at least 600 mg per day. The following major conclusions can be drawn from the recent controlled clinical trials:

1.) Short term treatment for 3 weeks using 600 mg i.v. per day reduced the chief symptoms of diabetic polyneuropathy (Ziegler *et al.*, 1995).

2.) Oral treatment for 4-7 months tends to reduce neuropathic deficits and improves cardiac autonomic neuropathy (Ziegler and Gries, 1997;Ziegler *et al.*, 1999).

3.) LA (1,200 mg p.o. for 4 weeks) lowered plasma glucose levels in patients with type II diabetes (Konrad *et al.*, 1999).

4.) Preliminary data over 2 years indicate possible long-term improvement in motor and sensory nerve conduction in the lower limbs (Reljanovic *et al.*, 1999).

5.) Clinical and postmarketing surveillance studies have revealed a highly favourable safety profile of the drug (Coleman Michael D., 2001).

6.) A pivotal long-term multicenter trial (NATHAN I study) of oral treatment with LA is being conducted in North America and Europe using a clinically meaningful and reliable primary outcome measure that combines clinical and neurophysiological assessment.

Hyperglycaemia, microangiopathy, and excessive oxidant stress are recognized causes of nerve damage in diabetic states. Recently, some protective mechanisms of LA were suggested in this context.

Besides its antioxidant activity, described in detail in B.3.5, LA has been shown to be involved in signal transduction processes concerning glucose metabolism. First, LA was described to stimulate glucose uptake in different cells (e.g. adipocytes, muscle cells) in an insulin-like manner by rapid translocation of the glucose transporters (GLUT)1 and GLUT4 from an internal membrane fraction to the plasma membrane (Moini *et al.*, 2002;Yaworsky *et al.*, 2000;Rudich *et al.*, 1999). Thereby, enhanced phosphatidylinositol-3-kinase (PI3-K)- as well as Akt-activity were suggested responsible for this LA effect. Konrad *et al.* (Konrad *et al.*, 2001a) reported that also p38 MAPK is involved in insulin- and LA-dependent stimulation of glucose uptake. This study also confirmed the unique pathway of LA and insulin in the increase of glucose uptake *via* GLUT4 translocation, and showed a causal link to forced PI3-K- and Akt-activity. It was described that also p38 MAPK activity is increased by LA- and insulin-treatment. Concerning the role of kinase activation, activated p38 MAPK was proposed to stimulate GLUT4 activation, but not GLUT4 translocation. The latter was suggested to be mediated by the PI3-K/Akt pathway.

3.5 Further properties

Away from its use in the therapy of diabetic disorders, exogenous administration of LA has been shown to exert other interesting effects in different pharmacological topics and models. This chapter will especially focus on described LA effects touching this work.

3.5.1 ROS-scavenging capacity and metal chelation

There is general agreement about the antioxidant properties of LA. Many, mostly *in vitro* studies show that LA scavenges hydroxyl radicals, hypochlorous acid, peroxynitrite, and singlet oxygen. It does not appear to scavenge hydrogen peroxide or superoxide radical and probably does not scavenge peroxyl radicals (Suzuki *et al.*, 1991;Biewenga *et al.*, 1997;Biewenga and Bast, 1995;Packer *et al.*, 1995;Packer and Tritschler, 1996;Packer, 1998). DHLA, the reduced form of LA, additionally catches superoxide radical. Metabolites of LA, such as bisnorlipoic acid, β-hydroxynorlipoic acid, and tetranorlipoic acid are also described exerting antioxidative properties (Biewenga *et al.*, 1997).

Besides its direct radical scavenging action LA may have an antioxidant effect in biological systems through transition metal chelation. Antioxidant activity

thereby is obtained when a complex is formed in which the metal is shielded and coordination sites for O_2 are occupied. It has been found that LA forms stable complexes with Mn^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+}, with the complex being almost entirely formed with the carboxylate group (Biewenga *et al.*, 1997;Packer, 1998). Furthermore, LA was described to reduce Cd^{2+}-induced toxicity in isolated hepatocytes, although the authors speculated that the effect was due to the conversion of LA to DHLA, which was the true chelating agent (Packer *et al.*, 1995).

3.5.2 Influence of LA on GSH metabolism

Increase in cellular GSH synthesis after LA administration has been reported for different cell lines, such as Jurkat T cells, human erythrocytes, glial cells, neuroblastoma cells, lymphocytes, and rat and mouse liver (Han *et al.*, 1995;Han *et al.*, 1997;Busse *et al.*, 1992;Arivazhagan *et al.*, 2001). The most detailed findings concerning the mechanism of LA increasing *de novo* synthesis of GSH were suggested by Han *et al.* as follows:

A prerequesite for the obtained result is the low redox potential of the DHLA/LA redox couple of -0.32 V, which is more negative than the potential of cysteine/cystine (-0.22 V). Therefore, DHLA is able to directly reduce cystine to cysteine, which is regarded as the limiting factor of glutathione synthesis (Lu, 1999).

Thus, after adding LA to the cells, it is quickly absorbed and reduced to DHLA by the enzyme systems already mentioned in B.3.3.2.1, which is released to the extracellular space. DHLA subsequently reduces cystine to cysteine, which is in turn taken up again by the cells 10-times faster than cystine, and is therefore immediately available for GSH synthesis (Figure 7). An important note is that simple reduction of present GSSG to GSH by DHLA could not explain the significant effects obtained, due to only small intracellular amounts of GSSG (Han *et al.*, 1997).

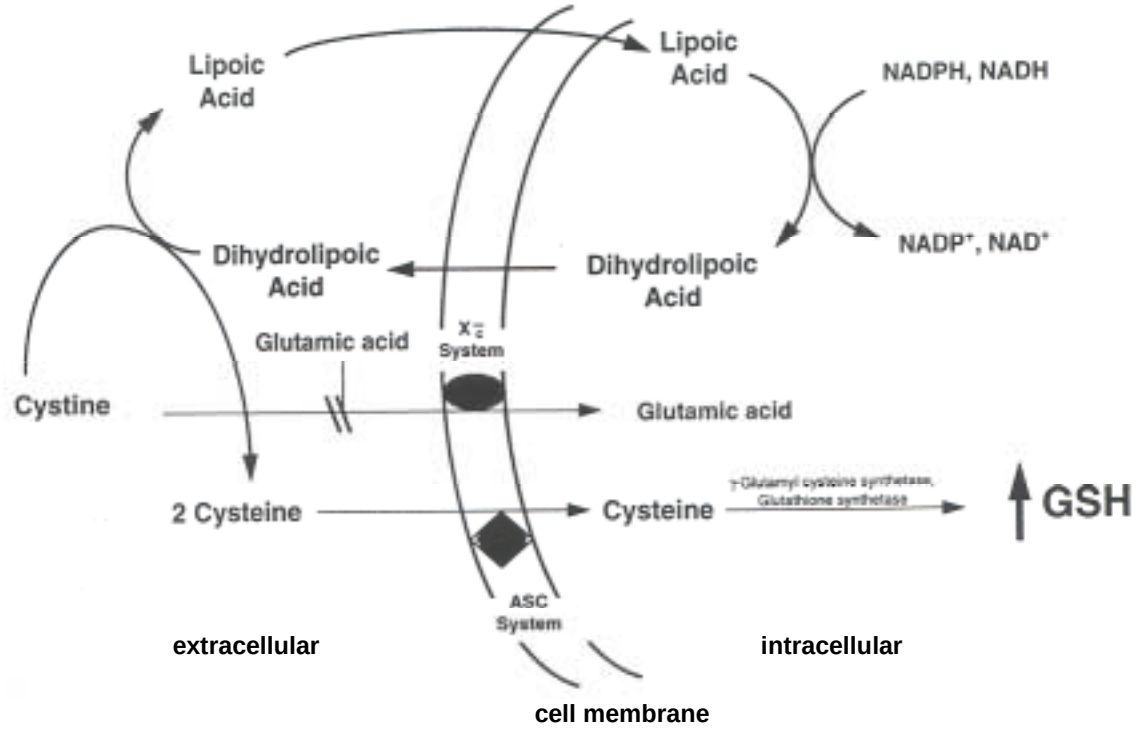


Figure 7: modified illustration of transport and reducing systems involved in intracellular GSH synthesis and possible mechanism for its stimulation by exogenously added LA (Han* et al.*, 1997).

3.5.3 Inhibition of NF-κB

There are several lines of evidence that ROS are involved in activation of NF-κB (see B.2.1.2) (Arrigo, 1999;Tsoulfas and Geller, 2001). Many researchers have reported antioxidants, including 2-mercaptoethanol, GSH, vitamin C, L-cysteine, DHLA, and LA to inhibit NF-κB activation (Saliou *et al.*, 1999;Suzuki *et al.*, 1992;Packer and Suzuki, 1993;Packer, 1998). Activation of NF-κB by antioxidants is interrupted by scavenging ROS, which represent the initiating stimulus for I-κB phosphorylation and therefore translocation of NF-κB into the nucleus (see B.2.1.2).

3.5.4 Influence of LA on ATP metabolism

LA was described to modulate the availability of energy equivalents in diabetic rat hearts (Strodter *et al.*, 1995) and in rat heart mitochondria (Zimmer *et al.*, 1995). As possible mechanisms for this LA effect reduced ATPase activity (LA and DHLA) and an increase in ATP synthase activity (especially DHLA) were

suggested (Zimmer *et al.*, 1991).

3.5.5 Involvement of LA in liver diseases

LA has been intensely investigated as a therapeutic agent in a number of conditions related to liver disease, including alcohol-induced damage, mushroom poisoning, Cd^{2+}-intoxification, CCl_4 poisoning, and hyperdynamic circulation in biliary cirrhosis (Cohen M.R. *et al.*, 1971;Muller and Menzel, 1990;Bludovska *et al.*, 1999;Vancini B., 1959;Marley *et al.*, 1999;Bustamante *et al.*, 1998). Beyond the antioxidative properties of LA, however, few is known about potential cellular and molecular targets of LA in liver disease.

In summary, the pharmacological profile of LA lead to the hypothesis that LA might protect from hepatic IRI.

C. Materials & Methods

1. Materials

1.1 Chemicals

Racemic α-lipoic acid (LA) was a gift from ASTA Medica (Batch No. 9811071, purity: 100%, Frankfurt/Main, Germany). All other materials were purchased from either Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany) and had the quality of “pro analysi”, if not stated otherwise.

1.2 Animals

Male Sprague-Dawley rats were purchased from Charles River (Sulzfeld, Germany) and housed in a temperature- and humidity-controlled room under a constant 12-hour light-dark cycle. All perfusions were performed with rats weighing 220-280 g, having free access to chow (Ssniff-Diet, Ssniff Spezialdiäten GmbH, Soest, Germany) and water up to the time of the experiments. The study was registered with the local animal welfare committee.

2. Methods

2.1 Isolated perfused rat liver system

2.1.1 Procedure

Rats were anaesthetized with Narcoren® (sodium pentobarbital, 50 mg/kg body weight, i.p., Merial GmbH, Hallbergmoos, Germany), 0.1 ml heparine-sodium (25000 I.U./5 ml, Braun-Melsungen AG, Melsungen) was injected into the *vena femoralis* to prevent blood clotting. After incision of the abdominal wall, *vena portae* was cannulated with a 14-gauge intravenous catheter and the liver was perfused at a constant flow rate of 2.6 - 3.4 ml x min^{-1} x (g liver)$^{-1}$ (membrane pump: Prominent® beta/4). Then *vena cava inferior* was cannulated *via* the right

atrium and ligated above the right *vena renalis*. After cannulating the bile duct with polyethylene-10 tubing, the liver was dissected from the gastrointestinal tract to exclude any nerval interaction ("isolated perfused rat liver").

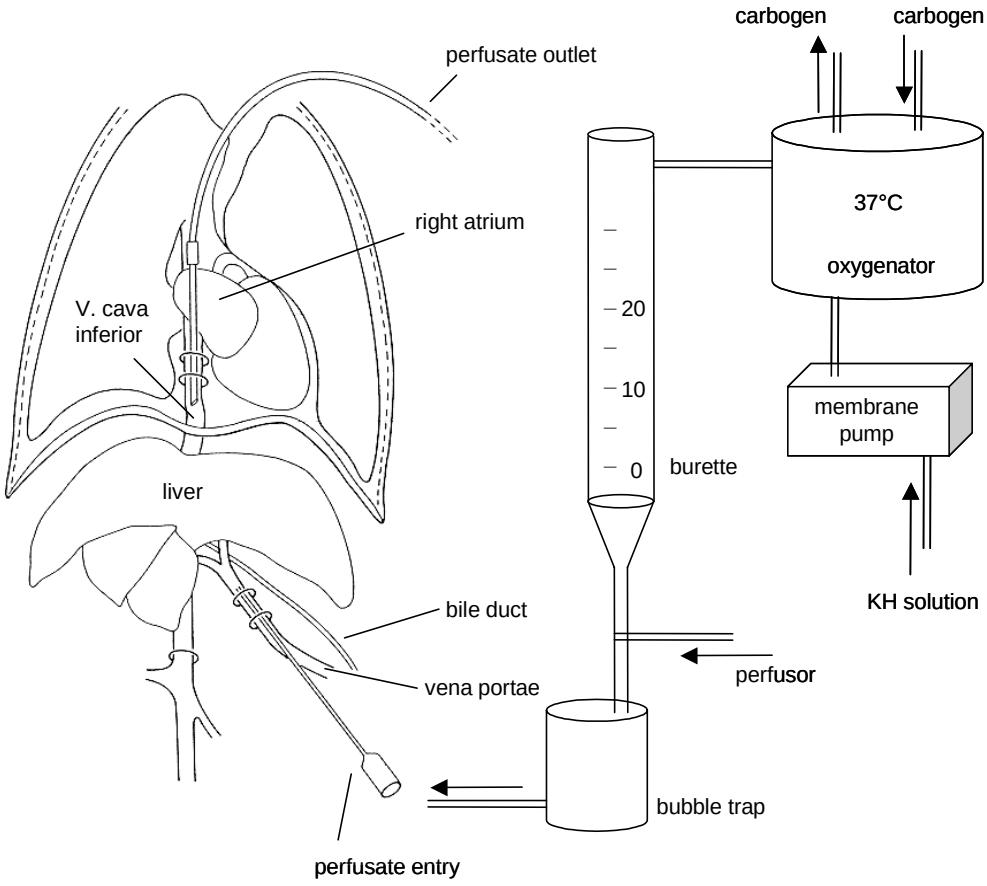


Figure 8: isolated rat liver perfusion technique - modified schematic description of the system (Lindl T., 1994).

In all experimental groups, livers were perfused with hemoglobin- and albumin-free, Krebs-Henseleit (KH) solution (pH 7.4, 37°C) in a nonrecirculating fashion. The perfusion medium was warmed and gassed with Carbogen (95% O_2 and 5% CO_2, Messer Griesheim GmbH, Krefeld, Germany) by a tube oxygenator. Substances were infused *via* perfusors® (Braun Melsungen AG, Melsungen, Germany) into the KH solution before entering a bubble trap on the way to the liver. Perfusate and bile were collected during perfusion, whereas perfusate was immediately cooled on ice. Bile ran directly into 1 ml tubes and was determined volumetrically every 5 min with a Hamilton® syringe as µl x min^{-1} x (g liver)$^{-1}$ (Hamilton Bonaduz AG, Bonaduz, Switzerland). Portal pressure was read from a burette, which served as hydrostatic column (Sies, 1978;Wolkoff *et al.*, 1987). It was determined every 5 min throughout the whole perfusion time and was

expressed as cm water as described previously (Wolkoff *et al.*, 1987;Gores *et al.*, 1986;Bilzer *et al.*, 1999;Bilzer *et al.*, 1994).

At the end of the perfusion the liver was dissected and measured pressure was subtracted from the pressure during reperfusion. A schematic description of the perfusion apparatus and the operative procedure is shown in Figure 8.

Krebs-Henseleit solution (KH, pH 7.4):

NaCl	118 mM
KCl	4.8 mM
KH_2PO_4	1.2 mM
$MgSO_4$ x 7 H_2O	1.2 mM
$NaHCO_3$	25 mM
$CaCl_2$ x 2 H_2O	1.5 mM

The solution was prepared with dist. water.

2.1.2 Continuous liver perfusion

The perfusion model was established according to Bilzer (Bilzer M., 1997). In order to characterize base levels of the applied parameters, continuous perfusions were performed.

In continuous liver perfusions, control livers were perfused with KH solution for 120 min. In LA treated livers a stock solution of LA (1 mg/ml KH solution) was infused by a perfusor® at a constant flow rate of 3.7 ml/h to reach a final concentration of 10 µM LA in the perfusing KH solution.

LA stock solution:

10.0 mg LA were dissolved in 10.0 ml KH solution using an ultrasonic bath (~15 min). The solution was prepared freshly each time due to photo instability of LA in aqeous solutions and filtered (0.2 µm) into perfusor® syringes (Braun Melsungen AG, Melsungen, Germany).

2.1.2.1 Experimental protocol

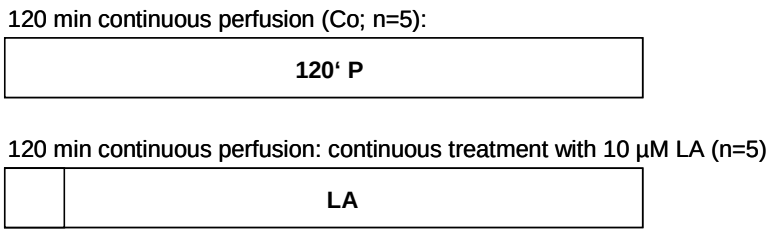


Figure 9: livers were perfused for 120 min in absence (Co) or presence of 10 µM LA, which was added continuously after a 10 min equilibration time of the perfusion system.

2.1.3 Short time infusion of H_2O_2

Continuous perfusions were performed with a short time infusion (15 min) of H_2O_2 with a final concentration in the KH medium of 500 µM (see Figure 10).

H_2O_2 stock solution:

1.0 ml of H_2O_2 was diluted with 65.0 ml of dist. water and filled into a perfusor® syringe (Braun Melsungen AG, Melsungen, Germany).

2.1.3.1 Experimental protocol

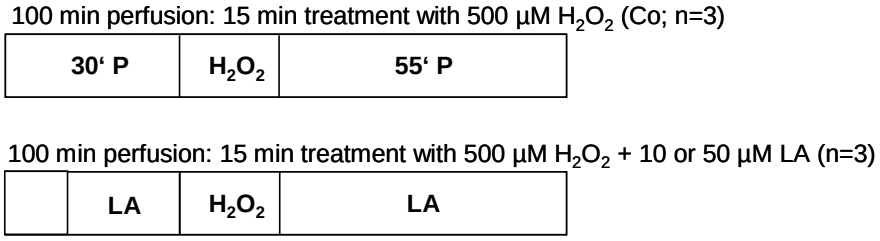


Figure 10: livers were perfused for 100 min with KH solution in absence (Co) or presence of LA (10 or 50 µM), which was added continuously, starting 20 min prior to a 15 min lasting infusion of 500 µM H_2O_2.

2.1.4 Warm ischemia and reperfusion

The following different treatment protocols were performed to study the influence of LA on IRI of the rat liver after 60 min of warm ischemia.

Livers were perfused for 30 min with KH solution (Pre-I), then perfusion was stopped for 60 min keeping the livers at 37°C. This period of warm ischemia (WI) was followed by up to 90 min of reperfusion (R). Three different schemes administering LA (10, 50 or 100 µM) were distinguished: *“Continuous application”*, i.e. applying LA continuously starting 20 min prior to ischemia,

"preconditioning" by infusing LA only 20 min prior to ischemia and *"postischemic treatment"*, i.e. LA administration during the reperfusion period (see Figure 11).

The role of PI-3 kinase in LA preconditioning was investigated by simultaneous application of the selective PI-3 kinase inhibitor wortmannin (WM 100 nM, Alexis Biochemicals, Grünberg, Germany).

2.1.4.1 Experimental protocol

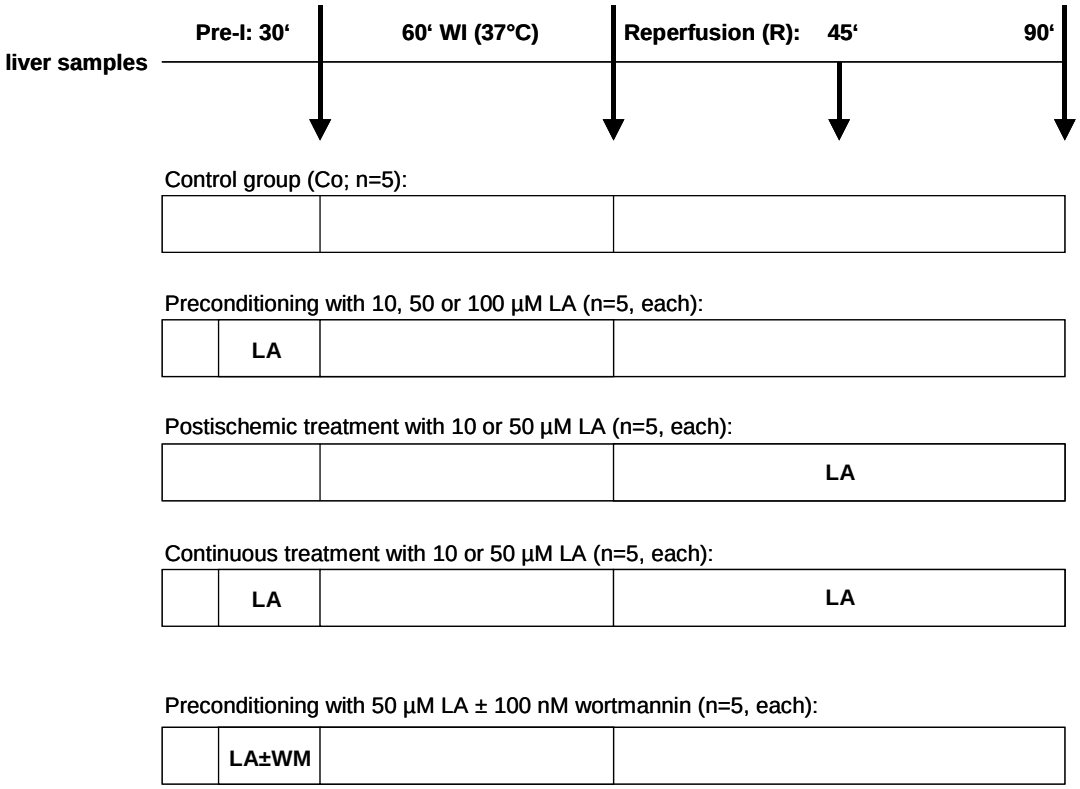


Figure 11: experimental protocol. *Livers were perfused for 30 min (Pre-I). After 60 min of ischemia at 37°C (WI), livers were reperfused (R) for 45 or 90 min. Thereby, three different LA treatment protocols were distinguished: "continuous application", i.e. administration of 10 or 50 µM LA continuously starting 20 min prior to ischemia. "Preconditioning", applying 10, 50 or 100 µM LA 20 min prior to ischemia and "postischemic application", by infusing 10 or 50 µM LA during reperfusion. In additional experiments, livers were perfused for 30 min in the presence or absence of 100 nM wortmannin (WM) with or without 50 µM LA, which were given 20 min prior to ischemia. After ischemia (WI, 60 min) livers were reperfused for 90 min. Liver samples were taken at the time points indicated by arrows.*

WM stock solution:

1 mg WM (Alexis Biochemicals, Grünberg, Germany) was dissolved in 1 ml DMSO (100%) and aliquots were stored at -20°C until the day of the experiment. 50 µl of the aliquot were diluted with 11.62 ml of KH solution, resulting in a final DMSO concentration of lower than 0.5%. This stock solution was filled into a perfusor® syringe.

2.1.5 Sample preparation

2.1.5.1 Liver tissue

At the end of perfusion livers were dissected, weighed in a wet state, and snap frozen in liquid nitrogen. After reducing the livers to small pieces in liquid nitrogen they were stored at -80°C until further analysis.

2.1.5.2 Perfusate

Perfusate samples were collected at different perfusion or reperfusion time points for immediate determination of lactate dehydrogenase (LDH) and purine nucleoside phosphorylase (PNP) activities or stored at -80°C for further examination.

2.2 Determination of enzyme activities

In a photometric enzyme activity assay the decrease or increase of an absorbing substance, i.e. change of absorbance versus time is recorded. The slope of the resulting curve is proportional to the enzyme activity, which is calculated out of these data.

2.2.1 Lactate dehydrogenase (LDH) activity

The principle of this assay is the LDH catalyzed conversion reaction of pyruvate to lactate. Thereby, NADH is oxidized to NAD^+, resulting in a NADH decrease, which is measured photometrically (Lambda Bio 20 photometer, Perkin Elmer) according to Bergmeyer (Bergmeyer HU, 1974). For calculation of LDH activity the extinction coefficient of NADH was used ($\varepsilon_{365\ nm}$ = 3.34 L x $mmol^{-1}$ x cm^{-1}). Enzyme activities are expressed as mU x min^{-1} x $(g\ liver)^{-1}$.

2.2.1.1 Reaction

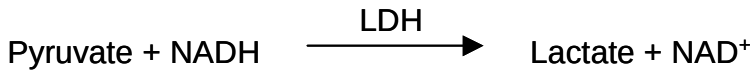


Figure 12: LDH-catalyzed reaction of pyruvate to lactate.

2.2.1.2 Solutions

Phosphate buffer:

K_2HPO_4 (8.74 g/L) and KH_2PO_4 (6.8 g/L) solutions were mixed until a pH of 7.5 was reached. 66 mg of pyruvate per L buffer were added.

NADH solution:

10 mg of NADH-Na_2 were dissolved in 1 ml of 0.5% of a $NaHCO_3$ solution.

2.2.1.3 Assay procedure

The following solutions were pipetted into volume-reduced cuvettes (1.5 ml, PESKE, Aindling-Pichl, Germany). The measurement was started after a short equilibration time (~30 s). Absorbance was monitored for 1 min at room temperature (RT).

	Reference:	**Sample:**
Phosphate buffer:	1,000 µl	500 µl
Perfusate:	----	500 µl
NADH solution:	----	10 µl

In cases of high LDH activity (e.g.: 2’ R) 1:10 dilutions of the perfusate with phosphate buffer were used.

2.2.2 Purine nucleoside phosphorylase (PNP) activity

The principle of this assay is the PNP-catalyzed conversion reaction of inosine to hypoxanthine, coupled to a second enzyme, xanthine oxidase, converting hypoxanthine to uric acid. The resulting increase of uric acid was measured

photometrically according to Bergmeyer (Bergmeyer HU, 1974). For calculation of PNP activity the extinction coefficient of uric acid was used ($\varepsilon_{293\ nm}$ = 12.5 L x $mmol^{-1}$ x cm^{-1}). Enzyme activities are expressed as mU x min^{-1} x $(g\ liver)^{-1}$.

2.2.2.1 Reactions

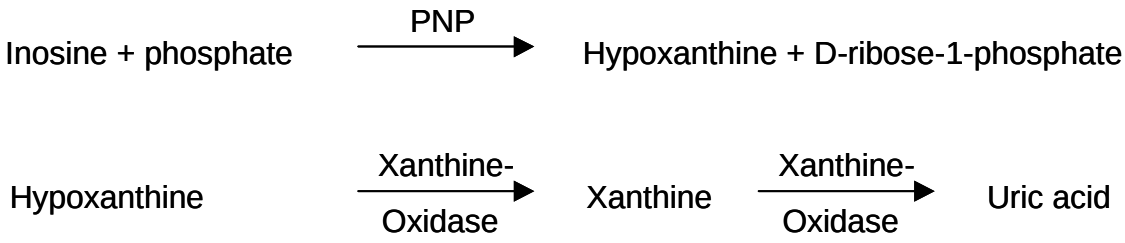


Figure 13: PNP- and xanthine oxidase-catalyzed reaction of inosine to uric acid.

2.2.2.2 Solutions

Phosphate buffer:

K_2HPO_4 (87.4 g/L) and KH_2PO_4 (68 g/L) solutions were mixed until a pH of 7.4 was reached.

Inosine solution:

13.41 mg of inosine were dissolved in 10 ml dist. water.

Xanthine oxidase (X-1875: 50 U/1.5 ml):

Freshly prepared dilution of xanthine oxidase (final concentration: 0.2 U/ml) in phospate buffer (see above).

2.2.2.3 Assay procedure

The following solutions were pipetted into cuvettes and mixed immediately. The measurement was started after a short equilibration time (30 s). Absorbance was monitored for 1 min at RT.

	Reference:	**Sample:**
Phosphate buffer:	200 µl	200 µl
Xanthine oxidase solution:	200 µl	200 µl
KH solution:	1,400 µl	----
Dist. water:	200 µl	----
Perfusate:	----	1,400 µl
Inosine solution :	----	200 µl

In cases of high PNP activity (e.g.: 2' R) 1:10 dilutions of the perfusate with phosphate buffer were used.

2.2.3 Caspase-3-like activity in liver tissue

The activity of caspase-3-like proteases was determined fluorimetrically using the artificial tetrapeptide substrate Ac-DEVD-AFC (amino acid sequence DEVD: asp-glu-val-asp), based on the reports of Thornberry et al. (Thornberry, 1994) and Hentze et al. (Hentze *et al.*, 2000). Thereby, the fluorophor 7-amino-4-trifluoromethylcoumarin (AFC) is liberated by caspase-3-like cleavage of the substrate after its aspartate residue, resulting in an increased fluorescence (*Figure 14*). Because other caspases, such as caspase-2 and -7 show similar substrate specificity, this assay does not exclusively detect caspase-3 activity and is therefore termed caspase-3-like activity. Nevertheless, caspase-3 is the predominant caspase out of the caspase-3-like proteases in the liver extract (Hengartner, 2000).

2.2.3.1 Reaction

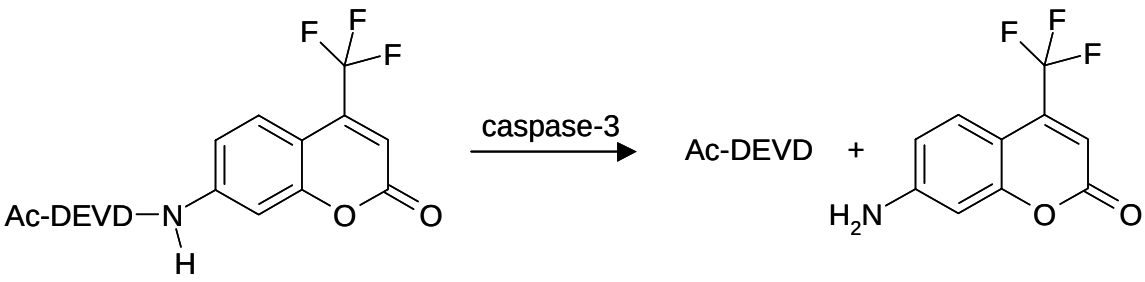


Figure 14: liberation of AFC by caspase-3-cleavage of Ac-DEVD-AFC.

2.2.3.2 Solutions

Extraction buffer:

HEPES pH 7.5	25 mM
$MgCl_2$	5 mM
EGTA	1 mM

Substrate buffer:

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

2.2.3.3 Assay procedure

Frozen liver tissue (~100 mg) was homogenized in 900 µl of ice cold extraction buffer with a Potter (Braun Biotech). Homogenates were subsequently centrifuged for 15 min at 20,160 x *g* and 4°C. Supernatants were stored at -80°C until analysis. The assay was carried out in duplicates on microtiter plates (Greiner GmbH, Frickenhausen, Germany) by mixing 10 µl of the liver cell extract with 90 µl of substrate buffer. Extraction buffer served as blank. Generation of 7-amino-4-trifluoromethylcoumarin (AFC) was determined kinetically by fluorescence measurement using the fluorescence plate reader BMG Fluostar (SLT Labinstruments, Crailsheim, Germany). Excitation wavelength was 385 nm, emission wavelength 505 nm. Enzyme activity was

calculated using an external AFC standard curve. Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above.

2.2.3.4 Determination of protein concentration: Pierce-assay

The Pierce-assay is based on the combination of the biuret reaction (proteins with Cu^{2+} in alkaline solution) with bicinchonic acid. The reaction results in a purple colored complex measured photometrically at λ = 562 nm.

Solutions:

Reagent A: Na_2CO_3, $NaHCO_3$, BCA reagent, Na-tartrate in 0.2 M NaOH.

Reagent B: 4% aqueous solution of $CuSO_4$.

50 parts of reagent A were mixed with 1 part of reagent B. 200 µl out of this solution were added to 10 µl of sample, incubated at 37°C for 30 min and measured photometrically at λ = 562 nm (SLT Spectra, SLT Labinstruments, Crailsheim, Germany). Protein content of cellular extracts was quantified using standard solutions of bovine serum albumin (BSA) from 0-2,000 µg/ml.

2.3 Enzymatical measurement of ATP

In substrate kinetics, enzyme reactions are used to determine the concentration of one specific substance in a mixture. During reaction, substrate (here: ATP) is converted into a photometrically measurable product (here: NADPH).

The principle of this assay is the complete conversion of ATP in the sample *via* a two-stage enzyme reaction forming NADPH. The assay is terminated after 15 min, when present amounts of ATP are completely consumed and a maximal NADPH concentration is reached (see Figure 16), i.e. no further increase of absorbance at the corresponding wavelength. Thereby, for each mol ATP, 1 mol NADPH is generated. Quantification of the ATP levels was performed using the extinction coefficient of NADPH ($\varepsilon_{365\ nm}$ = 3,500 L x mol^{-1} x cm^{-1}) according to Bergmeyer (Bergmeyer HU, 1974).

2.3.1 Reactions

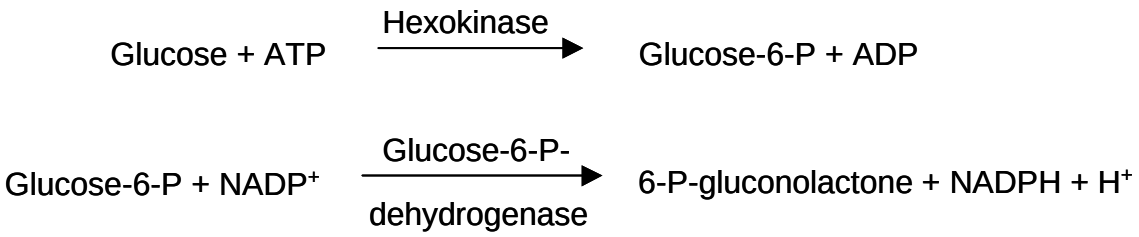


Figure 15: conversion reactions during ATP measurement.

2.3.2 Solutions

Triethanolamine (TEA)-HCl buffer (pH 7.5-7.6):

4.65 g of triethanolamine-HCl were dissolved in approx. 200 ml of dist. water. 11 ml of 1 M NaOH solution were added. The solution was subsequently replenished to 500 ml with dist. water.

$MgCl_2$-solution:

0.81 g of $MgCl_2$ were dissolved in 40 ml of dist. water.

Glucose-solution:

3.6 g of glucose were dissolved in 40 ml of dist. water.

$HClO_4$-solution (6% w/v):

2.08 ml of $HClO_4$-solution (70%) were diluted to 40 ml with dist. water.

K_2CO_3-solution:

28.8 g of K_2CO_3 were dissolved in dist. water.

$NADP^+$-solution:

27 mg of Na_2-$NADP^+$ were dissolved in 3 ml of dist. water.

Glucose-6-phosphate-dehydrogenase suspension (G-6-P-DH) (140 U/mg):

Enzymatic suspension in 3.2 M ammonium sulfate (Roche Diagnostics GmbH, Mannheim, Germany).

Hexokinase (HK) suspension (140 U/mg):

Enzymatic suspension (450 U/mg) in 3.2 M ammonium sulfate (Roche

Diagnostics GmbH, Mannheim, Germany), therefore a 1:3.21 dilution of the purchased hexokinase solution was necessary.

2.3.3 Sample preparation

1.) 1,000 µl of the $HClO_4$-solution were added to frozen liver pieces (approx. 300 mg) and homogenized on ice with a Potter (Braun Melsungen AG, Melsungen, Germany) at 8.75 x *g*.

2.) 5 min centrifugation of the homogenate at 20,160 x *g* and 4°C.

3.) 500 µl of the supernatant were treated with 45 µl of the K_2CO_3-solution to reach a final pH of 7.4 (pH control). The neutralized solution was kept on ice for 10 min.

4.) 5 min centrifugation of the precipitated $KClO_4$ (20,160 x *g*, 4°C) and immediate analysis of the supernatant.

2.3.4 Assay procedure

The following solutions were pipetted into cuvettes in the mentioned order. An auto zero was carried out after adding the glucose-solution.

	Reference:	**Sample:**
TEA buffer:	885 µl	885 µl
NADP-solution:	50 µl	50 µl
$MgCl_2$-solution:	100 µl	100 µl
Dist. H_2O:	300 µl	----
G-6-P-DH suspension:	5 µl	5 µl
Glucose-solution:	150 µl	150 µl
Dist. H_2O:	6.89 µl	6.89 µl
HK suspension:	3.11 µl	3.11 µl
Supernatant:	----	300 µl

2.3.5 Typical measurement

To calculate the values for ATP levels in rat liver, ΔA was read at the endpoint of the transformation reaction.

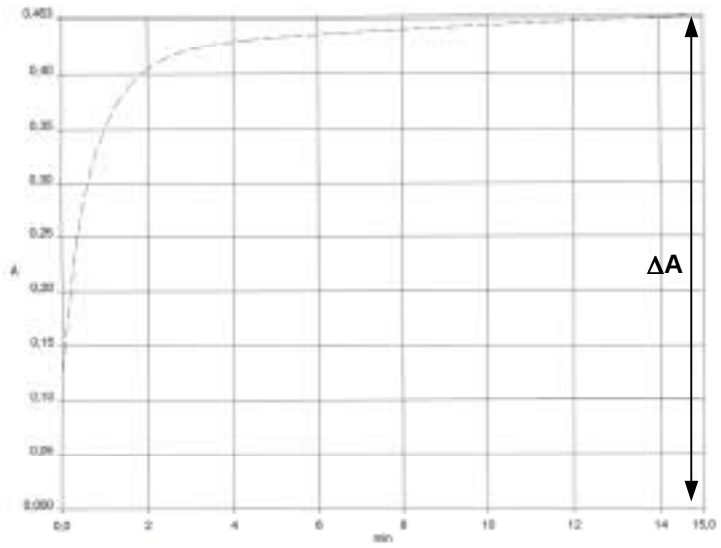


Figure 16: typical curve for the enzymatic conversion of ATP attained from a liver sample. *The graph shows absorbance of the cumulative NADPH formation with an endpoint after 15 min reaction time.*

2.3.6 Calibration curve

To assure that the analysis is in a linear range for the ATP concentrations found in rat liver, calibration curves were performed with ATP standard solutions (0.05-1.0 mM). All values obtained for rat livers were inside this range.

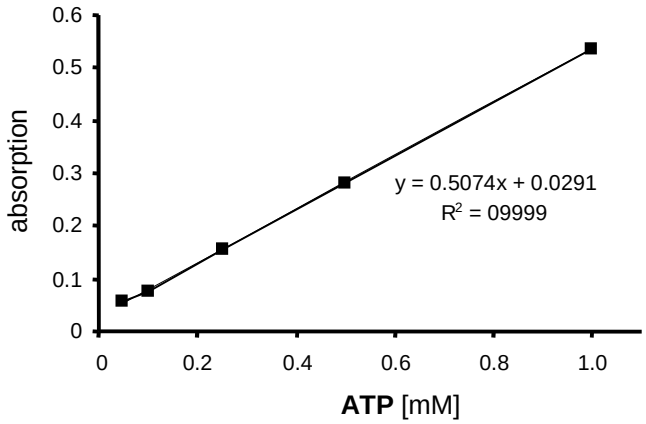


Figure 17: representative calibration curve with ATP standard substance, indicating the relevant range for ATP concentrations found in rat liver.

2.4 Analysis of LA and tetranorlipoic acid by HPLC

The analysis of these data was was performed by ASTA Medica. Quantification of LA and its metabolite tetranorlipoic acid (TNLA) were carried out by Alexander Kraft (ASTA Medica, Frankfurt/Main, Germany).

2.4.1 Sample preparation

2.4.1.1 Liver tissue

Liver tissue was homogenized with a Potter in 0.1 M Na_2HPO_4/EDTA 0.1 M, pH 7.0. 100 µl of the homogenate were deproteinized with 200 µl acetonitrile and centrifuged (12,000 x *g*, 10 min, 4°C). 100 µl of the supernatant were injected on the column.

2.4.1.2 Perfusate

Perfusate was diluted (1:10, 1:100, 1:200, as necessary) with elution buffer (63% KH_2PO_4, pH 2.3 and 37% acetonitrile) and 100 µl were directly injected for measurement.

2.4.2 Instruments and procedure

LA and TNLA were separated by 1 ml/min isocratic elution (63% KH_2PO_4, pH 2.3/37% acetonitrile) on a LiChrospher (RP-18) 5-µm column (4 x 250 mm; Merck/Hitachi, Darmstadt, Germany) at ambient temperature with a Hewlett Packard HPLC system (1100 series: bin pump G1312A, autosampler G1329A). Electrochemical detection was performed by ESA Coulochem II (Chelmsford, USA).

2.5 Quantification of cysteine, glutathione, oxidized cysteine, and oxidized glutathione by HPLC

2.5.1 General considerations

Many methods have been described to determine thiols and disulfides in different biological materials. A common problem of existing methods is their inability to simultaneously quantify the reduced and oxidized forms of several

thiols. In addition, the system has to be sensitive enough to guarantee detection of very low amounts of thiols and their corresponding oxidation products in complex matrices, such as liver tissue.

The present method is based on the derivatization of free thiols with the fluorescence dye monobromobimane. The method exploits the ability of $NaBH_4$ to reduce disulfides, of N-ethylmaleimide (NEM) to block free thiols, and of dimethyl sulfoxide (DMSO) to stabilize the reduction process. Therefore, the procedure fulfills the above mentioned criteria allowing quantification of thiols separately from disulfides, and in addition being very sensitive due to fluorescence detection.

The underlying procedure was originally described for plasma samples by Svardal et al. (Svardal *et al.*, 1990) and Mansoor et al. (Mansoor *et al.*, 1992) and was modified for measurement of liver and perfusate samples.

Chromatographic conditions were modified from that published by Newton et al. (Newton *et al.*, 1981).

2.5.2 Solutions

Solution I:

5-Sulfosalicylic acid		25 g
Dithioerythritol (DTE)		3.856 mg
Dist. H_2O	ad	50 ml

Solution II:

DMSO	6.4 ml
Dist. H_2O	3.45 ml
NaCl	29.8 mg
HBr (48%)	157.5 µl

Solution III:

53 mg $NaBH_4$ (1.4 M) were dissolved in 1 ml of 0.05 N NaOH (prepared freshly for each analysis).

Solution IV:

1.27 ml N-ethylmorpholine (1.0 M) were mixed with 8.73 ml of dist. water.

Solution V:

5.42 mg monobromobimane (20 mM) were dissolved in 1 ml of acetonitrile (Merck, HPLC grade).

Solution VI:

15 mg N-ethylmaleimide (NEM, 120 mM) were dissolved in 1 ml of PBS.

Solution VII:

905.8 µl $HClO_4$ solution (70%) were diluted to 10 ml with dist. water.

Solution VIII:

Solution I was diluted 1:10, i.e. final concentrations: 5-sulfosalicylic acid 5%, DTE 50 µM.

2.5.3 HPLC conditions

2.5.3.1 Elution solvents for HPLC:

Solvent A: 10% methanol/0.25% acetic acid.

Solvent B: 80% methanol/0.25% acetic acid.

The aqueous solution was adjusted to pH 3.9 with NaOH before mixing with methanol.

2.5.3.2 Chromatography

Separation of labelled monobromobimane derivatives was carried out on a Merck/Hitachi HPLC system (pump L6200A, autosampler AS-2000A, fluorescence detector F1000). A LiChrospher 100 (RP-18) 5-µm column (4 x 125 mm; Merck/Hitachi, Darmstadt, Germany) was used at 15°C and a flow rate of 1.2 ml/min. The elution profile was as follows: 0-10 min, 2-20% B, linear gradient; 10-13 min, 20-100% B, linear gradient; 13-18 min, 100% B, column regeneration; 18-19 min, 100-2% B, linear gradient; 19-25 min, 2% B, column equilibration for next injection. The excitation wavelength was 390 nm, emission wavelength 465 nm.

2.5.4 Sample preparation

2.5.4.1 Liver tissue

Liver samples were prepared according to Anderson et al. (Anderson, 1985).

1.) Approximately 500 mg of frozen liver tissue were weighed exactly and 5.0 ml of solution VIII were added immediately and homogenized on ice with a Potter at 1,250 rpm.

2.) Homogenate was centrifuged for 5 min at 20,160 x *g* (4°C) and supernatant was stored at -80°C until analysis.

3.) After thawing on ice homogenate was centrifuged for 10 min at 20,160 x *g* (4°C).

4.) A: For analysis of thiols, 180 µl of the supernatant and 20 µl of solution VIII were pipetted together. The solution was vortexed and derivatized as described under C.2.5.5.1.

B: Analyzing disulfides seperately from thiols needed a further step. Thereby, to 180 µl of the solution (see 4. A), 20 µl of solution VI was added (reaction time: 1 min). In this step present thiols react with NEM to a stable product (Asensi *et al.*, 1994;Akerboom and Sies, 1989). This procedure masks thiols, which therefore do not react in the later derivatization reaction and are not falsely quantified as disulfides (*Figure 20*, Figure 22). Derivatization was performed after reduction of the disulfides as described under C.2.5.5.2.

2.5.4.2 Perfusate

A: Perfusate was thawed on ice, 20 µl of solution I were added to 180 µl perfusate. After vortexing, precipitated protein was centrifuged for 2 min at 20,160 x *g* and 4°C. The supernatant was derivatized as described under C.2.5.5.1.

B: Analyzing disulfides, as already mentioned, to 180 µl of the supernatant (see A), 20 µl of solution VI were added (reaction time: 1 min). Derivatization was performed after reduction of the disulfides as described under C.2.5.5.2.

2.5.5 Derivatization procedures and chromatograms

For analysis of thiol or disulfide samples, the following derivatization procedures were distinguished:

2.5.5.1 Thiols

Derivatization procedure for thiols consisted of adjusting the pH value to 9.0 with N-ethylmorpholine before adding the fluorescence label monobromobimane. Addition of solution I protected thiols from oxidation without reducing disulfides (Svardal *et al.*, 1990). A representative chromatogram of labelled thiols is shown in Figure 19.

Derivatization reaction:

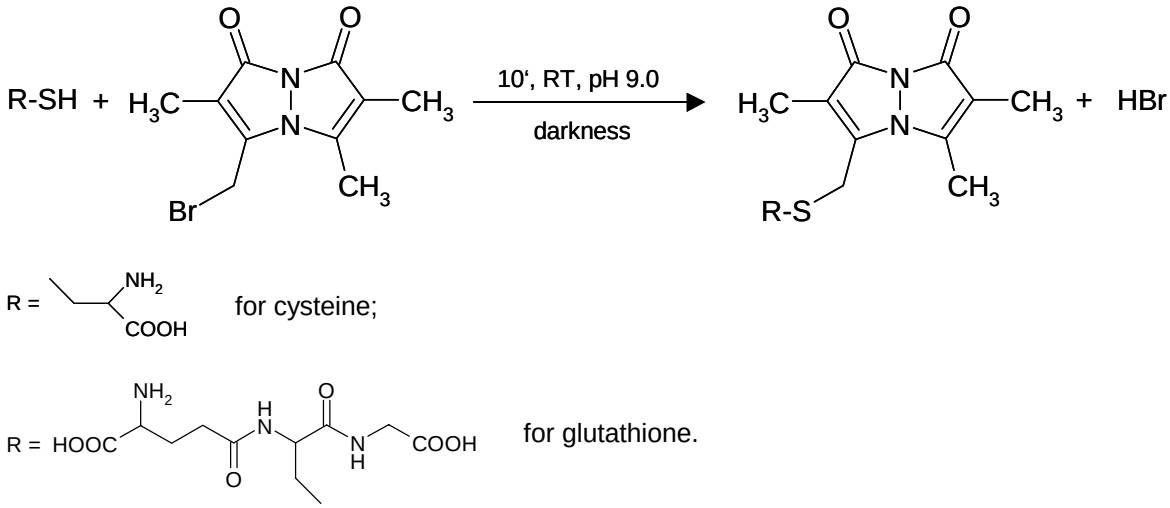


Figure 18: derivatization reaction of thiols (R-SH) with monobromobimane (Yang* et al.*, 1995).

Procedure:

30 µl of a thiol sample (see above)

+ 30 µl of solution VIII

+ 160 µl dist. H_2O

+ 50 µl solution IV (pH 9.0, pH control)

+ 10 µl solution V (10 min reaction time)

+ 20 µl solution VII

The solution with the generated monobromobimane derivatives was

immediately filled in HPLC-vials (brown glass) and subsequently injected in the HPLC system. Injection volume for liver samples was 5 µl. For perfusate, different injection volumes were necessary: Pre-I: 30 µl; 2' R: 5 µl; 10' R: 20 µl; 20' R/50' R/90' R: 30 µl.

Representative chromatogram of labelled thiols derived from liver tissue:

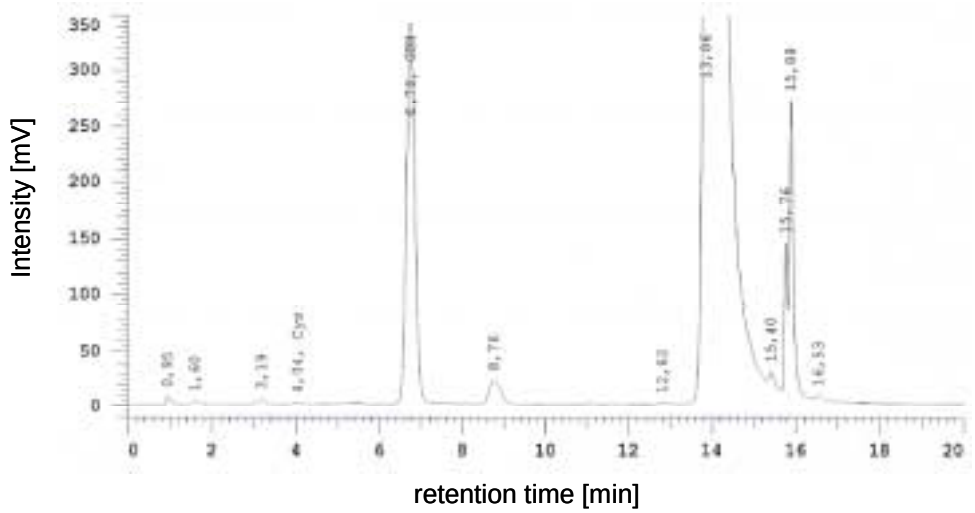


Figure 19: representative chromatogram of monobromobimane-labelled thiols derived from liver tissue. *Retention time for CYS-derivative: 4.04 min and for GSH-derivative: 6.76 min.*

2.5.5.2 Disulfides

The derivatization procedure for mixed disulfides is much more sophisticated than that for thiols: Excess of NEM is immediately inactivated by addition of $NaBH_4$, whereas thiols are not liberated from thiol-NEM adducts. The optimal amount of $NaBH_4$ used (1.4 M) for the reduction of disulfides was titrated by Svardal et al. and confirmed by own experiments (data not shown). DMSO and NaCl (solution II) were utilized to increase yield of the reduction reaction (Svardal *et al.*, 1990).

NEM-reaction:

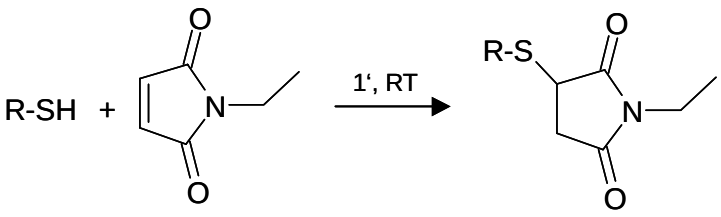
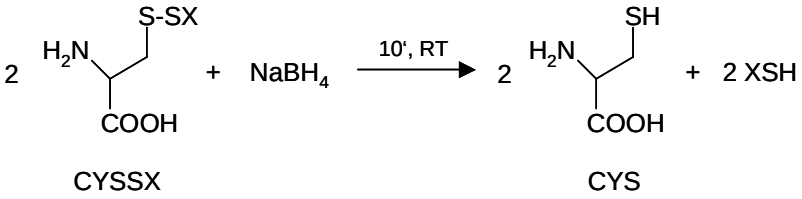


Figure 20: masking reaction of thiols (R-SH) with N-ethylmaleimide (NEM). *A representative chromatogram of NEM-masked thiols is shown in* **Figure 22***.*

Reduction reaction:



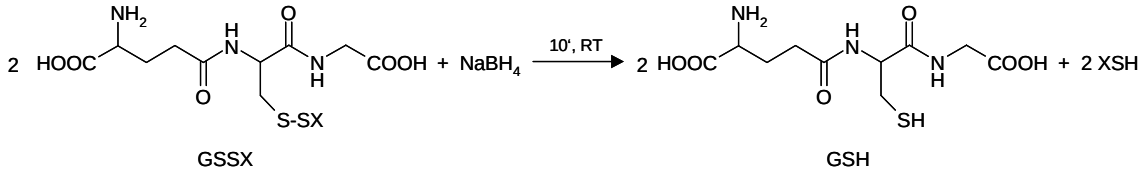


Figure 21: reduction of mixed disulfides with $NaBH_4$.

Reduction with sodium borohydride liberates the thiols CYS, GSH, and XSH, whereas XSH represents substances like homocysteine, homoglutathione, cysteinylglycine, etc., as well as cysteine and glutathione themselves.

Procedure:

30 µl of a disulfide sample (see above)

+ 30 µl of solution III

+ 160 µl of solution II (10 min reaction time)

+ 50 µl solution IV (pH 9.0, pH control)

+ 10 µl solution V (10 min reaction time)

+ 20 µl solution VII

As described above, the solution with the monobromobimane adducts was

immediately transferred into HPLC-vials and injected in the HPLC system. Injection volume for liver samples was 10 µl. For perfusate, different injection volumes were necessary: Pre-I: 30 µl; 2' R: 10 µl; 10' R: 20 µl; 20' R/50' R/90' R: 30 µl. Representative chromatogram of labelled disulfides after reduction is shown in Figure 22.

Chromatogram of thiols after reaction with NEM and monobromobimane:

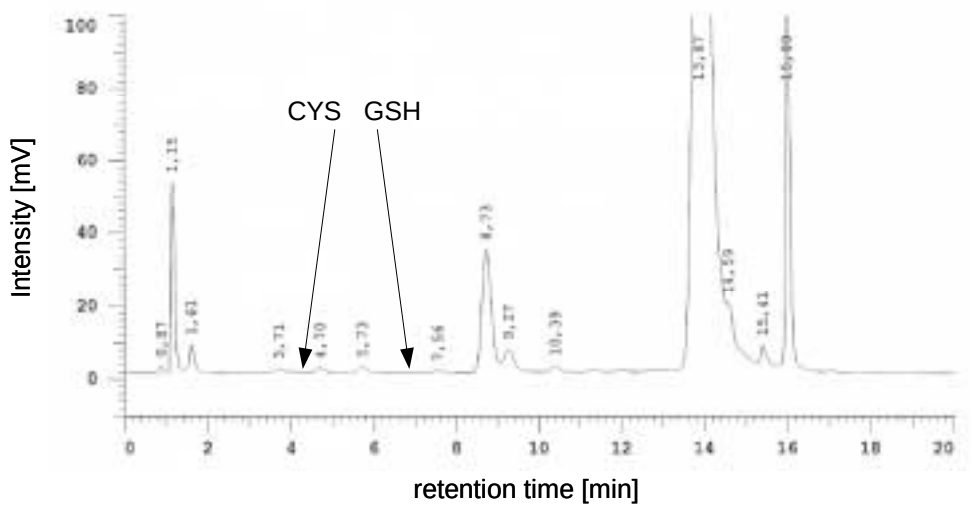


Figure 22: chromatogram of NEM-labelled thiols, demonstrating a quantitative NEM-reaction within 1 min, completely protecting from monobromobimane-labelling, i.e. no peaks appear for CYS- and GSH-derivatives.

Representative chromatogram of labelled disulfides after reduction derived from perfusate:

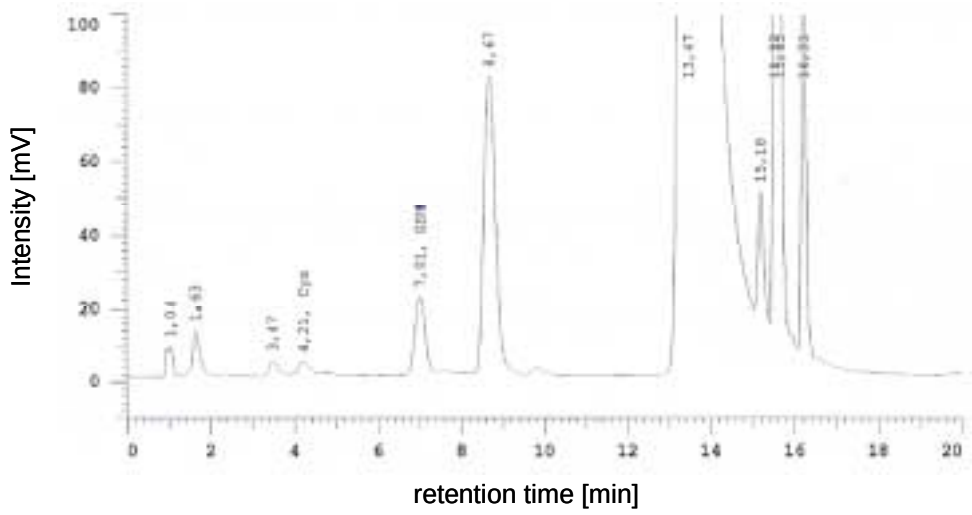


Figure 23: representative chromatogram of monobromobimane derivatives derived from perfusate after reduction of disulfides.

Retention time for CYS-derivative: 4.21 min and for GSH-derivative: 7.01 min.

2.5.6 Calibration curves

The HPLC-system was calibrated by injecting different concentrations of standard solutions. Therefore, standard substances (CYS, GSH, Na-GSSG) were dissolved in dist. water, except cystine, which was dissolved in 42.5% H_3PO_4 first and then diluted with dist. water. Due to rapid autoxidation in solution, dilutions of cysteine and glutathione were permanently kept on ice and prepared directly before labelling with monobromobimane.

Because of the complex constitution of mixed disulfides in biological material no exact standard substance does exist. Therefore, cystine and oxidized GSH (GSSG) were chosen to reflect conditions for typical disulfide reductions. Cystine and GSSG standard solutions were also treated with solution I as described for samples. Derivatization procedure for standards was equal to that described for samples (see above 2.5.5.1, 2.5.5.2).

Calibration curves were performed once a week without switching off the HPLC-system throughout the week. Further standard injections followed every 10th sample and were compared to the calibration curves. All of the values determined for liver and perfusate samples were in the linear range of the calibration curves.

Representative calibration curves for each standard substance:

cysteine (CYS) **glutathione (GSH)**

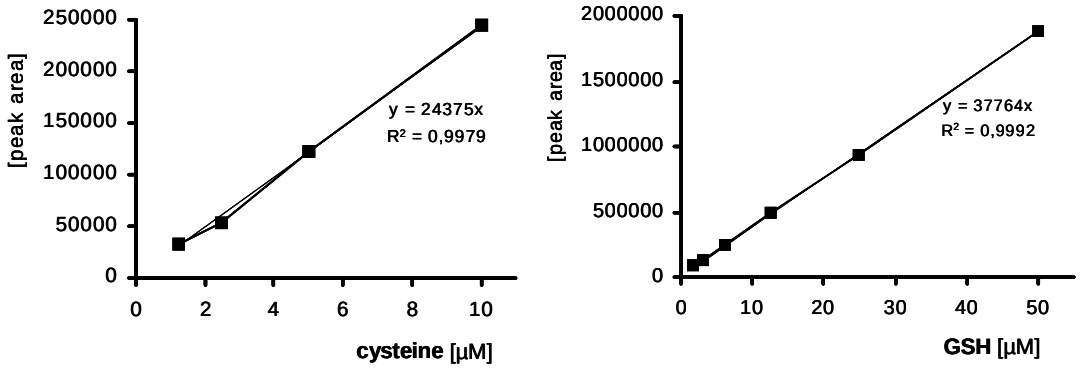


Figure 24: representative calibration curves for the standard substances cysteine and glutathione.

cystine **oxidized glutathione (Na-GSSG)**

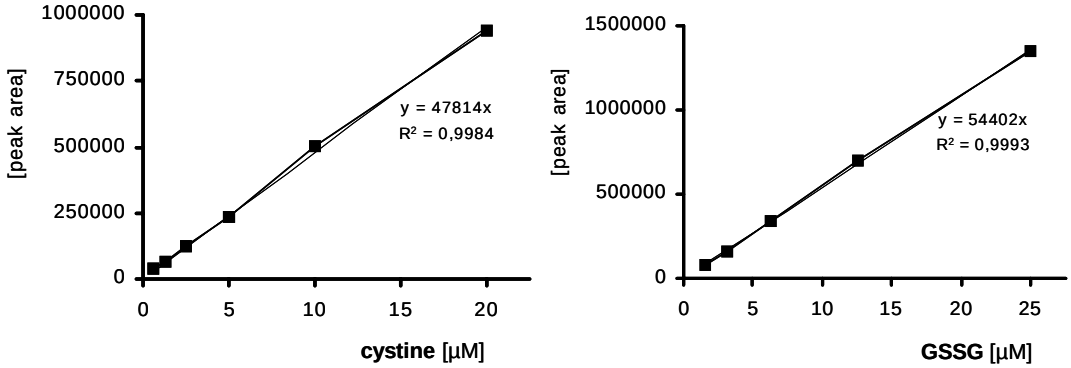


Figure 25: representative calibration curves for the standard substances cystine and oxidized glutathione (Na-GSSG).

All values for thiols (CYS, GSH) in liver are expressed as µmol/g liver, in perfusate as nmol x min^{-1} x $(g\ liver)^{-1}$. Values for mixed disulfides (CYSSX and GSSX) in liver are expressed as µmol cystine or GSSG/g liver, in perfusate as nmol cystine or GSSG x min^{-1} x $(g\ liver)^{-1}$.

2.6 Detection of transcription factors by EMSA

The **e**lectrophoretic **m**obility **s**hift **a**ssay (EMSA) provides a method for detecting DNA-binding proteins (Kiemer, 2002). This method can be used in the study of sequence-specific DNA-binding proteins such as transcription factors, e.g. nuclear factor κB (NF-κB) and activator protein 1 (AP-1). The assay is based on the observation that complexes of protein and DNA migrate through a non-denaturating polyacrylamide gel more slowly than free oligonucleotides.

The gel shift assay was performed by incubating nuclear cell extracts with ^{32}P end-labelled consensus oligonucleotides of NF-κB and AP-1. The shift between free and protein bound oligonucleotide appears after non-denaturating polyacrylamide gel electrophoresis. The specificity of the DNA-binding protein for the putative binding site was established by competition experiments using non-radioactive consensus oligonucleotides or other unrelated DNA sequences, e.g. AP-2 (data not shown).

2.6.1 Solutions

Buffer A:

HEPES (pH 7.9)		10 mM
KCl		10 mM
EDTA		0.1 mM
EGTA		0.1 mM
H_2O dist.	ad	50 ml
DTT		1 mM and
PMSF		0.5 mM were added immediately before use.

Buffer B:

HEPES (pH 7.9)		20 mM
NaCl		0.4 mM
EDTA		1 mM
EGTA		1 mM
Glycerol		25% (v/v)
H_2O dist.	ad	50 ml
DTT		1 mM and
PMSF		0.5 mM were added immediately before use.

STE buffer:

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

prepared in dist. H_2O.

2.6.2 Preparation of nuclear extracts

Preparation of nuclear extracts is achieved by swelling the cells first, adding a

hypotonic buffer and lysing the cells with Nonidet P-40, a nonionic detergens. Extraction of nuclear proteins from nuclei is achieved by the addition of hypertonic buffer.

2.6.2.1 Procedure

Nuclear extracts were prepared from frozen liver sections using a modification of the method described by Dignam et al. (Dignam *et al.*, 1983). Thereby, tissue samples were homogenized in 3 ml of ice-cold hypotonic buffer A with a Potter at 1,250 rpm. The homogenate was transferred to a polypropylene centrifuge tube and after a 10 min incubation on ice, centrifuged at 1,000 x *g* for 10 min at 4°C. The cell pellet was suspended in 1.4 ml of ice-cold buffer A. 90 µl of a 10% solution of Nonidet P-40 were added followed by 10 sec of vigorous vortexing. The suspension was incubated on ice for 10 min and then centrifuged at 12,000 x *g* for 45 sec at 4°C. The supernatant was removed and the nuclear pellet was extracted with 200 µl of hypertonic buffer B by shaking for 30 min at 4°C. The extract was centrifuged at 12,000 x *g* for 10 min at 4°C and the supernatant was aliquoted (20 µl) and frozen at -80°C. After a 1:100 dilution with dist. water, protein concentration of the nuclear extracts was determined by the Lowry-assay (see 2.6.3).

2.6.3 Determination of protein concentration: Lowry-assay

Protein content of the nuclear extract was determined photometrically according to the method of Lowry (Lowry OH, 1951). In this method the biuret reaction is combined with the Folin-Ciocalteau-phenol reagent resulting in a higher sensitivity of the colorimetric assay.

2.6.3.1 Solutions

Reagent A:

10 g Na_2CO_3 were dissolved in 500 ml of 0.1 M NaOH.

Reagent B:

0.5 g K/Na-tartrate and 0.25 g $CuSO_4$ were dissolved in 50 ml of dist. water.

Solution 1:

50 ml of reagent A were mixed with 1 ml of reagent B.

Solution 2:

50 ml of Folin-Ciocalteu's phenol reagent plus were diluted with 50 ml of dist. water.

2.6.3.2 Procedure

Protein solution was diluted with dist. water as described in 2.6.2.1. 200 µl of the sample were mixed with 1 ml of solution 1. After a 10 min incubation time at RT, 100 µl of solution 2 were added, vortexed, and incubated for at least 30 min in the dark. Absorbance was measured photometrically at 500 nm (Lambda Bio 20 photometer, Perkin Elmer GmbH, Überlingen, Germany). Calculation of protein concentration was performed by creating calibration curves using bovine serum albumine in aqueos dilutions from 0-100 µg/ml.

2.6.4 Radioactive labelling of oligonucleotides

Radioactive labelling of oligonucleotides was attained by using the enzyme T4 polynucleotide kinase (Promega, Heidelberg, Germany), which catalyzes the transfer of the terminal phosphate of ATP to the 5' hydroxyl termini of polynucleotides.

2.6.4.1 Used oligonucleotides

The two following double-stranded oligonucleotide probes containing a consensus binding-sequence for either

NF-κB: 5'-AGT TGA GGG GAC TTT CCC AGG C-3' or

AP-1: 5'-CGC TTG ATG AGT CAG CCG GAA-3' (1.75 pmol/µl Promega, Heidelberg, Germany) were used.

2.6.4.2 Labelling procedure

The following constituents were pipetted together on ice and incubated for 10 min at 37°C.

Oligonucleotide (1.75 pmol/µl)	2 µl
T4 polynucleotide kinase buffer	1 µl
[γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml)	5 µl
H_2O (nuclease free)	1 µl
T4 polynucleotide kinase (5-10 U/µl)	1 µl

The reaction was stopped by adding 1 µl of 0.5 M EDTA.

Separation of non-incorporated [γ-^{32}P]ATP (Amersham, Braunschweig, Germany) from labelled oligonucleotide was achieved by using a NucTrap® Column (NucTrap® Probe Purification Column, Stratagene Europe, Amsterdam, Netherlands). Thereby, the mixture was replenished to 70 µl with STE buffer and brought up onto a NucTrap® Column, which had been equilibrated before with 140 µl of STE buffer. The solution was pushed through the column. This process was repeated once again with 70 µl of STE buffer and once with air. Labelled oligonucleotide was found in the eluate. Labelling efficiency was measured with a β-counter LS 6500 (Beckman, Krefeld, Germany).

2.6.5 DNA binding reaction and gel electrophoresis

Binding reaction of transcription factors to the labelled oligonucleotides was performed in presence of the nucleic acid polymer poly (dI-dC), a double stranded, alternating copolymer, which is utilized to reduce nonspecific binding of proteins to the radiolabelled DNA fragment. Therefore, gels and electrophoresis buffers with low ionic strength were chosen and non-denaturating conditions were necessary to avoid disaggregation of the complexes due to lability of protein-DNA interactions. In addition, an effect called “caging” occurs in the gel matrix, preventing dissociation of protein and DNA by the surrounding gel.

2.6.5.1 Solutions

5x binding buffer:

Glycerol (100%)		20% (v/v)
$MgCl_2$		0.5 mM
EDTA		2.5 mM
NaCl		250 mM
Tris-HCl		50 mM
H_2O dist.	ad	50 ml

DTT (dithiothreitol) was added prior to use (final concentration: 2.5 mM).

10x TBE buffer:

Tris-base		53.9 g
Boric acid		~27.5 g (pH 8.3)
EDTA-Na_2		3.72
H_2O dist.	ad	500 ml

Gel loading buffer:

Tris-HCl (pH 7.5)	250 mM
Bromphenol blue	0.2%
Glycerol	40%

Non-denaturating gel 4.5%:

10 x TBE	1 ml
PAA (30%)	3 ml
Glycerol (100%)	500 µl
H_2O dist.	15.5 ml
TEMED	10 µl
APS 10% (m/v)	150 µl

Gel was prepared using a 30% solution of polyacrylamide/0.8% bisacrylamide (Rotiphorese Gel 30, Carl Roth GmbH+Co, Karlsruhe, Germany).

Solution 3:

DTT 1 M	1 ml
5x binding buffer	450 µl
Loading buffer	50 µl

2.6.5.2 Procedure

10 µg nuclear protein were incubated for 10 min at room temperature in a 14 µl reaction volume containing:

H_2O dist.	x µl
Poly(dI-dC) (2 µg/µl)	1 µl
Solution 1	3 µl
10 µg protein	10 µl – x µl

Then 1 µl of 5 x 10^4 cpm radiolabeled oligonucleotide probe was added and mixed thoroughly.

Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4.5% non-denaturing polyacrylamide gel in 0.25x TBE buffer at 100 V. The gel was autoradiographed with an intensifying storage phosphor imaging screen at -80°C for up to 3h, depending on the radioactivity of the labelled oligonuclotide. Specificity of the DNA-protein complex was confirmed by competition with a 100-fold excess of either unlabeled NF-κB or AP-1 cosensus sequence. Signal detection and quantification was performed by phosphorimaging (Cyclone Storage Phosphor Screen, Canberra-Packard GmbH, Dreieich, Germany).

2.7 Western blot

Quantification of phospho-p38 mitogen activated protein kinase (phospho-p38 MAPK) and phospho-Akt quantitatively (= phospho-protein kinase B) was performed by Western blot analyses. In order to determine cytosolic as well as intracellularly bound proteins whole liver cell lysates were made. After aquiring protein concentration of the liver cell extracts, proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), blottet on a polyvinylidenfluoride (PVDF) membrane and then protein bands were visualized by chemiluminescence after incubating with different antibodies.

2.7.1 Sample preparation

Approx. 100 mg liver tissue was weighed and 1 ml of ice cold lysis buffer was added immediately. After centrifugation for 10 min at 20,160 x *g* and 4°C, 200 µl of the supernatant was diluted with 800 µl of lysis buffer. Protein content of the obtained liver cell extract was determined using the method of Lowry et al. (Lowry OH, 1951) (see also 2.6.3),

2.7.1.1 Solutions

Lysis buffer:

EDTA	2 mM
NaCl	137 mM
Glycerol	10%
$Na_4P_2O_7$ x 10 H_2O	2 mM
Tris	20 mM
Triton X-100	1%
$C_3H_7Na_2O_6P$ x H_2O	20 mM
NaF	10 mM

Lysis buffer was supplemented with 2 mM Na_3VO_4 , 1 mM PMSF and with a 25-fold stock solution of the protease inhibitor mix Complete® (final dilution: 1/25, Boehringer Mannheim, Mannheim, Germany).

5x Sample buffer:

3.125 M Tris-HCl pH 6.8	100 µl
Glycerol 100%	500 µl
SDS 20%	250 µl
DTT 16%	125 µl
Pyronin Y 5%	5 µl
H_2O demin.	20 µl

2.7.2 SDS-polyacrylamide electrophoresis (SDS-PAGE)

Separation of proteins was achieved by denaturating SDS-PAGE in a vertical

apparatus (Mini PROTEAN 3, BioRad Laboratories Ltd., Hertfortshire, England). Molecular weight standard (Cruz Marker SC2035, Santa Cruz Biotechnology, Heidelberg, Germany) was used in each experiment to assure detection of the demanded protein.

2.7.2.1 Discontinuous Electrophoresis

Discontinuous electrophoresis represents a two step electrophoresis with two different gels: resolving and stacking gel. The resolving gel with small pores (pH 8.8) and the stacking gel (pH 6.8) with large pores. In the electrode buffer terminating ions with low mobilities (e.g. glycine, which has a low net charge at the pH value of the stacking gel), in the gel exclusively leading ions with high mobility (e.g. Cl^-) are used.

Entering the stacking gel first, protein zones become concentrated. Because of the large pores in the gel matrix, mobilities are dependent on the net charge, not on the size of the molecule. Reaching the resolving gel frictional resistance for proteins increases, resulting in a slower migration towards the anode, and becoming higher concentrated and fractioned. Cl^- and glycine, unaffected by the gel matrix, generate a new front and move ahead of the proteins.

2.7.2.2 Solutions

Resolving gel 10%:

PAA solution 30%	5 ml
1.5 M Tris-HCl, pH 8.8	3.75 ml
SDS 10%	0.15 ml
H_2O dist.	6.1 ml
10 min of vacuum degassing	
TEMED	15 µl
APS	75 µl

Stacking gel:

PAA solution 30%	1.7 ml
1.5 M Tris-HCl, pH 6.8	1 ml
SDS 10%	0.10 ml
H_2O dist.	7.0 ml
10 min of vacuum degassing	
TEMED	20 µl
APS	100 µl

Gels were prepared using a 30% solution of polyacrylamide/0.8% bisacrylamide (Rotiphorese Gel 30, Carl Roth GmbH+Co, Karlsruhe, Germany).

Electrophoresis buffer:

Tris		3 g
Glycine		14.4 g
SDS		1 g
H_2O	ad	1,000 ml

2.7.2.3 Procedure

First, the resolving gel was filled into the vertical gel cassette with an overlay of demin. water. After polymerisation, the aqueous phase was removed, stacking gel was added and slot formers were immediately put in (polymerisation time: at least 1 h at RT).

Prior to electrophoresis protein samples were denatured and proteins were charged negatively by adding 5x sample buffer (4 parts of protein sample mixed with 1 part 5x sample buffer) and boiling for 5 min at 95°C. Samples (100 µg protein) and molecular weight standard were filled into the slots and were separated electrophoretically after being filled up to 30 µl with 1x sample buffer. Stacking was carried out at 100 V for 21 min. Proteins were resolved at 200 V for 36 min.

2.7.3 Coomassie staining

In order to control sample loading and blotting efficiency gels were stained with Coomassie blue.

2.7.3.1 Solutions

Staining solution:

Coomassie blue	0.3%
Acetic acid (100%)	10% (v/v)
Ethanol (96%)	45% (v/v)
in demin. water	
Filtration of the solution.	

Destaining solution:

Acetic acid (100%)	10% (v/v)
Ethanol (96%)	30% (v/v)
in demin. water	

2.7.3.2 Staining procedure

To control consistent blotting, gels were stained for 30 min in Coomassie staining solution. Gels were then destained twice for 10 min with destaining solution and stored over night in demin. water.

2.7.4 Protein transfer and immunological detection

To quantify the proteins of interest they had to be immobilized and then visualized. Therefore, SDS-PAGE separated proteins were electroblotted from the gel onto a polyvinylidenfluoride (PVDF) membrane (Immobilon-P™, 0.45 µm pore size, Millipore Corporation, Bedford, USA). Proteins were detected by a first specific antibody (overnight, 4°C) and a second immunoglobulin antibody binding to the heavy chain of the first antibody (1 h, RT). The second antibody was coupled to horseradish peroxidase, which catalyzes the chemiluminescence producing oxidation of luminol and H_2O_2. This light emission was enhanced and prolonged by a chemical enhancer (e.g. phenols in Renaissance® chemiluminescence reagent plus, NEN, Köln, Germany) and recorded by a Kodak image station (Eastman Kodak Company, Rocester,USA).

2.7.4.1 Solutions

Anode buffer I:

Tris		30 g
Methanol		200 ml
H_2O demin.		800 ml

Anode buffer II:

Tris		3 g
Methanol		200 ml
H_2O demin.		800 ml

Cathode buffer:

ε-amino-n-caproic acid		5.2 g
Methanol		200 ml
H_2O demin.		800 ml

TBS-T pH 8.0 (washing buffer):

Tris		3 g
NaCl		11.1 g
Tween 20		2.0 ml
H_2O demin.	ad	1000 ml
Adjusting pH to 8.0		

2.7.4.2 Semidry blotting

Proteins were transferred between two horizontal electrodes (Biometra fastblot B43, Göttingen, Germany) from the gel onto a PVDF-membrane using the semi-dry blotting technique, which is fast and needs only a small volume of buffer (Kyhse-Andersen, 1984). Thereby, anions migrate at the same speed, so that a regular transfer takes place.

2.7.4.2.1 Procedure

The blotting membrane was cut to the size of the resolving gel and deposited in methanol, demin. water, and anode buffer II for 5 min each. On the anode a bubble free stack of soaked blotting papers was placed, consisting of 6 sheets

drenched in anode buffer I and 3 sheets in anode buffer II. Onto this stack the membrane and the gel were put onto and covered with 9 sheets of blotting paper soaked with cathode buffer.

55 min of blotting was conducted at a current of 0.8 mA/cm^2 blotting surface and 115 V (power supply: Consort E835, Merck, Darmstadt, Germany). Then the membrane was dried for 30 min at 80°C. To block free binding sites, the membrane was incubated in a 5% (m/v) solution of low fat milk powder (Blotto, BioRad Laboratories Ltd.) in TBS-T for at least 1 h at RT.

2.7.4.3 Antibodies

2.7.4.3.1 p38 MAPK

For phospho-p38 MAPK Western blotting, a polyclonal rabbit phospho-p38 MAPK antibody (Thr 180/Tyr 182, Cell signalling, Frankfurt/Main, Germany) was used as first antibody. The secondary antibody was a horseradish peroxidase (HRP) conjugated polyclonal anti-rabbit IgG antibody (Dianova, Hamburg, Germany).

Reprobes of the blots were performed with a polyclonal rabbit total-p38 MAPK antibody (Cell signalling, Frankfurt/Main, Germany) in order to control loading differences.

2.7.4.3.2 Akt

For phospho-Akt Western blotting, a polyclonal rabbit phospho-Akt (Ser 473, Cell signalling, Frankfurt/Main, Germany) served as first antibody. The second antibody was a horseradish peroxidase (HRP) conjugated polyclonal anti-rabbit IgG antibody (Dianova, Hamburg, Germany).

Reprobes of the blots were performed with a polyclonal rabbit total-Akt antibody (Cell signalling, Frankfurt/Main, Germany) in order to control loading differences.

2.7.4.4 Immunological detection

The primary antibodies were diluted 1:1,000 in 1% Blotto in TBS-T or with 5% bovine serum albumine (BSA, for phospho-p38 MAPK antibody). Then the membrane was incubated with the primary antibody overnight at 4°C by shaking gently. After 4 washing steps for 10 min with TBS-T, the secondary antibody

(1:10,000 dilution with 1% Blotto in TBS-T) was added to the membrane for 1 h at RT. After another 3 washing steps for 10 min, two detection solutions were mixed (1:1, Renaissance® Chemiluminescence Reagent Plus, NEN lifescience, Cologne, Germany) as described in the manufacturer's manual. The membrane was then incubated with this mix for 1 min. Chemiluminescence detection and quantification was performed with a Kodak image station (Eastman Kodak Company, Rocester, USA).

2.8 Cell culture

Cell culture experiments were performed to study later IRI events as previously investigated, e.g. the inhibition of NF-κB target genes, such as iNOS and the corresponding NO output.

2.8.1 Medium and supplements

Since macrophages are known to develop tolerance to lipopolysaccharides (LPS) activation upon continuous exposure to endotoxin in the culture medium, low endotoxin containing media and supplements were used. The cell culture medium was Dulbecco's Modified Essential Medium (DMEM; PAN, Aidenbach, Germany), with 4 mM of L-glutamine and 10% of heat inactivated Fetal Calf Serum (FCS, Gibco-BRL, Eggenstein, Germany) added prior to use.

2.8.2 Cells

The murine macrophage cell line RAW 264.7 (American Type Culture Collection ATCC, TIB 71, Rockville, USA) was cultivated in DMEM with phenol red and was passaged once or twice per week. For investigations the cells were diluted and 10^4 cells/200 µl were seeded into a 96 well plate und cultivated for 3 days until the experiment.

2.8.3 Cultivation

2.8.3.1 Incubation

RAW 264.7 macrophages were cultivated in an incubator (Heracell, Heraeus, Kendro Laboratory Products, Munich, Germany) at 37°C and 5% CO_2 in fully humidified air. The cells grew in an adherent monolayer in polystyrole flasks in

an area of 75 or 150 cm^2 or in 96 well plates (PESKE, Aindling-Pichl, Germany).

2.8.3.2 Passaging

The cell culture was splitted and transferred into new flasks dependent on the growing velocity of the macrophages (once or twice per week). Thereby approx. 5 ml of medium were added to the cell line and cells were detached from the bottom of the flask by knocking strongly. After counting, cells were diluted (1:5 or 1:10) and passaged. Cells were used until the 20^{th} passage. After that, fresh cells were taken from a liquid nitrogen store.

2.8.3.3 Cell counting

Determination of cell concentration was performed in a Neubauer counting chamber.

2.8.3.4 Freezing

The macrophages were stored in liquid nitrogen (-196°C). To avoid bursting of the cells during freezing dimethylsulfoxide (DMSO) was added to the medium. The freezing medium was sterile filtrated after mixing.

Freezing medium:

DMSO	10 ml
FCS	20 ml
Medium	70 ml

2.8.3.5 Thawing

Cells stored in cryo tubes were taken from the liquid nitrogen, thawed as fast as possible and transferred into a 75 cm^2 cell culture flask. Medium was changed after 24 h to remove dead cells.

2.9 Photometrical detection of nitrite production

2.9.1 Treatment of cells

Cells were treated with bacterial lipopolysaccharide (LPS, E. coli, serotype 055:B5, 1 µg/ml) in the absence or presence of various concentrations of LA (5-500 µg/ml). LA was first dissolved in ethanol and further diluted with medium.

Final ethanol concentrations on the cells were < 0.1% and shown not to affect LPS induced NO production. After 20 h of stimulation, the concentration of nitrite, a stable metabolite of NO, was measured in the culture supernatant by the Griess assay (Green *et al.*, 1982). In a different set of experiments the NO donor sodium nitroprusside (SNP, 1 mg/ml) was added to the cells in the presence or absence of LA and nitrite accumulation was measured after 1.5, 2.5, and 4.5 h.

2.9.2 Griess-assay

The stable NO-oxidation product nitrite can be determined photometrically after its reaction to an azo-dye (Green *et al.*, 1982). Quantification of LPS-induced nitrite formation was carried out using a calibration curve of different nitrite concentrations in medium (0-100 µM).

2.9.2.1 Solutions

Sodium nitrite:

The standard curve was performed with different concentrations of sodium nitrite dissolved in medium (0-100 µM). The calibration solutions were prepared freshly for each experiment and measured together with cell supernatants on the same 96 well plate.

Griess-reagents:

A: sulfanilamide 1% (m/v) in H_3PO_4 5% (v/v)

B: naphthylethylenediamine 0.1% (m/v) in dist. H_2O

2.9.2.2 Procedure

After 20 h of stimulation with LPS in absence or presence of LA, 100 µl of the cell supernatants were pipetted in triplicates on a 96 well plate. After addition of 90 µl of the Griess-reagents A and B each, the absorption was measured at λ = 550 nm on a SLT Spectra plate reader (SLT Labinstruments Deutschland GmbH, Germany) and converted into nitrite concentrations.

2.10 Photometrical determination of cell viability

In order to exclude toxic effects of LA and its metabolites cell viability had to be

examined. A parameter for cell viability is the mitochondrial respiratory activity, which was determined by the MTT-test.

2.10.1 MTT-assay

Mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the corresponding formazan was determined as an indicator of cell viability (Mosmann, 1983).

2.10.1.1 Solutions

Phosphate buffered saline (PBS):

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

After adjusting the pH value to 7.4 the solution was autoclaved for 40 min.

MTT-solution:

5 mg MTT were dissolved in 1 ml PBS, sterile filtrated and stored at -20°C.

2.10.1.2 Procedure

After removing the supernatant for nitrite determination, the cells were incubated with MTT solution for 10 min at 37°C and solubilized 2 h in DMSO. The extent of formazan production was determined photometrically at 550 nm.

2.11 Statistical analysis

All data are expressed as the mean ± SEM (n=number of organs). Unless stated otherwise, all experiments were performed with n=5 organs per treatment group. Statistical significance between groups was determined with one sample or student's t-test. A p value < 0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism® (Version 3.02, GraphPad Software Inc., San Diego, USA). Statistical significance was divided as recommended by GraphPad Prism software as follows: *** represents p-values < 0.001, ** represents p-values < 0.01, and * represents p-values < 0.05.

D. Results

1. Validation of the liver perfusion system

Validation of the isolated perfused rat liver system was carried out according to Bilzer (Bilzer M., 1997) by undertaking continuous liver perfusions and perfusions with a short time infusion of H_2O_2 in the absence or presence of LA.

1.1 Continuous liver perfusion

Continuous perfusions were performed in order to describe basal levels of the employed parameters (hepatic enzyme release, portal pressure, and bile flow) and to determine, whether LA affects these parameters of liver cell viability and hepatic function (Gores *et al.*, 1986;Bilzer M., 1997).

1.1.1 No influence of LA application on LDH and PNP efflux

The most common indicator of liver tissue damage is the cellular enzyme release. Measurement of intracellular enzymes, such as lactate dehydrogenase (LDH) and purine nucleoside phosphorylase (PNP) in the perfusate reflects integrity of cell membranes and therefore cell viability. Marked increase in their activities suggest cell damage leading to a release of these proteins from the cytosol (Bilzer M., 1997;Fiegen *et al.*, 1997;Gores *et al.*, 1986;Rao *et al.*, 1990;Jaeschke *et al.*, 1988).

In continuous perfusions LDH and PNP release increased slightly without affecting liver function and viability (*Figure 26*). Administration of 10 µM LA, beginning 10 min after canulation of the portal vein, showed no significant influence on LDH and PNP efflux rates (*Figure 26*).

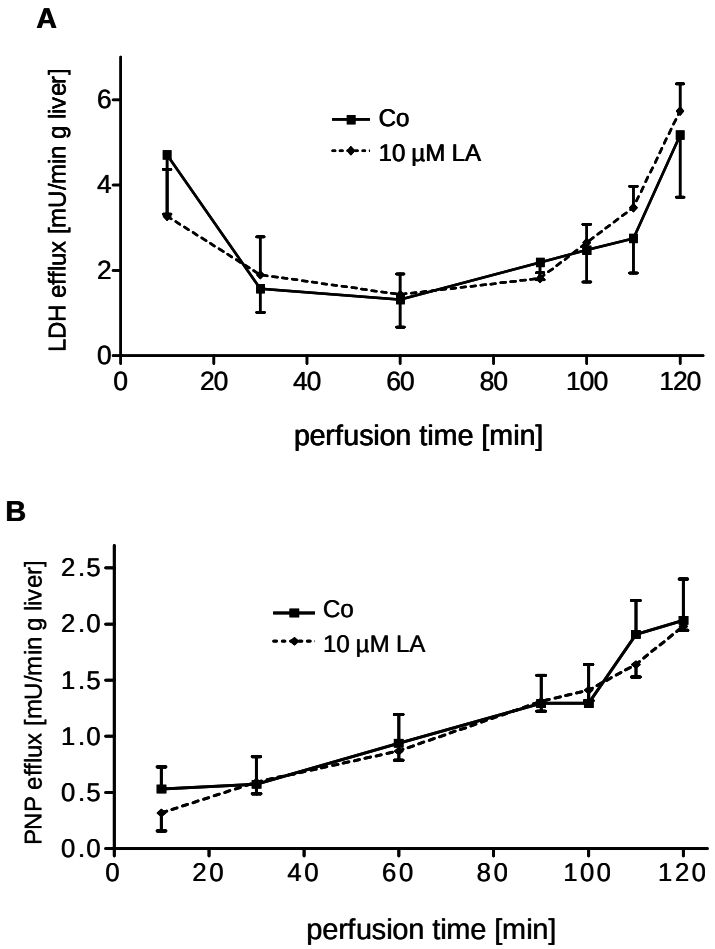


Figure 26: LA application did not influence enzyme efflux. *Livers were perfused for 120 min in the absence (Co) or presence of 10 µM LA, which was added continuously 10 min after canulating the portal vein. Analysis of LDH (panel A) and PNP (panel B) activity in perfusate was carried out at different perfusion time points as described in "Methods". Data are expressed as enzyme activity in mU x min^{-1} x (g liver)$^{-1}$ and show means ± SEM of n=5 experiments in each group.*

1.1.2 No influence of LA administration on portal pressure

Portal pressure is a marker for hemodynamic disturbances in the liver.

No significant difference in portal pressure was obtained by applying 10 µM LA after a 10 min equilibration time of the perfusion system.

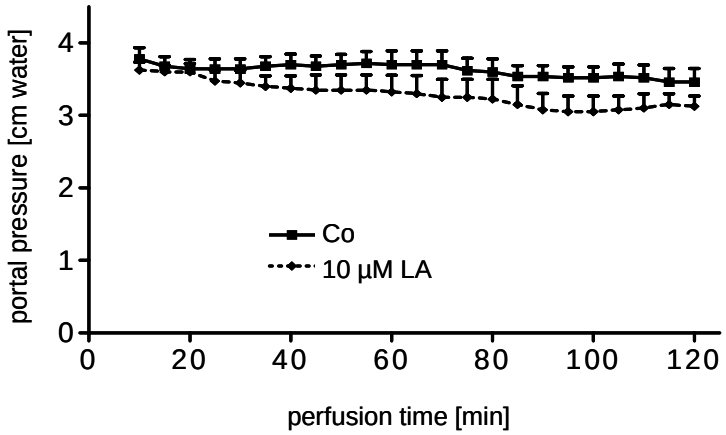


Figure 27: LA application did not influence portal pressure. *Livers were perfused for 120 min in the absence (Co) or presence of 10 µM LA, which was added continuously 10 min after canulating the portal vein. Portal pressure was monitored throughout perfusion as described in "Methods". Data are expressed as cm water and represent means ± SEM of n=5 experiments in each group.*

1.1.3 No influence of LA application on bile flow

Bile flow is an indicator of liver function (Fiegen *et al.*, 1997;Gores *et al.*, 1986;Yin *et al.*, 1998;Settaf *et al.*, 2000;Bilzer *et al.*, 1999;Bilzer *et al.*, 1994) .

Administration of 10 µM LA, continuously applied after a 10 min equilibration time significantly increased bile flow at some specific perfusion time points (Figure 28).

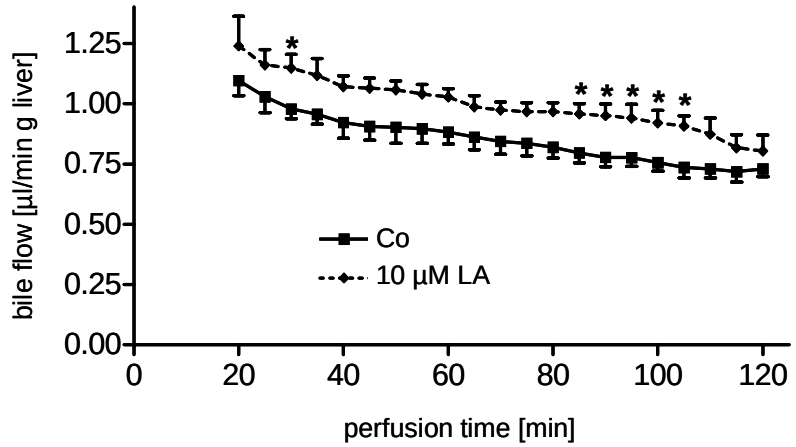


Figure 28: LA application enhanced bile excretion.

Livers were perfused for 120 min in the absence (Co) or presence of 10 µM LA, which was added continuously 10 min after canulating the portal vein. Bile flow was determined collecting bile every 5 min as described in "Methods". Data are expressed as µl x min^{-1} x (g liver)$^{-1}$ and represent means ± SEM of n=5 experiments in each group.

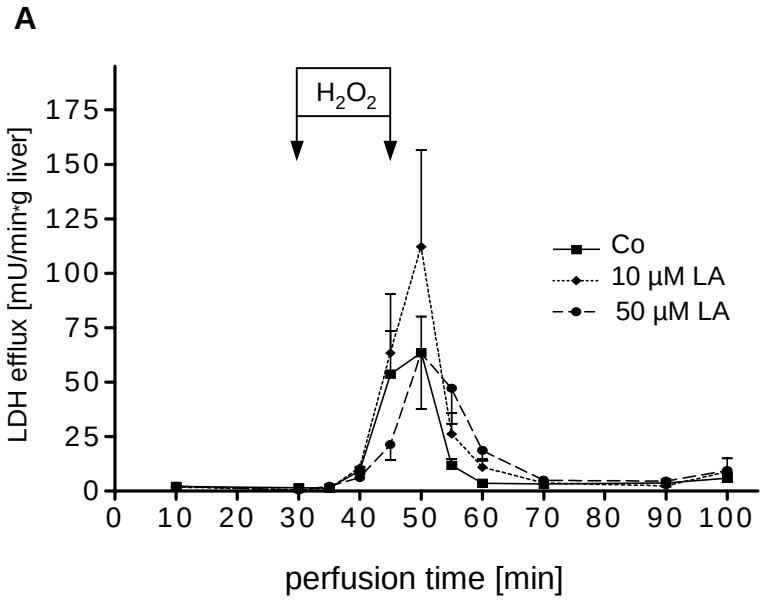
2. Short time infusion of H_2O_2

ROS production is well described to occur during reperfusion after hepatic ischemia. As preliminary experiments approaching ischemia and reperfusion studies, perfusions were performed with a short time infusion of H_2O_2, which is known as a typical member of oxygen radicals during reperfusion (Bilzer and Gerbes, 2000;Rauen *et al.*, 1994;Jaeschke and Farhood, 1991;Okuda *et al.*, 1992).

As a model of ROS production, perfusions with short time infusion of H_2O_2 should elucidate, whether the antioxidant LA is able to reduce H_2O_2-induced liver cell injury.

2.1 No influence of LA application on LDH and PNP efflux

Continuous addition of LA (10 or 50 µM) 10 min after canulating the portal vein did not influence the increased enzyme release of livers stressed by a short time infusion of 500 µM H_2O_2 (Figure 29).



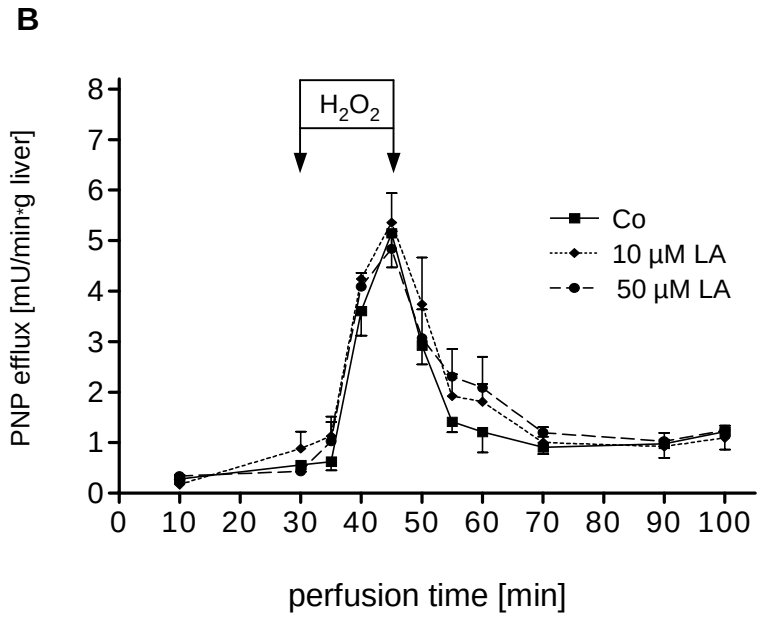


Figure 29: influence of LA application on enzyme efflux after infusion of H_2O_2. *Livers were perfused for 100 min in the absence (Co) or presence of LA (10 or 50 µM), which was added continuously 10 min after canulating portal vein. After 30 min of perfusion, 500 µM H_2O_2 was infused for 15 min. Analysis of LDH (panel A) and PNP (panel B) activity in perfusate was carried out at different perfusion time points as described in "Methods". Data are expressed as enzyme activity in mU x min^{-1} x (g liver)$^{-1}$ and show means ± SEM of n=3 experiments in each group.*

2.2 No influence of LA administration on portal pressure

Application of LA (10 or 50 µM), starting 10 min after canulating portal vein, significantly increased the already enhanced portal pressure at specific time points after infusion of H_2O_2 (Figure 30).

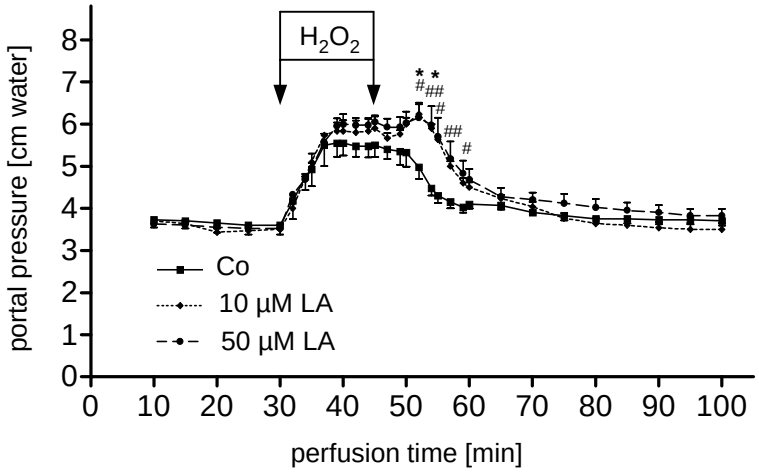


Figure 30: influence of LA application on portal pressure after infusion of H_2O_2. *Livers were perfused for 100 min in the absence (Co) or presence of LA (10 or 50 µM), which was added continuously 10 min after canulating portal vein. After 30 min of perfusion, 500 µM H_2O_2 was infused for 15 min. Portal pressure was monitored throughout perfusion as described in "Methods". Data are expressed as cm water and represent means ± SEM of n=3 experiments in each group. *(#) p<0.05 represent significant differences in the values between untreated and 50 µM (10 µM) LA treated livers.*

2.3 No influence of LA application on bile flow

LA (10 or 50 µM), added continuously 10 min after canulating portal vein, did not significantly alter the H_2O_2 induced decrease of bile flow (Figure 31).

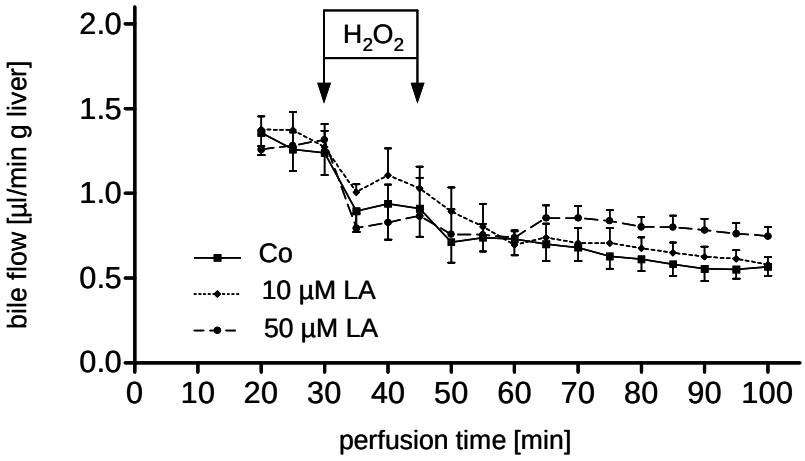


Figure 31: influence of LA application on bile flow after infusion of H_2O_2.

Livers were perfused for 100 min in the absence (Co) or presence of LA (10 or 50 µM), which was added continuously 10 min after canulating portal vein. After 30 min of perfusion 500 µM H_2O_2 was infused for 15 min. Bile was collected every 5 min as described in "Methods". Data are expressed as µl x min^{-1} x (g liver)$^{-1}$ and represent means ± SEM of n=3 experiments in each group.

3. LA reduces IRI of the rat liver

3.1 LA attenuates postischemic sinusoidal efflux of LDH and PNP

In order to investigate an effect of LA on IRI, three different treatment protocols were employed, i.e. continuous, preischemic as well as postischemic administration of LA (Figure 11). Liver cell damage was determined again by measuring release of the cytosolic enzymes LDH and PNP into perfusate. Except for continuous application of LA, LDH and PNP efflux (*Figure 32*, *Figure 33*, *Figure 34*) were significantly reduced in livers treated with 50 µM LA compared to untreated controls. By lowering the dose of LA to 10 µM only little effect on tissue protection could be seen. Raising the dose to 100 µM LA in the pretreatment protocol reduced enzyme release only in the initial reperfusion period.

Comparison of the three treatment protocols showed that preconditioning with LA was the most efficient treatment protocol: enzyme release of livers preconditioned with LA was reduced almost throughout reperfusion. Postischemic and to an even lesser extent, continuous application of LA, reduced LDH and PNP release only at some specific time points during reperfusion. Thus, preconditioning with LA seems to be superior to its postischemic or continuous application.

3.1.1 Continuous LA administration

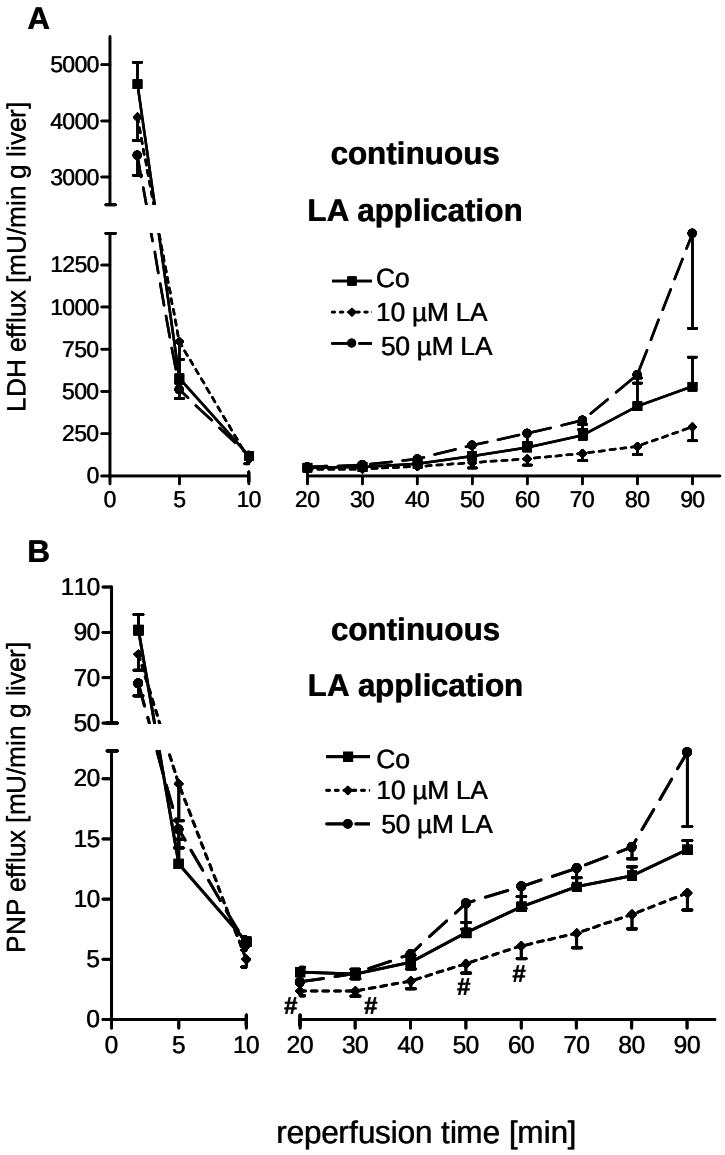


Figure 32: influence of continuous LA application on LDH- and PNP efflux. *Livers were perfused for 30 min in the absence (Co) or presence of LA (10 or 50 µM), which was added 20 min prior to ischemia and continuously after ischemia (60 min, 37°C) during 90 min of reperfusion. Analysis of LDH (panel A) and PNP (panel B) activity in perfusate was carried out at different reperfusion time points as described in "Methods". Data are expressed as enzyme activity in mU x min^{-1} x (g liver)$^{-1}$ and show means ± SEM of n=5 experiments in each group. $^{\#}$ p<0.05 represents significant differences in the values between untreated and 10 µM LA treated livers.*

3.1.2 Preconditioning with LA

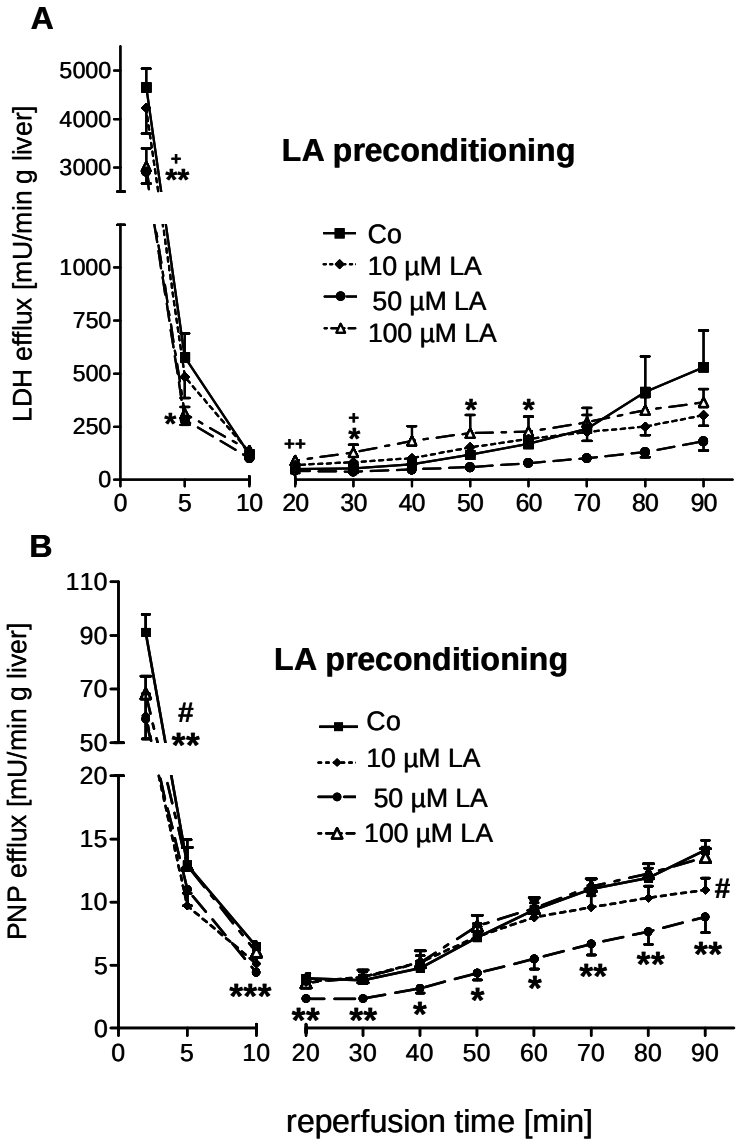


Figure 33: influence of LA preconditioning on LDH- and PNP efflux. *Livers were perfused for 30 min in the absence (Co) or presence of LA (10, 50 or 100 µM), which was added 20 min prior to ischemia (preconditioning). After 60 min of ischemia (WI, 37°C) livers were reperfused for up to 90 min. Analysis of LDH (panel A) and PNP (panel B) activity in perfusate was carried out at different reperfusion time points as described in "Methods". Data are expressed as enzyme activity in mU x min-1 x (g liver)-1 and show means ± SEM of n=5 experiments in each group. *** p<0.001, ** p<0.01, and *(#), [+] p<0.05 represent significant differences in the values between untreated and 50 µM (10 µM) [100 µM] LA treated livers.*

3.1.3 Postischemic LA application

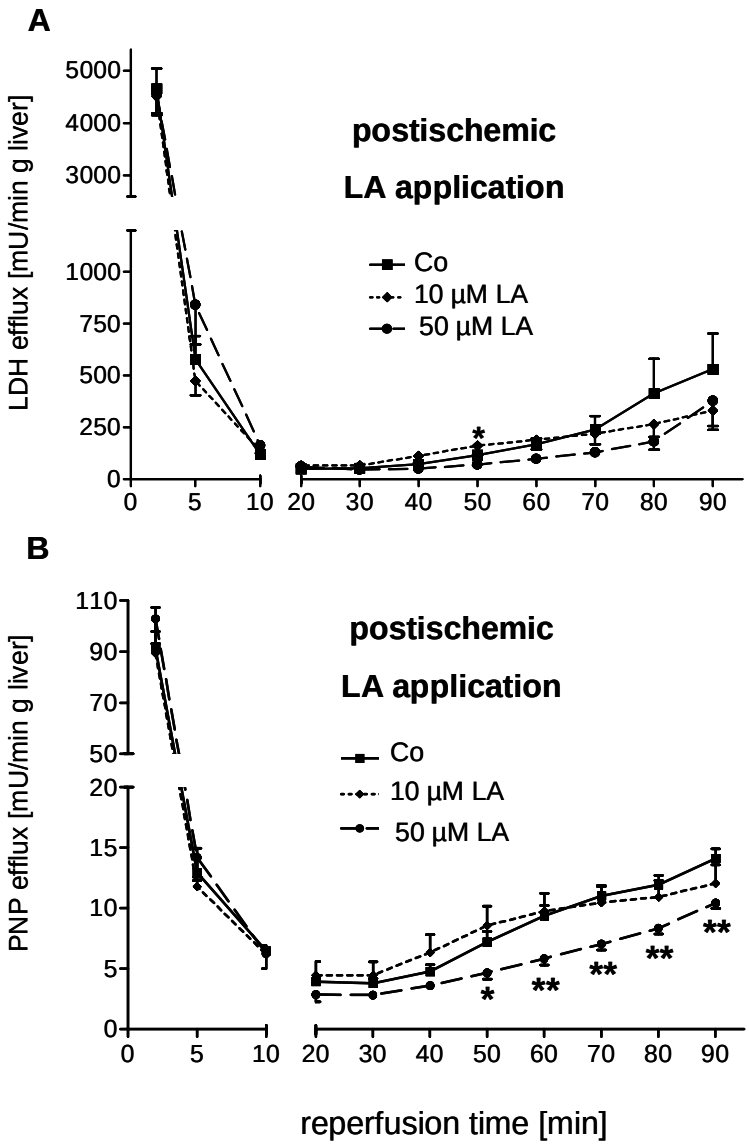


Figure 34: influence of postischemic LA administration on LDH- and PNP efflux. *Livers were perfused for 30 min. After ischemia (WI, 60 min, 37°C) livers were reperfused in the absence (Co) or presence of LA (10 or 50 µM, postischemic application). Analysis of LDH (panel A) and PNP (panel B) activity in perfusate was carried out at different reperfusion time points as described in "Methods". Data are expressed as enzyme activity in mU x min^{-1} x (g liver)$^{-1}$ and show means ± SEM of n=5 experiments in each group. ** p<0.01, and * p<0.05 represent significant differences in the values between untreated and 50 µM LA treated livers.*

3.2 LA preconditioning reduces portal pressure

Portal pressure was strongly increased in the early reperfusion period and slightly but significantly reduced by preconditioning livers with 50 and 100 µM LA (Figure 36). Treatment with 10 µM LA, as well as postischemic and continuous administration of 50 µM LA did not affect portal pressure upon reperfusion (*Figure 35*,*Figure 37*). These findings underline that pretreatment with LA represents the most effective strategy attenuating IRI.

3.2.1 Continuous LA administration

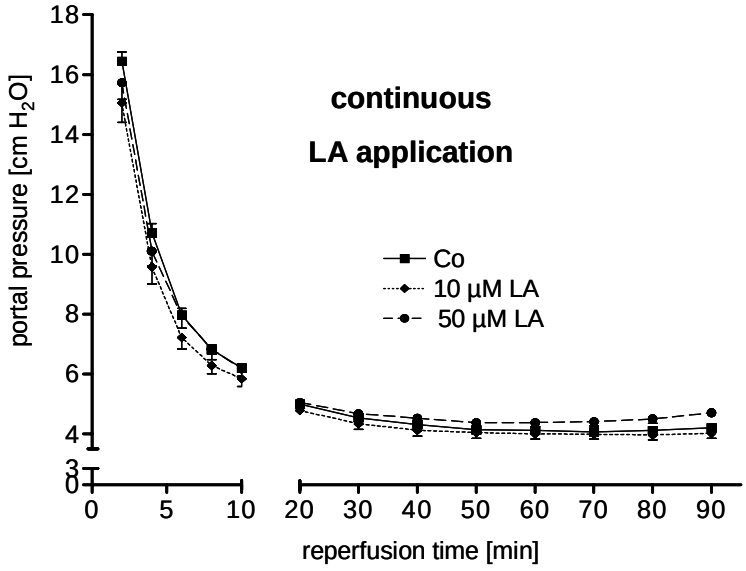


Figure 35: influence of continuous LA administration on portal pressure. *Livers were perfused for 30 min in the absence (Co) or presence of LA (10 or 50 µM), which was added 20 min prior to ischemia and continuously after ischemia (60 min, 37°C) during 90 min of reperfusion. Portal pressure was monitored throughout reperfusion as described in "Methods". Data are expressed as cm water and represent means ± SEM of n=5 experiments in each group.*

3.2.2 Preconditioning with LA

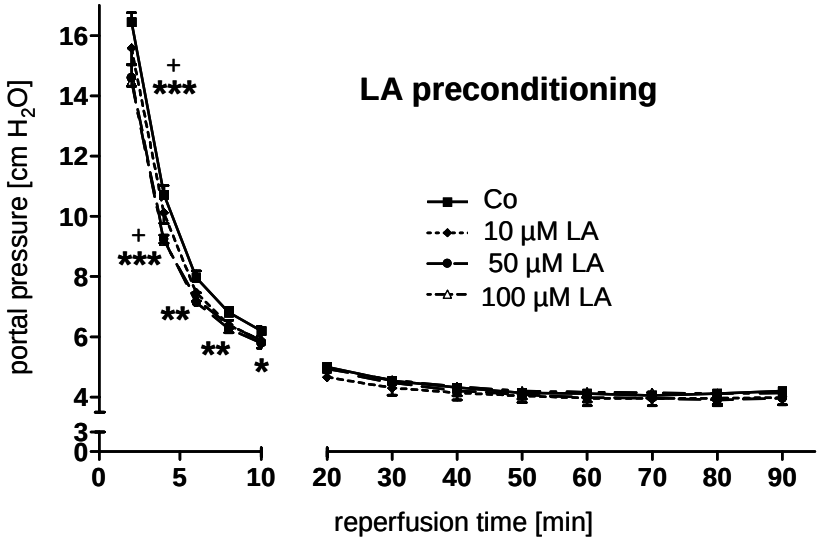


Figure 36: LA preconditioning reduces portal pressure. *Livers were perfused for 30 min in the absence (Co) or presence of LA (10, 50, or 100 µM), which was added 20 min prior to ischemia. After 60 min of ischemia (WI, 37°C), livers were reperfused for up to 90 min. Portal pressure was monitored throughout reperfusion as described in "Methods". Data are expressed as cm water and represent means ± SEM of n=5 experiments in each group. *** p<0.001, ** p<0.01, and * [+] p<0.05 represent significant differences in the values between untreated and 50 µM [100 µM] LA treated livers.*

3.2.3 Postischemic LA application

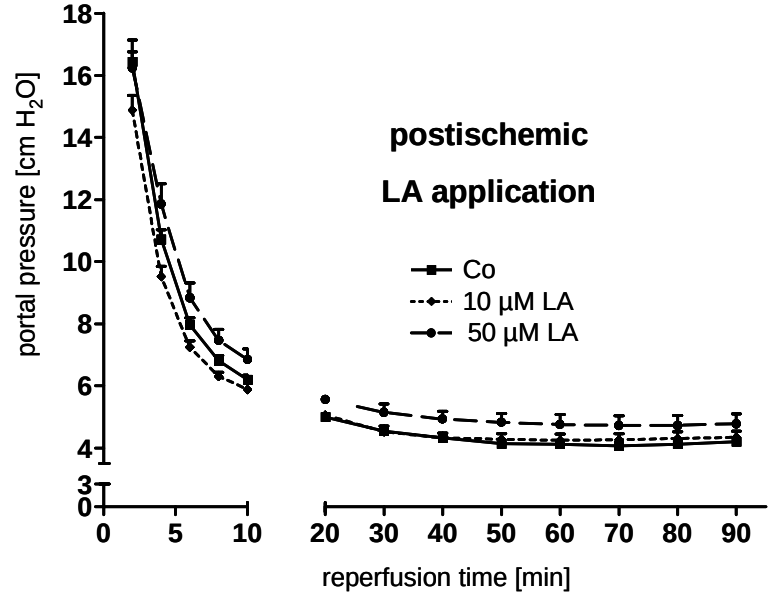


Figure 37: influence of postischemic LA application on portal pressure.

Livers were perfused for 30 min. After ischemia (WI, 60 min, 37°C) livers were reperfused in the absence (Co) or presence of LA (10 or 50 µM, postischemic application). Portal pressure was monitored throughout reperfusion as described in "Methods". Data are expressed as cm water and represent means ± SEM of n=5 experiments in each group.

3.3 No significant influence of LA treatment on bile flow

After 60 min of warm ischemia bile flow dramatically decreased during reperfusion to approx. 10% compared to preischemic levels. None of the treatment protocols and none of the different LA concentrations applied showed a significant increase of bile flow upon reperfusion. In some cases a tendency but no significance of an enhanced bile excretion was obtained (Figure 38,Figure 39, Figure 40).

3.3.1 Continuous LA administration

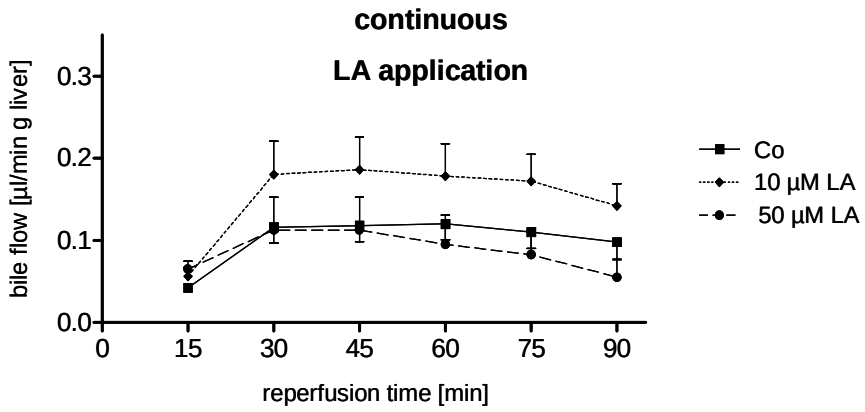


Figure 38: influence of continuous LA treatment on bile flow. *Livers were perfused for 30 min in the absence (Co) or presence of LA (10 or 50 µM), which was added 20 min prior to ischemia (60 min, 37°C) and continuously during reperfusion (90 min). Bile was collected every 15 min during reperfusion as described in "Methods". Data are expressed as µl x min^{-1} x (g $liver)^{-1}$ and represent means ± SEM of n=5 experiments in each group.*

3.3.2 Preconditioning with LA

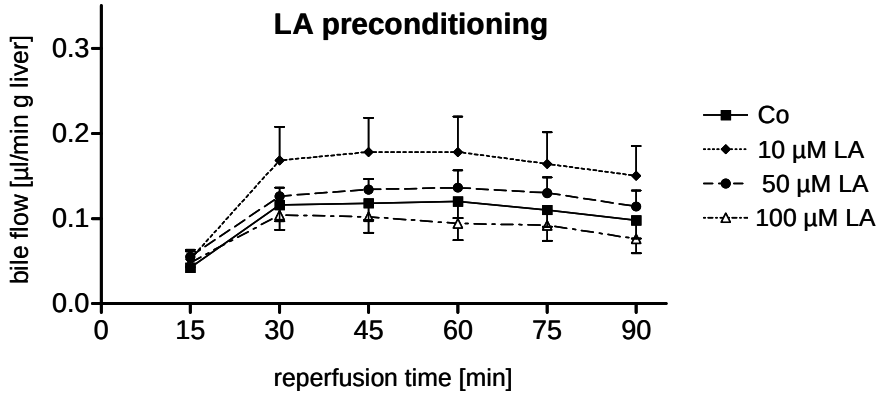


Figure 39: influence of LA preconditioning on bile excretion. *Livers were perfused for 30 min in the absence (Co) or presence of LA (10, 50 or 100 µM), which was added 20 min prior to ischemia. After 60 min of ischemia (WI, 37°C), livers were reperfused for up to 90 min. Bile was collected every 15 min during reperfusion as described in "Methods". Data are expressed as µl x min^{-1} x (g liver)$^{-1}$ and represent means ± SEM of n=5 experiments in each group.*

3.3.3 Postischemic LA application

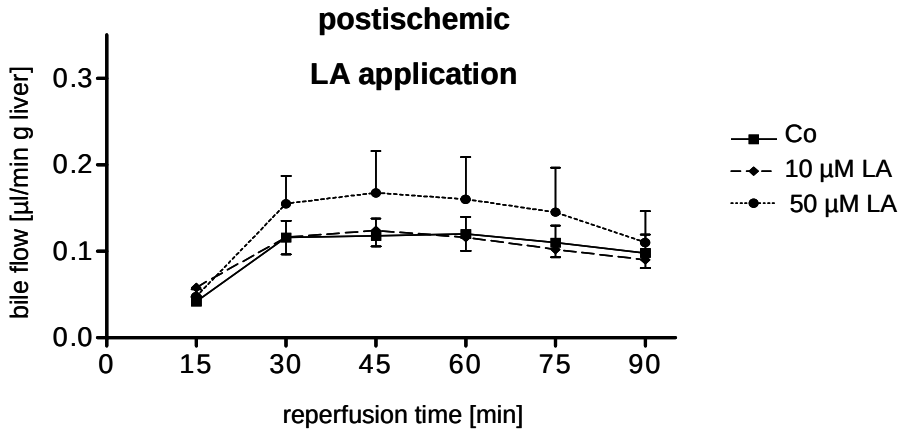


Figure 40: influence of postischemic LA administration on bile excretion. *Livers were perfused for 30 min. After 60 min of ischemia (WI, 37°C) livers were reperfused in the absence (Co) or presence of LA (10 or 50 µM, postischemic application). Bile was collected every 15 min during reperfusion as described in "Methods". Data are expressed as µl x min^{-1} x (g liver)$^{-1}$ and represent means ± SEM of n=5 experiments in each group.*

The results so far provided evidence that LA is a potent inhibitor of IRI in rat livers and emerged that preconditioning livers with LA is more effective than postischemic or continuous treatment.

Further investigations should now characterize the hepatoprotective action of

LA. These mechanistic studies were undertaken by applying the most promising LA treatment scheme - the preconditioning protocol.

3.4 LA metabolism during hepatic IRI

Before examining the mechansisms of LA preconditioning amounts of LA in livers and perfusates were determined in order to know how much LA is responsible for the shown preconditioning effect. In terms of interpreting further results it was also necessary to get information about possible metabolites of LA and their quantity.

Knowing the concentration of LA present in the different samples made it also possible to exclude any interaction with the applied assays.

3.4.1 LA metabolites

In the literature only little information is available about LA metabolites in rats. The publication of Spence *et al.* (Spence and McCormick, 1976) describes the identification of the following metabolites found in urine after intraperitoneal injection of LA: bisnorlipoic acid, β-hydroxybisnorlipoic acid, tetranorlipoic acid (TNLA), β-ketolipoic acid, and β-ketobisnorlipoic acid (see Figure 5). Peinado et al. (Peinado *et al.*, 1989) report that 30% of the radioactivity (^{35}S-labelled LA) administered to rat livers in a single-pass perfusion appear in liver tissue as metabolites of LA or LA itself (see also B.3.3.2).

In this work only two of the above mentioned metabolites of LA were detected in quantifiable concentrations in liver as well as in perfusate: LA and TNLA. Therefore, presence of other known metabolites and presence of dihydrolipoic acid (DHLA) could be excluded by the analyses performed.

In samples of untreated livers LA was not detectable.

3.4.2 LA and TNLA contents in liver after LA preconditioning

The amounts of LA and TNLA in livers peaked after applying LA 20 min prior to ischemia reaching maximal values of 38.6 ± 7.9 nmol LA/g liver, which was equivalent to 1.21 ± 0.26% of total LA amount administered and 36.9 ± 8.9 nmol TNLA/g liver (*Figure 41*). LA and TNLA contents decreased continuously in the course of the experiment, i.e. Pre-I, after WI, and after 45 and 90 min of reperfusion.

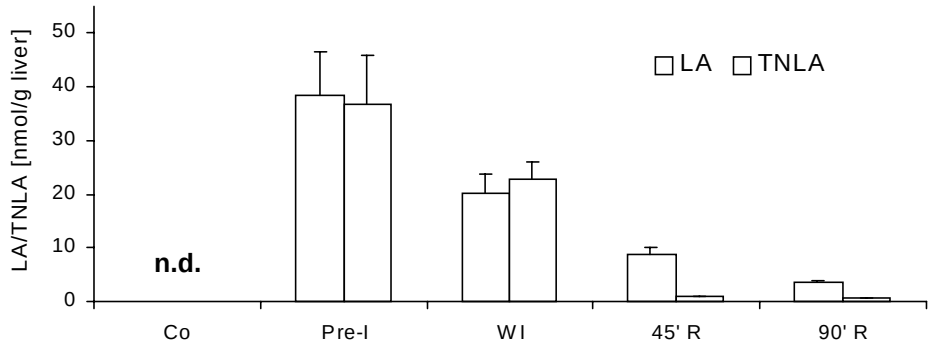


Figure 41: LA and TNLA contents in liver after LA preconditioning. *Livers were pretreated with 50 µM LA prior to ischemia (WI, 60 min, 37°C) followed by reperfusion for up to 90 min. LA and TNLA contents in liver were analyzed by HPLC (see "Methods") after 30 min of perfusion (Pre-I), 60 min of WI, 45 and 90 min of reperfusion (R). LA and TNLA contents in liver are expressed as nmol/g liver and are shown as means ± SEM of n=3 organs in each group. In untreated livers LA was not detectable (n.d.).*

3.4.3 LA and TNLA contents in perfusate after LA pre- or postischemic treatment

Direct intravenous administration of antioxidants during reperfusion has been described to reduce hepatic IRI (Bilzer *et al.*, 1999). Therefore, LA and TNLA contents in perfusate were measured to describe the differences of LA preconditioning versus postischemic treatment concerning their antioxidative potential in the vasculature. Figure 42 shows that measurable amounts of LA and TNLA in perfusate were present before, during, and initially after ischemia of LA pretreated organs. After 10 min of reperfusion only traces of TNLA were detectable. As expected, postischemic LA treatment showed a continuous presence and higher concentrations of both LA and TNLA in perfusate during reperfusion.

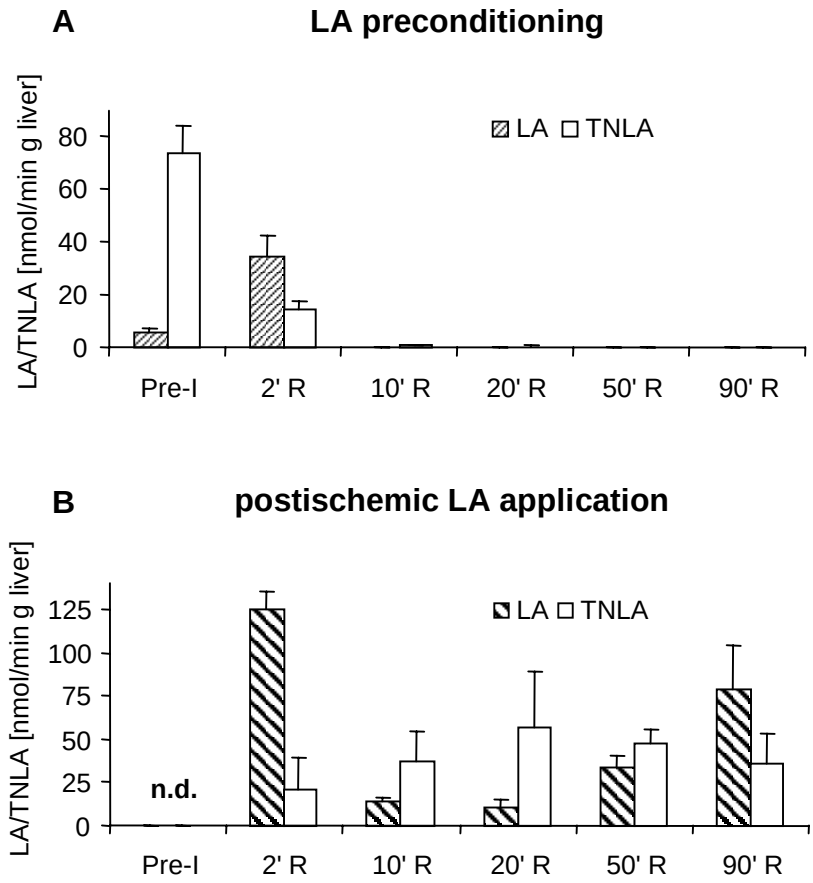


Figure 42: LA and TNLA contents in perfusate after LA pre- and postischemic treatment. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added either 20 min prior to ischemia (preconditioning) or given continuously after ischemia (60 min, 37°C) during 90 min of reperfusion (postischemic application). LA and TNLA contents in perfusate were analyzed by HPLC (see "Methods") at the time points indicated. LA and TNLA contents in liver are expressed as nmol/g liver and are shown as means ± SEM of n=3 organs in each group. In perfusates of untreated livers LA and TNLA were not detectable (n.d.).*

4. Mechanisms of LA preconditioning

In order to investigate the mechanisms of LA preconditioning, GSH metabolism was examined first. The facts that application of GSH has been shown to protect from IRI (Bilzer *et al.*, 1999) and that LA has been described to increase GSH concentration in different cell types (Han *et al.*, 1997) and in mouse and rat liver (Busse *et al.*, 1992;Arivazhagan *et al.*, 2001) lead to the hypothesis that the protective action of LA could be explained, at least in part, by increasing GSH.

4.1 CYS and CYSSX contents in liver

Due to reports in the literature stating (Han *et al.*, 1997) that LA increases GSH synthesis by improving cystine utilization the following experiments should show, if this thesis is also true for the applied IRI model (see B.3). Han and collegues described that GSH synthesis is increased by LA and DHLA, restoring the cysteine pool of cells, which is known as the limitating factor in GSH synthesis. *Figure 43* demonstrates that LA preconditioning did not significantly influence cysteine (CYS) as well as oxidized cysteine (CYSSX) levels in liver at any time point analyzed.

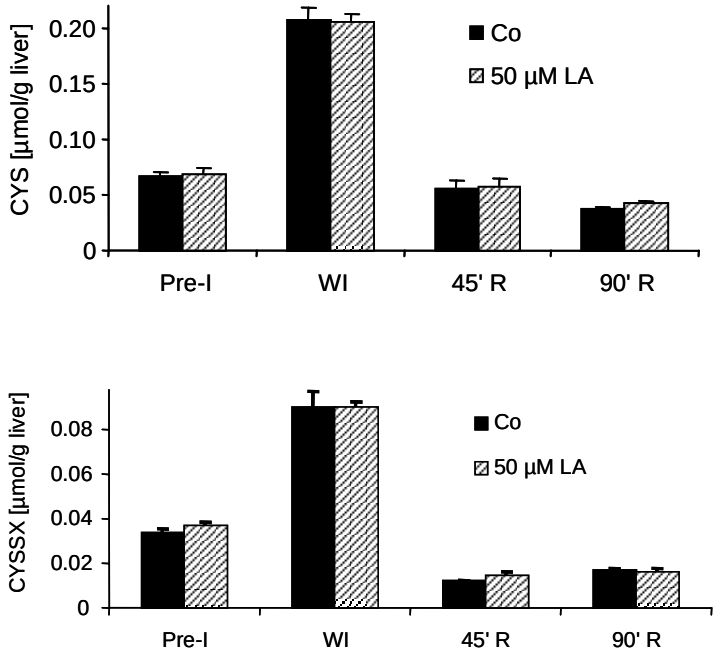


Figure 43: influence of LA pretreatment on cysteine (CYS) and oxidized cysteine (CYSSX) contents in liver.

Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (Pre-I). After 60 min of ischemia (WI, 37°C), livers were reperfused (R) for up to 90 min. CYS and CYSSX contents in liver were determined by HPLC (see "Methods") and are expressed as µmol/g liver. Data show means ± SEM of n=5 experiments.

4.2 CYS and CYSSX contents in perfusate

Also in perfusate cysteine concentrations remained unaltered by LA pretreatment compared to untreated control livers, whereas contents of oxidized cysteine in perfusate were significantly reduced during IRI (Figure 44).

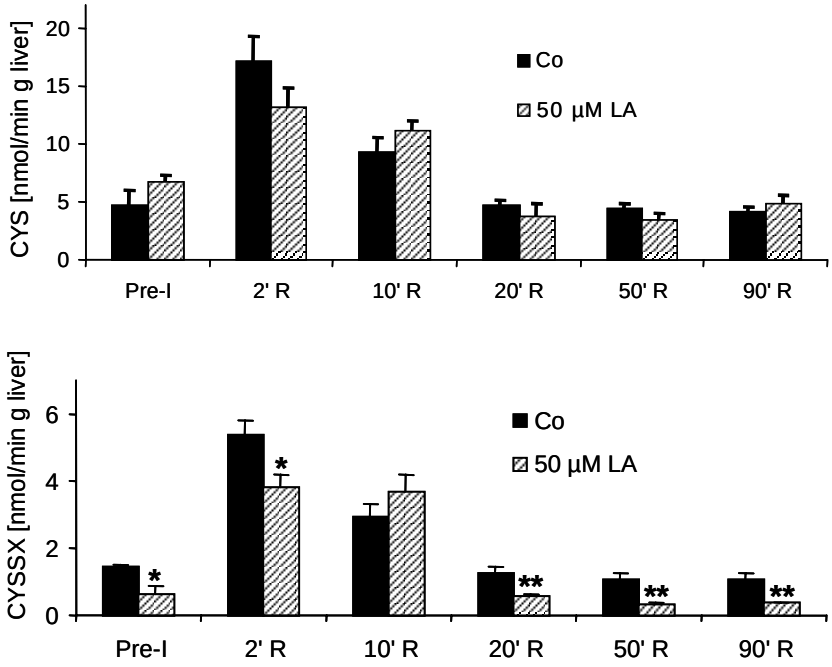


Figure 44: influence of LA pretreatment on CYS and CYSSX contents in perfusate. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (Pre-I). After 60 min of ischemia (WI, 37°C), livers were reperfused (R) for up to 90 min. CYS and CYSSX contents in livers were determined by HPLC (see "Methods") and are expressed as nmol x min^{-1} x (g liver)$^{-1}$. Data show means ± SEM of n=5 experiments. ** p<0.01, * p<0.05 represent significant differences to untreated livers.*

4.3 GSH and GSSX contents in liver

Interestingly, in contrast to the findings in the literature (Han *et al.*, 1997;Busse *et al.*, 1992), LA pretreatment significantly decreased GSH concentrations in liver during ischemia (WI) compared to untreated controls (*Figure 45*). At every other time point GSH values in LA preconditioned organs did not significantly differ from untreated livers.

Levels of oxidized GSH (GSSX) were significantly reduced by LA preconditioning in the preischemic period, whereas at each other time point GSSX concentrations were comparable to control organs (*Figure 45*).

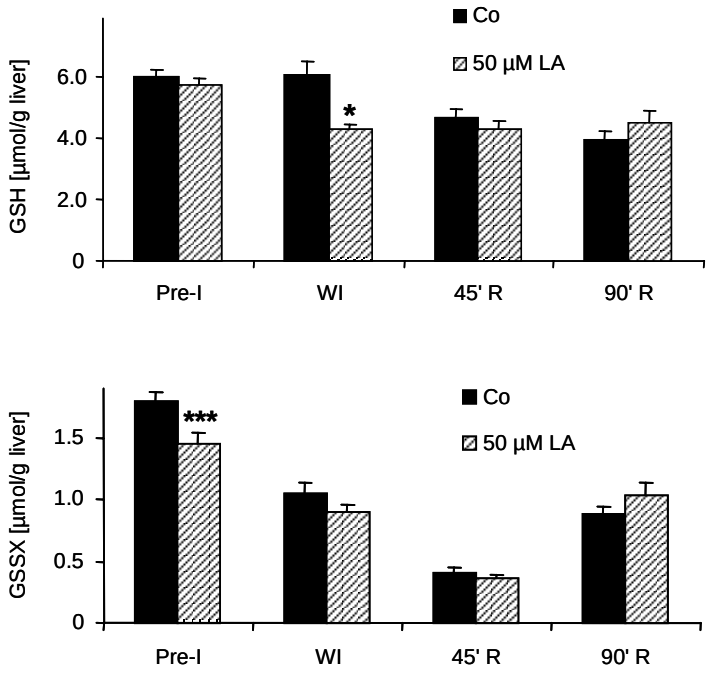


Figure 45: influence of LA pretreatment on GSH and GSSX contents in liver. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (Pre-I). After 60 min of ischemia (WI, 37°C), livers were reperfused (R) for up to 90 min. GSH and GSSX contents in liver were determined by HPLC (see "Methods") and are expressed as µmol/g liver. Data show means ± SEM of n=5 experiments. *** p<0.001, * p<0.05 represent significant differences to untreated livers.*

4.4 GSH and GSSX contents in perfusate

The amounts of GSH and GSSX found in perfusate of LA pretreated livers did not significantly differ from untreated controls at any time point analyzed (*Figure 46*).

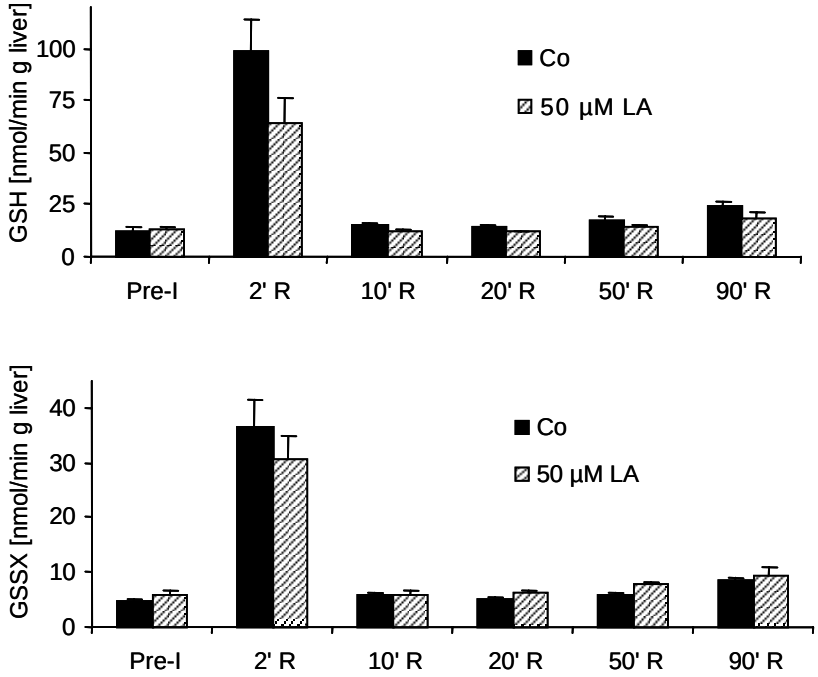


Figure 46: influence of LA pretreatment on GSH and GSSX contents in perfusate. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (Pre-I). After 60 min of ischemia (WI, 37°C), livers were reperfused (R) for up to 90 min. GSH and GSSX contents in perfusate were determined by HPLC (see "Methods") and are expressed as* nmol x min^{-1} x $(g\ liver)^{-1}$*. Data show means ± SEM of n=5 experiments.*

4.5 LA preconditioning reduces activation of NF-κB and AP-1

It is well known that the redox-sensitive transcription factors NF-κB and AP-1 are activated in IRI with the consequence of an increased expression of proinflammatory mediators (Tsoulfas and Geller, 2001;Fan *et al.*, 1999;Bradham *et al.*, 1997;Yamada *et al.*, 2000). Therefore, the effect of LA pretreatment on NF-κB and AP-1 activity was investigated. After 90 min of reperfusion NF-κB activation was significantly decreased in LA preconditioned livers (Figure 47, panel A), whereas activation at any earlier time point was not affected. AP-1 DNA binding activity was significantly reduced after WI and 90

min of reperfusion (Figure 47, panel B).

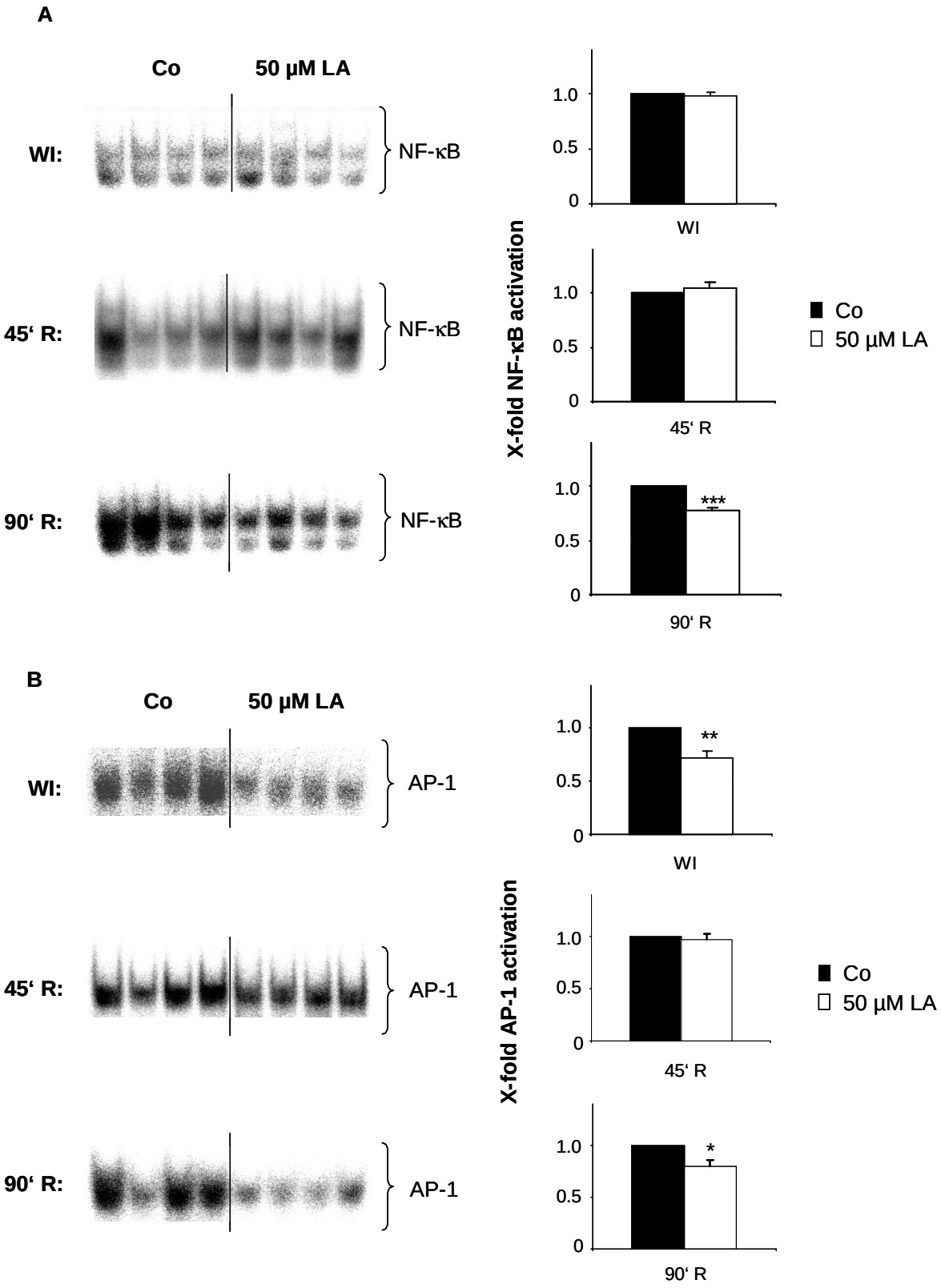


Figure 47: effect of LA preconditioning on postischemic NF-κB and AP-1 activation.

*Livers were untreated (Co) or preconditioned with 50 µM LA 20 min prior to ischemia. After 60 min of ischemia (WI), livers were reperfused for up to 90 min. Binding activity of nuclear protein to the radiolabeled consensus binding sequences of NF-κB (panel A) and AP-1 (panel B) were assessed by EMSA (see "Methods") after WI, 45 and 90 min of reperfusion (R). Data show one representative gel shift experiment for each time point on the left side. Bars on the right side show densitometrical analysis of the shifts, whereby NF-κB/AP-1 activation of LA preconditioned livers is expressed as x-fold of the values in the correlating untreated group. Data is shown as means ± SEM of n=4 livers in each group, analyzed in two independent shifts. *** p<0.001, ** p<0.01 and * p<0.05 represent significant differences in the values between untreated and LA pretreated livers.*

4.6 Elevated ATP content in LA pretreated livers

ATP content of livers is an indicator of their energetic status. As ATP depletion is a typical biochemical feature of ischemic periods (see B.2.1.1) it was interesting to see, whether LA administration influences this parameter. In the course of ischemia and reperfusion ATP content of livers significantly decreased (*Figure 48*). As shown in *Figure 48*, preconditioning with LA significantly increased ATP contents compared to control livers after WI and at the end of the reperfusion period.

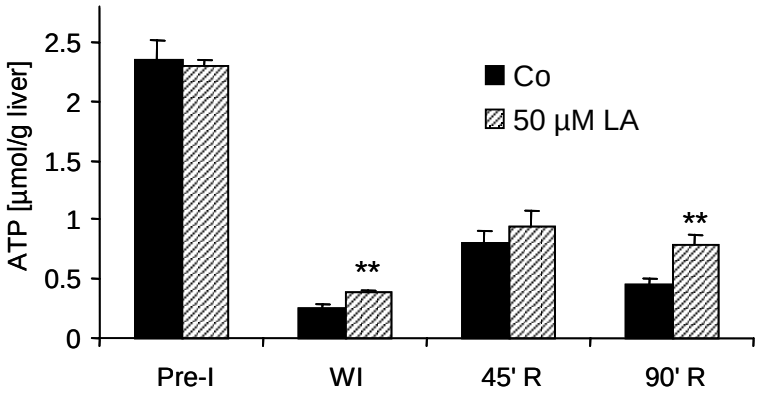


Figure 48: effect of LA preconditioning on ATP content in liver. *Livers were perfused in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (WI, 60 min, 37°C), followed by reperfusion for up to 90 min. ATP content in liver was analyzed after 30 min of perfusion (Pre-I), after 60 min of warm ischemia (WI), and after 45 and 90 min of reperfusion (R) as described in "Methods". Data are expressed as µmol/g liver and show means ± SEM of n=5 experiments. ** p<0.01 represents significant differences of the values between untreated and LA pretreated livers.*

4.7 Phospho-p38 MAPK in IRI

The role of p38 MAPK activation in IRI is controversially discussed (Abe *et al.*, 2000). There is evidence that ischemic and hypoxic preconditioning are mediated *via* the activation of p38 MAPK (Weinbrenner *et al.*, 1997;Maulik *et al.*, 1998;Nakano *et al.*, 2000;Ping and Murphy, 2000;Carini *et al.*, 2001).

Before evaluating a possible influence of LA treatment, the activation pattern of p38 MAPK in this model of IRI was investigated. *Figure 49* shows that phosphorylation of p38 MAPK slightly but significantly decreased during ischemia and reperfusion.

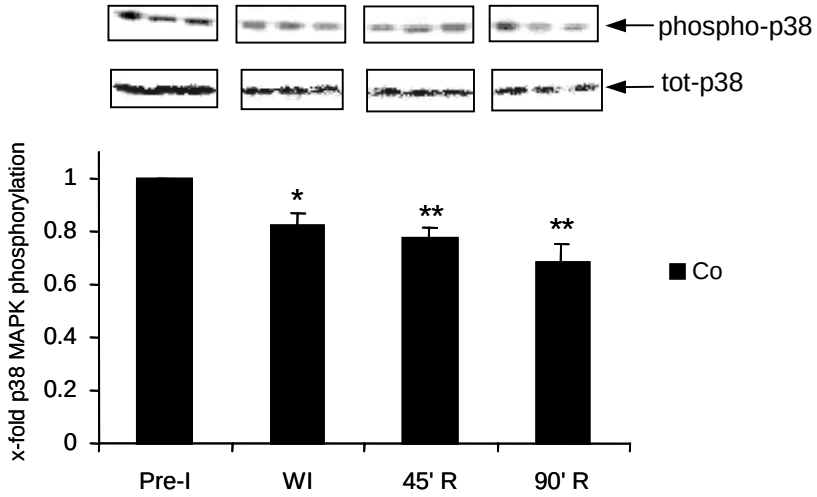


Figure 49: time course of p38 MAPK activation. *Livers were perfused for 30 min with KH solution prior to ischemia (WI, 60 min, 37°C). Western blot analysis with antibodies against phosphorylated (phospho-p38) and total p38 MAPK (tot-p38) (see "Methods") was performed after 30 min of perfusion (Pre-I), after 60 min of WI, and after 45 and 90 min of reperfusion. One representative Western blot is shown. Western blots were analyzed densitometrically, whereby p38 MAPK phosphorylation during ischemia and reperfusion was expressed as x-fold of the values in the preischemic group. Values are shown as means ± SEM of n=3 livers in each group, analyzed in two independent Western blots. ** p<0.01, * p<0.05 represent significant differences of the values between preischemic and postischemic untreated livers.*

4.7.1 LA preconditioning does not affect activation of p38 MAPK

In order to investigate p38 MAPK as a crucial kinase in LA preconditioning we determined its activation compared to untreated organs. Pretreatment of livers with LA, however, exerted no significant effect on phosphorylation of p38 MAPK (*Figure 50*). Thus, p38 MAPK activation does not seem to be involved in LA preconditioning.

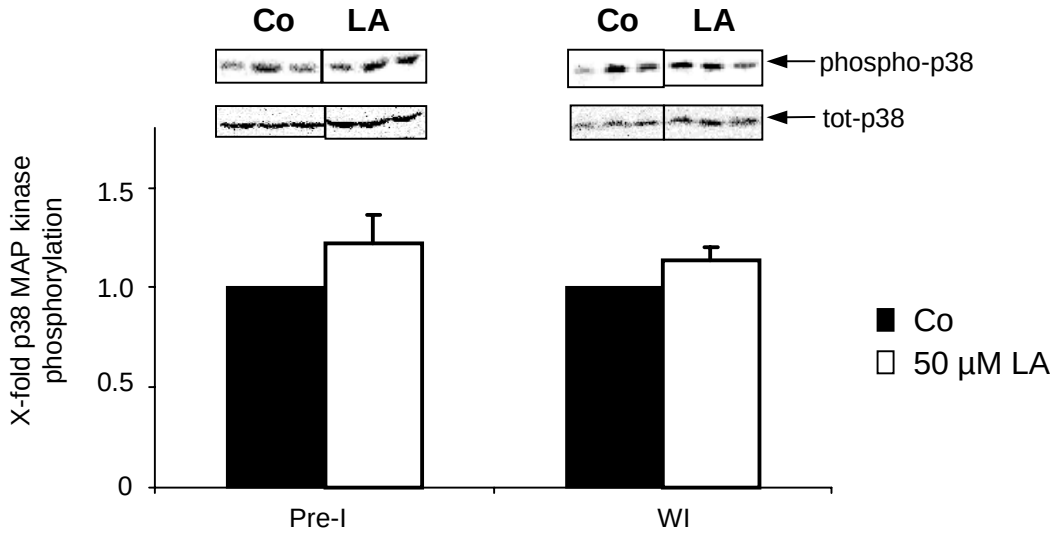


Figure 50: influence of LA preconditioning on p38 MAPK phosphorylation. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (WI, 60 min, 37°C). Western blot analysis with antibodies against phosphorylated (phospho-p38) and total p38 MAPK (tot-p38) (see "Methods") was performed after 30 min of perfusion (Pre-I) and after 60 min of WI. One representative Western blot is shown for each time point. Western blots were analyzed densitometrically, whereby p38 MAPK phosphorylation in LA preconditioned livers was expressed as x-fold of the values in the untreated group. Values are shown as means ± SEM of n=5 livers in each group, analyzed in two independent Western blots.*

4.8 Phospho-Akt in IRI

Activation of another kinase, Akt (protein kinase B), has been described possessing cytoprotective potential (Chan *et al.*, 1999;Leslie *et al.*, 2001), but has never been related to hepatic IRI before. *Figure 51* shows a tremendous increase of Akt phosphorylation during reperfusion compared to preischemic values.

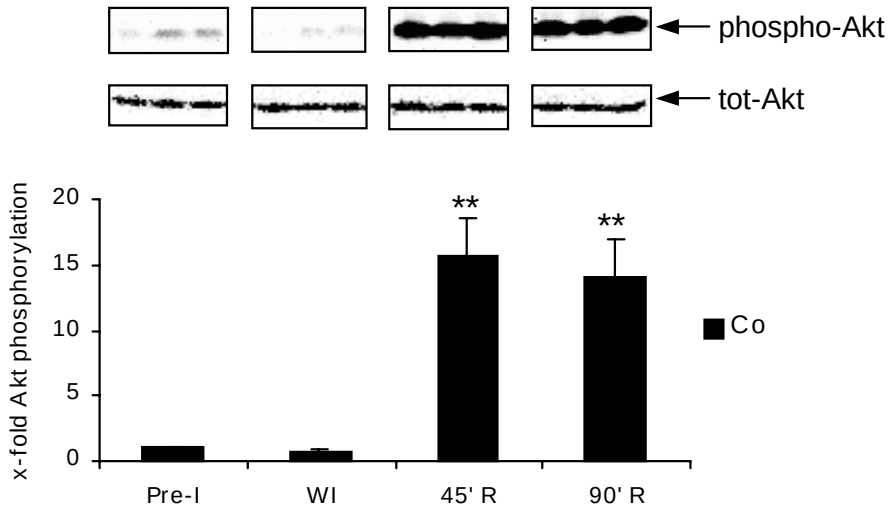


Figure 51: time course of Akt activation. *Livers were perfused for 30 min with KH solution prior to ischemia (WI, 60 min, 37°C). After ischemia livers were reperfused for up to 90 min. Western blot analysis with antibodies against phosphorylated (phospho-Akt) and total Akt (tot-Akt) (see "Methods") was performed after 30 min of perfusion (Pre-I), after 60 min of WI, and after 45 and 90 min of reperfusion. One representative Western blot is shown. Western blots were analyzed densitometrically, whereby Akt phosphorylation during ischemia and reperfusion was expressed as x-fold of the values in the preischemic group. Values are shown as means ± SEM of n=3 livers in each group, analyzed in two independent Western blots. ** p<0.01 represents significant differences of the values between preischemic and postischemic livers.*

4.8.1 LA preconditioning increases phosphorylation of Akt

Akt has been described being involved in ischemic preconditioning of the rat heart (Tong *et al.*, 2000) underlining the cytoprotective potential of this kinase. *Figure 52* shows that pretreatment of livers with LA significantly increased activation of Akt at the end of ischemia and reperfusion.

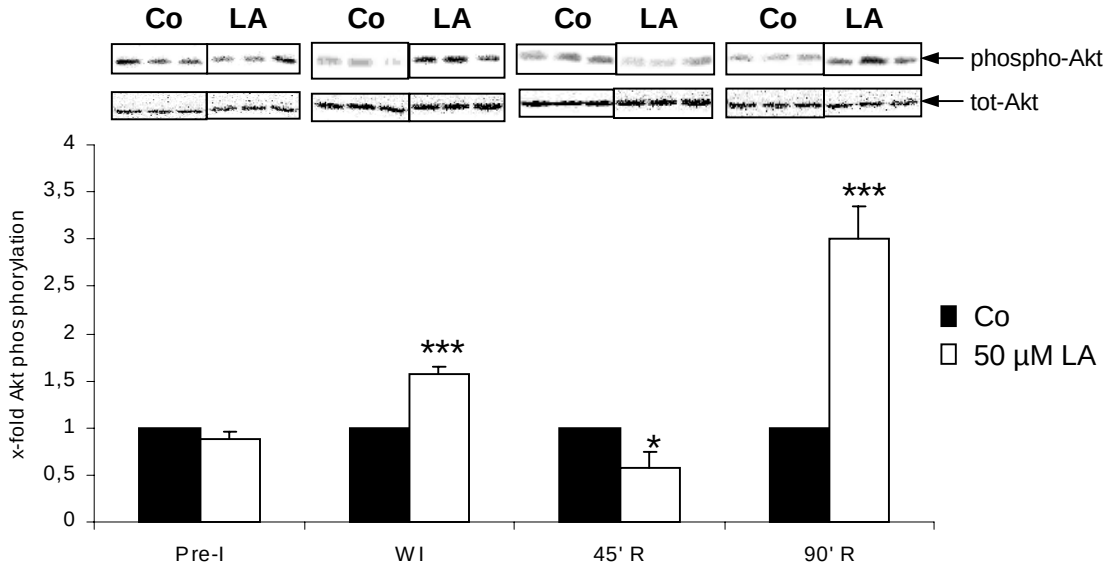


Figure 52: LA preconditioning effect on Akt (protein kinase B) activation. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA, which was added 20 min prior to ischemia (WI, 60 min, 37°C). Western blot analysis (see "Methods") was performed detecting phosphorylated (phospho-Akt) and total Akt (tot-Akt) after 30 min of perfusion (Pre-I), after 60 min of WI, and after 45 and 90 min of reperfusion (R). One representative Western blot is shown for each time point. Western blots were analyzed densitometrically, whereby Akt phosphorylation in LA preconditioned livers is expressed as x-fold of means in the untreated group. Values are shown as means ± SEM of n=5 in each group, analyzed in two independent Western blots. *** p<0.001, * p<0.05 represent significant differences to untreated livers.*

4.9 Inhibition of the PI-3K/Akt pathway abrogates the protective effect of LA preconditioning

In order to determine a causal relationship between Akt activation and hepatoprotection by LA preconditioning, livers were co-treated with LA and the PI-3 kinase inhibitor wortmannin (WM, 100 nM). Since Akt represents the downstream target of PI-3 kinase, it is well established that administration of WM blocks Akt activation (Gentilini *et al.*, 2000;Chan *et al.*, 1999;Tong *et al.*, 2000). LDH and PNP release were again taken as indicators for cell damage. Application of WM alone for 20 min prior to ischemia did not affect postischemic cell damage (data not shown). *Figure 53* shows that LDH and PNP efflux were significantly reduced in LA pretreated compared to untreated livers as also seen in *Figure 33*. Importantly, simultaneous treatment with WM blocked the protection of LA preconditioning. These data provide evidence that LA preconditioning reduces IRI *via* the PI-3K/Akt kinase pathway.

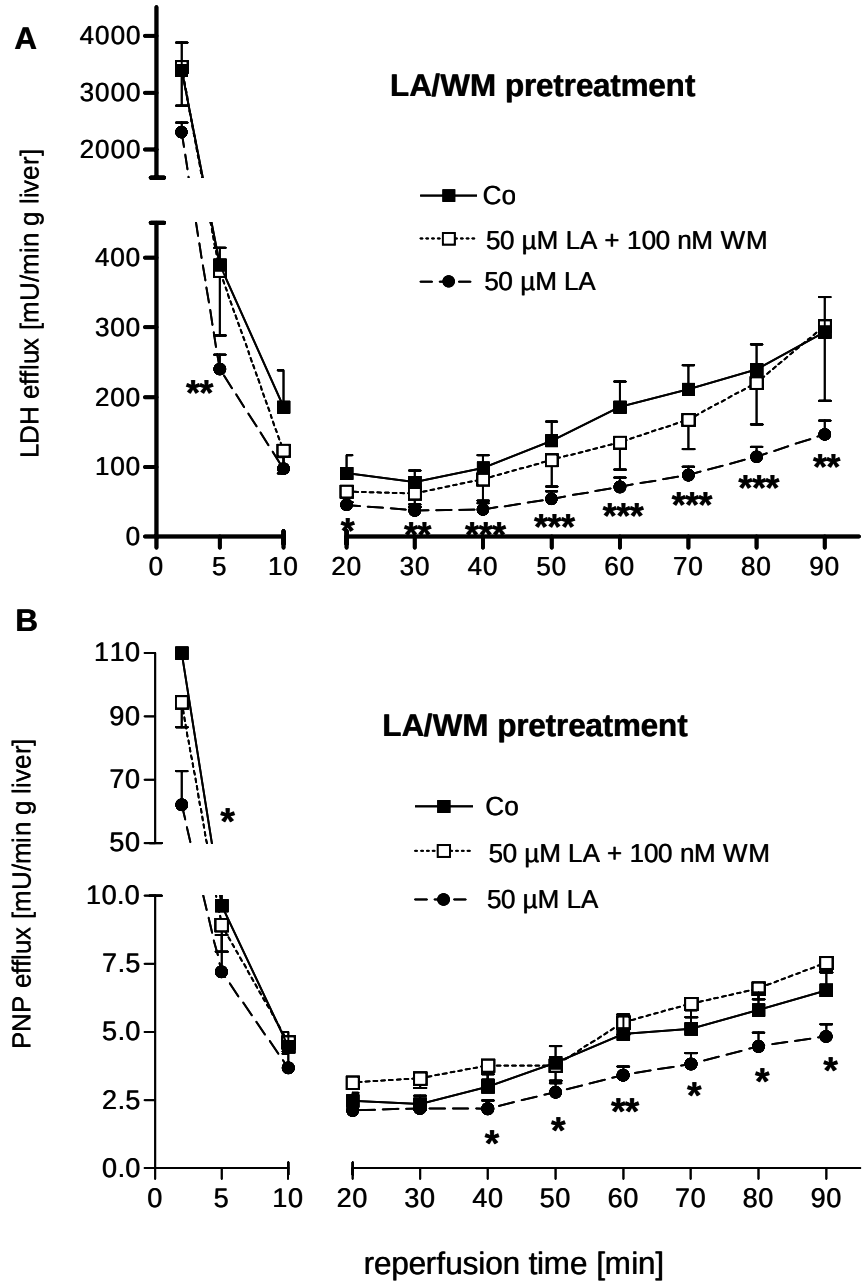


Figure 53: LDH- and PNP efflux after pretreatment with LA and WM. *Livers were perfused for 30 min in the absence (Co) or presence of 50 µM LA with or without 100 nM WM, which were added simultaneously 20 min prior to ischemia. After 60 min of warm ischemia (WI) livers were reperfused for 90 min. Analysis of lactate dehydrogenase (LDH, panel A) and purine nucleoside phosphorylase (PNP, panel B) activity was carried out immediately after collecting perfusate as described in "Methods". Data are expressed as enzyme activity in mU x min^{-1} x (g liver)$^{-1}$ weight and show means ± SEM of n=5 livers in each group. *** p<0.001, ** p<0.01, and * p<0.05 represent significant differences between LA treated and untreated livers.*

4.10 LA preconditioning does not alter caspase-3-like activity

Due to the fact that Akt is typically described as an antiapoptotic kinase (Chan *et al.*, 1999), the impact of LA pretreatment on apoptotic processes was determined. Activation of the effector caspase-3 is considered essentiel for most apoptotic processes (Gujral *et al.*, 2001;Cohen, 1997). Therefore, caspase-3-like activity is one potential target evaluating apoptotis in liver.

In fact, elevated caspase-3-like activity after 45 and 90 min of reperfusion was observed. However, LA preconditioning did not significantly affect caspase-3-like activity during reperfusion (see *Figure 54*).

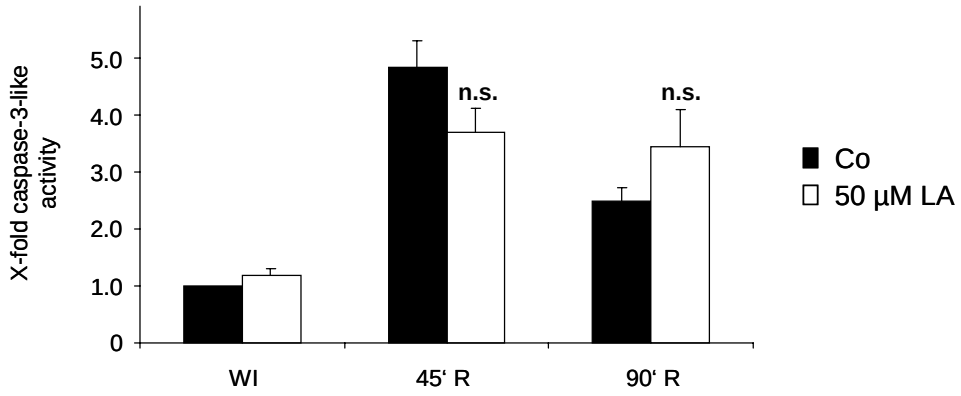


Figure 54: caspase-3-like activities during reperfusion in LA preconditioned livers. *Livers were untreated (Co) or preconditioned with 50 µM LA. 60 min of warm ischemia (WI) were followed by reperfusion (R) for up to 90 min. Caspase-3-like activity was determined as described in “Methods”. Data are expressed as x-fold caspase-3-like activity of untreated livers after 60 min of WI and show means ± SEM of n=5 experiments. n.s. expresses no statistical significance between untreated and LA pretreated livers.*

5. RAW 264.7 murine macrophages

The data shown so far provide mechanisms of LA action in IRI up to 90 min of reperfusion. However, there is overwhelming evidence that IRI continues, with Kupffer cells and neutrophils as the main sources for a proinflammatory response (Fan *et al.*, 1999). In this context, activated KC produce a huge amount of NO by iNOS induced after NF-κB activation during reperfusion (Hur *et al.*, 1999;Tsoulfas and Geller, 2001;Kiemer A.K., 2002).

As the inhibition of NF-κB at the end of reperfusion (see D.4.5) suggested an influence of LA also on later IRI events, such as inhibition of iNOS induction, a murine macrophage cell culture model (RAW 264.7) was chosen to get first hints on this hypothesis. The applied IRI model was unsuitable to determine late reperfusion events (>5 h) as already mentioned in the introduction (see B.2.1.5).

KC belonging to the mononuclear phagocyte system of the liver share many functions with macrophages. Upon inflammatory stimuli, such as lipopolysaccharide (LPS), KC as well as macrophages trigger signals for the production of a variety of bioactive substances. Both cell types lead to an increased NO production *via* activation of NF-κB coupled to the induction of iNOS after LPS treatment (Kiemer A.K., 2002). Therefore, RAW macrophages were used as a model to obtain first informations about the potential of LA to influence KC activation.

5.1 Inhibition of LPS-induced nitrite accumulation by LA

RAW 264.7 macrophages were stimulated with LPS (1 µg/ml) for 20 h to evoke iNOS induction. Co-treatment of cells with LA significantly reduced nitrite accumulation in doses ≥ 5 µg/ml in RAW 264.7 macrophages *(Figure 55*).

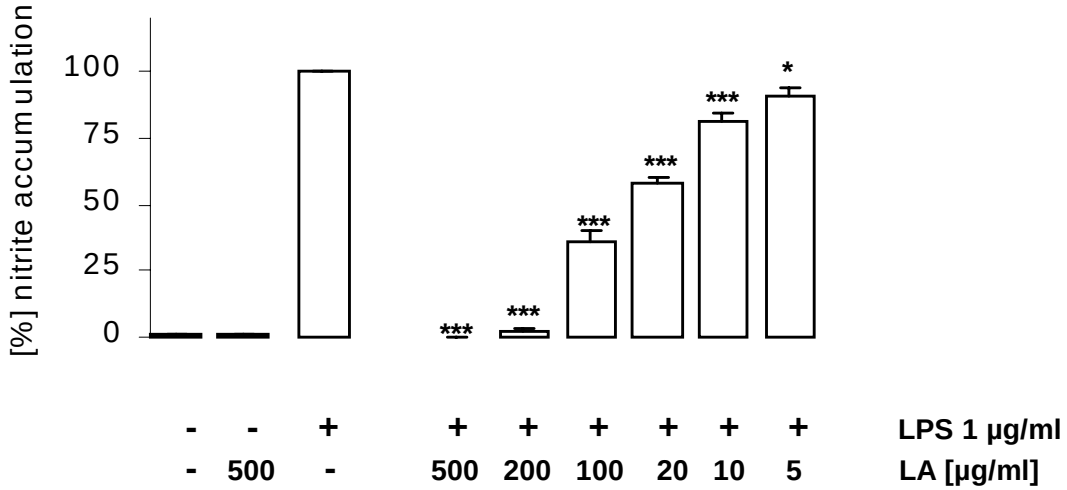


Figure 55: dose dependent inhibition of LPS-induced nitrite production in RAW 264.7 by LA. *RAW 264.7 macrophages were cultured for 20 h in either medium alone or in medium containing LPS (1 µg/ml) in the absence or presence of LA (5-500 µg/ml). Nitrite accumulation was determined as described in "Methods". Data are expressed as percentage of nitrite accumulation in cells activated with 1 µg/ml LPS (100%) and show means ± SEM. of one or two independent experiments performed in triplicates. *** p<0.001 and * p<0.05 represent significant differences compared to the values in LPS activated cells.*

5.2 Interaction of LA with NO produced *in situ*

In order to determine whether reduced nitrite concentrations in supernatants of LA treated cells were due to a direct reaction of LA with NO due to the radical scavenging properties of LA (Biewenga *et al.*, 1997;Biewenga and Bast, 1995;Packer *et al.*, 1997;Packer *et al.*, 1995) the following experiment was performed: The NO donor sodium nitroprusside (SNP) was added to the cells simultaneously with LA and nitrite accumulation was determined after 1.5, 2.5, and 4.5 h. These experimental conditions showed a slightly but significantly reduced nitrite concentration in the presence of LA after 1.5 h (*Figure 56*). LA did not have any influence on nitrite formation at later time points. These data suggest that LA to a certain extent directly interacts with NO and thereby inhibits nitrite formation.

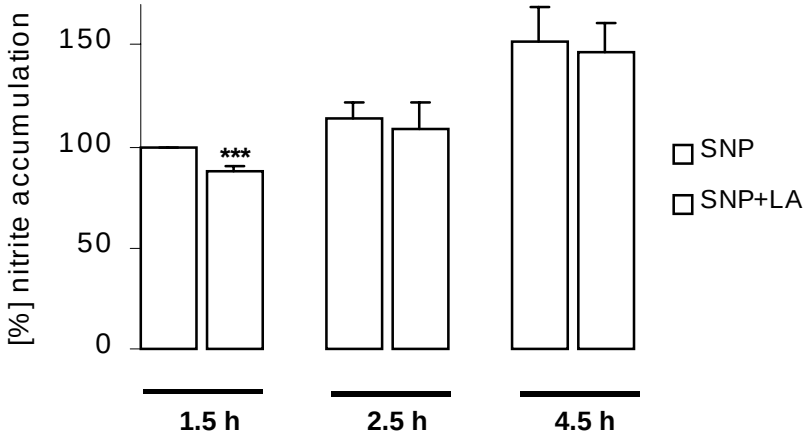


Figure 56: nitrite accumulation after administration of NO donor. *RAW 264.7 macrophages were either left untreated or treated with the NO donor SNP (1 mg/ml) in the presence or absence of LA (20 µg/ml). Nitrite accumulation was assessed as described under "Methods". Data are expressed as percentage of nitrite accumulation in cells activated with SNP (100%) and show means ± SEM. of two to four independent experiments performed in triplicates. *** p<0.001 represents significant differences compared to the values in cells treated with SNP for 1.5 h.*

5.3 Cell viability

The inhibitory effect of LA on NO synthesis was not due to cytotoxicity of LA in concentrations up to 500 µg/ml. As an indicator of cell viability, the mitochondrial respiratory activity of the cells was assessed. In RAW 264.7 macrophages viability of cells in the presence of LPS + LA was even significantly higher than in cells treated with LPS only (*Figure 57*), therefore, a decrease of nitrite accumulation, because of a reduced number of viable cells can be excluded.

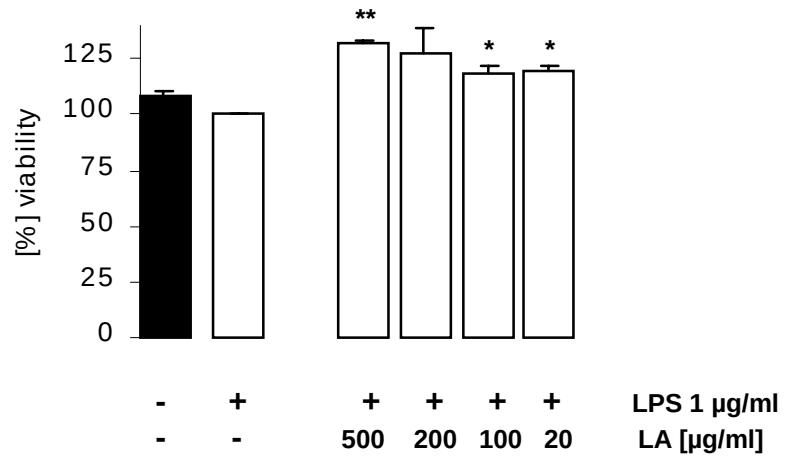


Figure 57: LA increased cell viability of RAW 264.7 macrophages. *RAW 264.7 macrophages were either left untreated or treated with LPS (1 µg/ml) in the absence or presence of LA (20-500 µg/ml). Cell viability was determined by MTT test as described in "Methods". Data are expressed as means ± SEM. of one or two independent experiments performed in triplicates. Viability of cells treated with LPS only was referred to as 100%. ** p<0.01 and * p<0.05 represent significant differences compared to the values in LPS activated cells.*

E. Discussion

1. Validation of the liver perfusion system

At the beginning of this study the system of the isolated perfused rat liver had to be established. Control parameters of the perfusion system were the common features describing liver cell viability and hepatic function: enzyme release, portal pressure, and bile flow.

In the literature enzyme efflux rates of approx. 2-15 mU x min^{-1} x (g liver)$^{-1}$ for LDH and 0.2-5 mU x min^{-1} x (g liver)$^{-1}$ for PNP (Bilzer M., 1997) were proposed and were perfectly in line with the values seen in the used perfusion system (see *Figure 26*). Data provided for portal pressure were also in agreement with previous reports, which describe values between 3-6 cm water (Dahl S., 1997;Bilzer M., 1997) for continuously perfused rat liver (see Figure 27). Concerning bile flow, Dahl and colleagues stated a decrease in isolated perfused rat livers from approx. 1.3 - 0.8 µl x min^{-1} x (g liver)$^{-1}$ within 2 h of perfusion (Dahl S., 1997;Gores *et al.*, 1986) reflecting the values obtained in this work (see Figure 28). On the basis of these results the perfusion system was considered as valid to perform further experiments.

The next step was to elucidate, whether administration of LA does affect the parameters used. Due to the fact that no information was available about LA in hepatic IRI so far and only little has been published about its use in the isolated perfused rat liver (Spence and McCormick, 1976;Peinado *et al.*, 1989), it was necessary to initially determine whether administration of LA influences the applied system. Performing continuous perfusions (D.1.1) showed that LA did not significantly alter enzyme release and portal pressure, indicating that LA administration did not influence liver cell viability and hepatic circulation in the perfusion system. A novel finding represents the obtained increase of bile flow in LA treated livers. LA has been described before to enter bile in rat liver perfusion (Borbe H.O. and Ulrich H., 1989), but not to enhance bile formation.

2. Short time infusion of H_2O_2

The fact that Kupffer cell activation during reperfusion leads to an increased sinusoidal ROS formation, a further preliminary set of experiments was done mimicking ROS production by infusing H_2O_2. H_2O_2 as a reactive oxygen intermediate was chosen for these experiments, because it is proven to be involved in ROS-induced hepatic IRI (Jaeschke *et al.*, 1991;Bilzer M., 1997). The experiments should clarify the question, whether LA is able to attenuate H_2O_2-induced liver cell injury. However, LA did not show any protection in H_2O_2-induced injury. Portal pressure in the LA treated groups even showed a significant increase after H_2O_2 application. This surprising result might be explained by the generation of a LA oxidation product having vasoconstrictive properties.

3. LA attenuates IRI of the rat liver

The main part of this study investigated the influence of LA on ischemia reperfusion injury (IRI) of the rat liver. Basis for choosing LA as a possible substance attenuating IRI was derived from its very promising pharmacological profile described in the introduction (see B.3). Among the properties of LA mentioned there, especially interesting concerning IRI are: the antioxidative potential of LA and its metabolites (B.3.5.1), the influence on energy metabolism (B.3.5.4), and very recently the findings of Konrad et al. (Konrad *et al.*, 2001) suggesting that LA specifically activates molecular signalling cascades of the cell (B.3.4). Additionally, LA is widely used in the therapy of diabetic disorders and is therefore documented in numerous clinical trials to be well tolerated (see B.3.4) (Coleman Michael D., 2001).

3.1 LA protects from IRI

This work shows for the first time that this established antidiabetic agent (Ziegler *et al.*, 1999) attenuates hepatic IRI. Pre- as well as postischemic, and continuous administration of LA were shown to reduce IRI. Focussing on the preconditioning effect of LA it was also shown for the first time that the survival kinase Akt is involved in protection from IRI of the rat liver. This assumption is based on two findings. *First,* LA increased phosphorylation of Akt and *second*,

most importantly the PI-3 kinase inhibitor wortmannin abrogated the protective effect of LA preconditioning.

Furthermore, additional effects of LA were observed, which should also be discussed in terms of its beneficial role in attenuating IRI.

3.2 Raised ATP availability

Preconditioning with LA lead to an increased ATP content in liver tissue during ischemia and reperfusion. The potency of LA to increase ATP levels has been reported before in rat heart mitochondria (Zimmer *et al.*, 1995) and diabetic hearts (Strodter *et al.*, 1995). Since ATP depletion occuring during ischemia (Jaeschke *et al.*, 1988;Gerbes *et al.*, 1998) leads to liver cell damage, the increased energy pool after LA pretreatment might contribute to the protective effect (*Figure 48*).

3.3 Inhibition of NF-κB and AP-1 activation

LA preconditioning also inhibited the activation of two pivotal redox-sensitive transcription factors, namely nuclear factor κB (NF-κB) and activator protein 1 (AP-1). LA has been previously reported to inhibit NF-κB binding activity in various cell culture models, supporting these data (Suzuki *et al.*, 1995). Activation of transcription factors, such as NF-κB and AP-1, has been linked to the pathophysiology of IRI by activating inflammatory cascades leading to organ damage (Ricciardi *et al.*, 2000;Banafsche *et al.*, 2001;Fan *et al.*, 1999;Zwacka *et al.*, 1998;Yamada *et al.*, 2000). Thus, inhibition of these proinflammatory mediators might contribute to a protective effect. However, in this study LA attenuated the transcription factors mainly at *late* reperfusion time points (Figure 47). Therefore, these mechanisms seem most likely not to be essential for the protective effect of LA, which is seen already at the *beginning* of the reperfusion period. For later IRI events, such as iNOS-induction after NF-κB activation, cell culture experiments should give further hints on LA influencing NF-κB target gene expression (D.5).

3.4 Postischemic LA application

The inhibitory effect of LA on the activition of the redox-regulated transcription factors NF-κB and AP-1 might be linked to a reduced ROS production during IRI. These direct antioxidative properties of LA are most likely responsible for the protection conveyed by postischemic application of LA. The fact that ROS production is highly increased during early reperfusion (Bilzer and Gerbes, 2000;Rauen *et al.*, 1994) might explain why application of potent antioxidants during reperfusion protects from IRI (Bilzer *et al.*, 1999). This hypothesis is supported by demonstrating an immense difference of LA- and TNLA-amounts in perfusate between pre- and postischemically administered LA (Figure 42), which represents a high sinusoidal antioxidative potential for postischemic treatment. Therefore, protection by postischemic administration might be due to the direct ROS scavenging properties of LA. In addition, data from the H_2O_2-experiments (see D.2) suggest that application of LA seems not to be involved in attenuating the H_2O_2-induced part of liver cell damage during IRI.

3.5 Influence on thiol/disulfide status

Besides such a direct interaction with ROS, antioxidant properties might also arise from the modulation of other redox dependent factors, such as GSH. In fact, LA increases GSH levels in various systems by improving cystine utilization (Han *et al.*, 1997). However, in this study an enhanced cysteine or GSH contents in liver or perfusate after applying LA could not be shown (see *Figure 43*, *Figure 45*) and therefore an indirect antioxidative action *via* GSH as hypothesized could be excluded. Pretreatment of livers with LA rather reduced than augmented hepatic GSH content (*Figure 45*). Interestingly, GSH depletion has been reported before to be hepatoprotective after different stimuli (Hentze *et al.*, 2000). The reduced contents of CYSSX in perfusate of LA pretreated livers might be expression of a reduced sinusoidal ROS formation during reperfusion of these livers (Figure 44).

3.6 Activation of kinases

The most meaningful effect of LA in this work surely is the increased Akt activation observed in LA preconditioned organs. Akt has been reported to possess cytoprotective potential as has also been described for p38 MAPK.

Interestingly, LA did not activate p38 MAPK in our model of IRI (*Figure 50*), although LA has previously been shown to activate p38 MAPK in muscle cells (Konrad *et al.*, 2001). Thus, LA does not confer its protection *via* this kinase which is known to mediate ischemic preconditioning of hearts (Weinbrenner *et al.*, 1997) as well as in hypoxic preconditioning of hepatocytes (Carini *et al.*, 2001).

Up to now, no information is available on a potential cytoprotective action of Akt in hepatic IRI. Only one work describes a role for Akt in ischemic preconditioning of rat hearts (Tong *et al.*, 2000). LA has been reported to activate Akt, an effect which was connected to its antihyperglycemic activity (Konrad *et al.*, 2001;Yaworsky *et al.*, 2000). This work here reports for the first time that activation of this survival kinase, as induced by LA pretreatment, is causally involved in attenuating hepatic IRI, since inhibition of PI-3 kinase by wortmannin abrogated the protective effect of LA (*Figure 53*). Therefore, LA-induced activation of Akt is pivotal for LA-mediated hepatoprotection. Anyway, as suggested by the time course of Akt, the tremendous postischemic activation of this survival kinase also in untreated livers might represent a key event of hepatic cells resisting cell death (*Figure 51*). The observation of an additionally increased Akt activation by LA pretreatment at the end of reperfusion might also demonstrate a forced stress resistance in these livers (*Figure 52*).

3.7 No influence on apoptotic cell death

The cytoprotective effect of Akt has been mainly attributed to its antiapoptotic potential so far (Chan *et al.*, 1999). However, hepatoprotection by LA does not seem to be involved in antiapoptotic processes of our model of IRI. The postischemic activation of caspase-3-like activity proposes an involvement of apoptotic cell death during IRI, but LA application did not influence these processes since caspase activities remained unaltered (*Figure 54*).

Therefore, this observation suggests that Akt activation might not only attenuate apoptotic processes but also protect from necrotic cell death, as recently also reported by Jin et al. in neuronal cells (Jin *et al.*, 2000).

4. Inhibition of NO production in RAW 264.7 macrophages by LA

Especially the reduced activation of NF-κB at the end of reperfusion (see D.4.5) suggested that LA is able to also influence later IRI events. Due to the fact that the isolated perfused rat liver is only suitable for perfusion times up to 5 h, a change of the experimental model was necessary to get first insights in possible later mechanisms. For this purpose, a RAW 264.7 macrophage model was used (see also D.5) to evaluate the influence of LA on NF-κB target gene expression. As a later reperfusion event, NO formation after NF-κB activation and subsequent iNOS induction has been described (Tsoulfas and Geller, 2001). The fact that iNOS knockout mice showed reduced liver cell damage after warm ischemia and reperfusion suggests that NO production is harmful to the reperfused liver (Lee *et al.*, 2001). Data provided in the present study, using RAW macrophages, gave first hints about the potency of LA to reduce NO formation (see *Figure 55*). A distinct effect of LA could be shown regarding iNOS induction by LPS, whereas LA treatment in concentrations > 200 µg/ml completely inhibited nitrite formation. Experiments with the NO donor sodium nitroprusside showed that a decreased NO formation might be partly explained by a NO scavenging effect of LA (*Figure 56*). However, this effect seems not to be strong enough to explain the complete inhibition of NO production after LPS stimulation. In conclusion, the macrophage experiments provide evidence for a role of LA to attenuate expression of NF-κB target genes (e.g. iNOS), which are discussed to aggravate IRI.

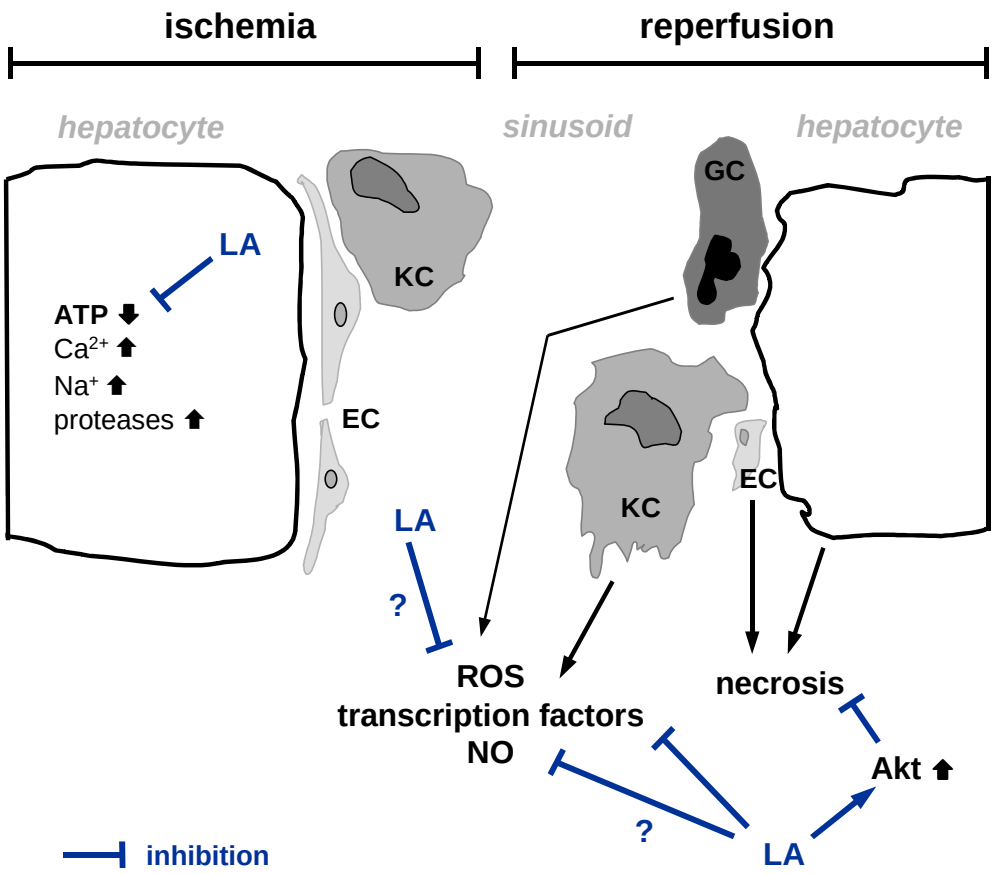


Figure 58: shown protective mechanisms of LA in hepatic IRI*. Kupffer cell (KC), endothelial cell (EC), granulocyte (GC), reactive oxygen species (ROS), nitric oxide (NO).*

To summarize, this work shows for the first time that LA reduces IRI of the rat liver. Thus, LA treatment might represent a new pharmacological approach in attenuating hepatic IRI. Even more importantly, it is shown that preconditioning with LA is mediated *via* the PI-3K/Akt pathway. Therefore, these data reveal activation of Akt as a new target mediating protection from hepatic IRI.

F. Summary

Ischemia reperfusion injury (IRI) represents a major clinical problem in liver transplantation and resection. Main pathophysiological events during this injury comprise depletion of ATP, Kupffer cell activation with subsequent formation of reactive oxygen species and inflammatory response. Depending on the severity of IRI, cell damage leads to necrotic or apoptotic liver cell death, resulting in organ dys- or even nonfunction.

The present work investigated the potential of α-lipoic acid (LA), a well established agent in the therapy of diabetic polyneuropathy, to reduce IRI of the rat liver. In the system of the isolated perfused rat liver, an experimental model of hepatic IRI, LA was utilized in different treatment protocols and concentrations.

As a key result of this study, pre- as well as postischemic treatment with LA was shown for the first time to markedly reduce hepatic IRI.

Further investigations characterized the underlying mechanisms of LA action, focussing on the preconditioning protocol. Thereby, LA was shown to increase hepatic ATP content during ischemia and reperfusion, suggesting a better energy availability in these organs. Activation of the redox-sensitive transcription factors NF-κB and AP-1 was reduced by LA, suggesting a decreased inflammatory response of the liver.

Most importantly, this work describes for the first time that the protein kinase Akt plays a crucial role in IRI. The cytoprotective kinase was activated by LA preconditioning during ischemia and reperfusion. Blocking Akt activation by simultaneous application of wortmannin, a selective PI-3 kinase inhibitor, abrogated the LA preconditioning effect, showing a causal involvement of Akt in LA-mediated protection from IRI. Therefore, Akt activation can be regarded as a new protective mechanism in hepatic IRI.

To conclude, LA treatment might represent a new pharmacological approach in attenuating IRI of the liver.

G. Appendix

1. Abbreviations

A	Ampere
APS	Ammonium persulfate
ATP	Adenosine-5’-triphosphate
BSA	Bovine serum albumine
C	Celsius
CCl_4	Carbon tetrachloride
Co	control
CoA	Coenzyme A
cpm	Counts per minute
CYS	Cysteine
CYSSX	Oxidized cysteine
dist.	Distilled
DMEM	Dulbecco’s Modified Essential Medium
DMSO	Dimethylsufoxide
DNA	Deoxyribonucleic acid
DTE	Dithioerythritol
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis(aminoethylether) tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FCS	Fetal Calf Serum
g	gram
g	Acceleration due to gravity
G-6-P-DH	Glucose-6-phosphate-dehydrogenase

GSH	Glutathione
GSSX	Oxidized glutathione
h	Hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	Hexokinase
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
i.v.	intravenous
iNOS	Inducible nitric oxide synthase
IRI	Ischemia reperfusion injury
KC	Kupffer cell
L	Liter
LA	Racemic α-lipoic acid
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
m	Milli
M	Molar
MEK	MAP/ERK kinase
mg	milligram
min	Minute
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	Dihydronicotinamide adenine dinucleotide
NADH-Na_2	Dihydronicotinamide adenine dinucleotitide disodium salt
NEM	N-ethylmaleimide
Nonidet	Non-ionic detergent
p.o.	Peroral
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PI-3K	Phosphatidylinositol-3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PNP	Purine nucleoside phosphorylase
Pre-I	Pre-ischemia
PVDF	Polyvinylidenfluoride
R	Reperfusion
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	second
SEM	Standard error of mean
SNP	Sodium nitroprusside
STE	Sodiumchloride/tris-HCl/EDTA
TBE	Tris, borate, EDTA buffer
TBS-T	Phosphate buffered saline solution with Tween
TEA	Triethanolamine
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF-α	Tumor necrosis factor α
TNLA	Tetranorlipoic acid
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl)-aminomethane
U	Unit
V	Volt
% (m/v)	Mass per volume per cent
% (v/v)	Volume per cent
µ	Micro

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I. Acknowledgements

This work was carried out between March 1999 and June 2002 at the Center of Drug Research, Department of Pharmacy, Pharmaceutical Biology, Mrs. Prof. Dr. A. M. Vollmar at the LMU Munich.

I'm indepted to Prof. Dr. Angelika Vollmar, for providing me the opportunity to perform this work in her laboratories. Her constant constructivity in discussions and her personal manner were always very encouraging. Making "points" makes sense!

Special thanks go to my thesis committee, especially to Prof Dr. E. Wagner for acting as the second examiner.

I would also like to thank my supervisor Dr. Alexandra Kiemer, who initiated and accompanied this study. Her advices and suggestions were useful and necessary throughout the work. Thank you also for critically reading the manuscript.

I'm much obliged to ASTA Medica, especially to Dr. Claudia Wicke and Alexander Kraft for providing α-lipoic acid and its analysis.

Thanks also to Dr. Andreas Baron for teaching me in perfusing livers.

I'm grateful to my collegues, all having the same problems. Thanks especially to Anke, Dr. Rainer Samtleben, HaPe and Thomäss.

I'd like to appreciate the motivated lab assistance of Wolfgang Rödl, Rita Socher, Anna Obenaus, Brigitte Weiss and Raima Yasar.

Thank you: Mama & Papa for everything. Micha for your support in private questions. Basti for showing me basic but relevant things in life. Erwin for doing family pioneer work among us brothers and sisters. Martina for car sharing. My grandmas and grandpas for continuous and generous - not only financial - support. Elke, for being with me.

Leiki and Wolfi for your friendship! Tobi, for not only being my bench neighbour. Hubsi for bearing 5 years with me in the Huber/Müller-WG, a marvelous time.

Special thanks to the PDM n.e.V., the Pharma soccer team and the Bayerische Apothekerauswahl for being vital institutions.

Curriculum vitae

Persönliche Daten

Name	Christian Müller
Geburtstag und -ort	13.06.1973 in Ingolstadt
Staatsangehörigkeit	Deutsch
Eltern	Anna (geb. Vollnhals) and Erwin Müller

Schulbildung

1983 - 1992	Christoph-Scheiner-Gymnasium Ingolstadt
08.07.1992	Abitur

Studium

WS 93/94 - WS 96/97	Pharmaziestudium an der Ludwig-Maximilians-Universität München
09/1995	1. Staatsexamen
10/1997	2. Staatsexamen
11/1997 - 04/1998	Praktikum in der Kugel-Apotheke, München
05/1998 - 10/1998	Praktikum bei der Hexal AG, Holzkirchen
30.11.1998	3. Staatsexamen
17.12.1998	Approbation als Apotheker
02 - 03/1999	Tätigkeit als Apotheker in der Kugel-Apotheke, München
03/1999 - 06/2002	Dissertation am Zentrum für Pharmaforschung, Department Pharmazie, Lehrstuhl für Pharmazeutische Biologie unter der Leitung von Frau Prof. Dr. A. M. Vollmar