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PROTECTION FROM HEPATIC ISCHEMIA/REPERFUSION INJURY AND IMPROVEMENT OF LIVER REGENERATION BY α-LIPOIC ACID

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ABSTRACT—The aim of this study was to characterize the *in vivo* action of lipoic acid (LA) in hepatic ischemia/reperfusion injury (IRI) and its effects on liver regeneration involving the investigation of mechanisms of action and effects on animal survival. Two groups of rats were compared: one group received 500 μmol α-LA injected *via* the inferior vena cava 15 min before the induction of 90 min of selective ischemia. The untreated group received vehicle. Influence of LA on IRI of the liver was determined in short- and long-term experiments. Cellular damage was decreased under preconditioning conditions with LA. Caspase 3, 8, and 9 activities were significantly lower in the LA group accompanied by a decrease in terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive hepatocytes. Electron micrographs in the untreated group showed massive mitochondrial damage. The survival rate as end point of liver function was markedly increased after pretreatment with LA. Increased levels of tumor necrosis factor α was shown by enzyme-linked immunosorbent assay as well as real-time reverse transcription–polymerase chain reaction in the LA group accompanied by increased mitotic index and Ki-67 staining in liver tissue. Attenuation of IRI of the rat liver *in vivo* by LA is accompanied by reduction of necrosis and apoptosis-related cell death, whereas liver regeneration is increased.

KEYWORDS—Liver surgery, ischemic tolerance, liver failure, preconditioning of the liver, apoptosis

INTRODUCTION

Hepatic ischemia/reperfusion injury (IRI) occurs primarily in the course of extended liver resection procedures necessitating the performance of a prolonged Pringle maneuver after liver transplantation as well as after cardiogenic or hemorrhagic shock. IRI may not only cause severe prolonged disturbances of liver function or liver failure, but also involve other organ systems, especially the lung and the kidneys (1–3) and is therefore of highest clinical importance.

The loss of mitochondrial adenosine triphosphate (ATP) production is thought to be closely related to ischemia. ATP depletion results in disrupted cellular volume control and disintegration of mitochondria. Some researchers have suggested that these phenomena may be responsible for cell death after longer periods of anoxia (4, 5). There is a broadly shared consensus that reactive oxygen species (ROS), which are produced under conditions of prolonged warm ischemia and subsequent reperfusion, play an important role in the induction of IRI (5, 6). Under these conditions, tumor necrosis factor α (TNF-α) plays a key role in signaling the pathway of IRI. TNF-α is primarily expressed by Kupffer cells, the resident macrophages of the liver, during inflammatory processes, such as IRI (7, 8). Despite its role as a mediator of cell damage and inflammation, TNF-α is also known to be one of the major growth factor of hepatic regeneration (9).

A number of substances capable of scavenging cytotoxic oxygen radicals have been studied in both animal models and humans, and a hepatoprotective effect has been shown for several substances, such as α-tocopherol, glycine, or glutathione (10–12). In addition, ischemic preconditioning (IPC) is one of the most effective strategies to decrease liver injury in both animals and human studies (13). Interestingly, however, in case of small liver remnants, the protective effects of IPC were lost (14), and in this context, IPC also seems to impair liver regeneration.

In view of the fact that preconditioning strategies in liver surgery needs to be rapid, readily performed, and without side effects, we aimed to characterize the potency of α-lipoic acid (LA) in pharmacological preconditioning of the liver. LA is a disulfide with coenzyme function in mitochondrial multienzyme complexes. The significant reduction potential of α-LA defines the substance as a radical scavenger with an antioxidant effect (15, 16). In addition to these antioxidant effects, LA has been shown to activate specific cytoprotective cell signaling pathways (17). The mechanism of action of LA in IRI of the liver *in vivo* are, as yet, completely unknown.

The current study should therefore clarify the mechanism of action of LA in ischemia/reperfusion of the liver *in vivo* and test the impact of LA on liver regeneration.

MATERIALS AND METHODS

Animals

Male Brown Norway rats weighing 175 to 200 g (Harlan, Paderborn, Germany) were used. All animals had access to water and rat chow ad libitum (Global Diet; Harlan); *APS Guiding Principles in the Care and Use of Animals* were adhered to. The animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published

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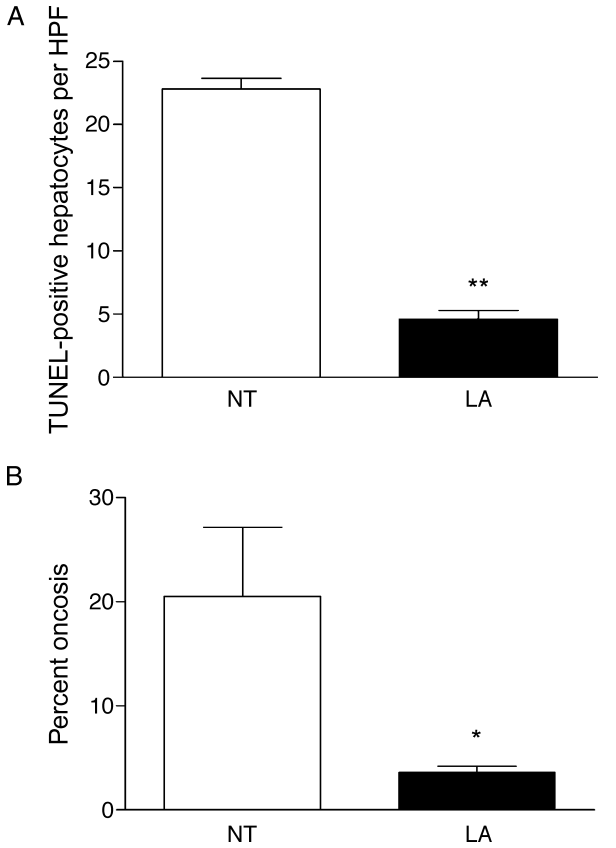

FIG. 1. **A, TUNEL-positive hepatocytes: percentage of TUNEL-positive hepatocytes as described in Materials and Methods after 90 min of ischemia and 1 h of reperfusion.** Values are given as means ± SE (n = 10). $^{**}P < 0.01$ NT versus LA. B, Percentage of oncosis as described in Materials and Methods after 90 min of ischemia and 1 h of reperfusion. Values are given as means ± SE (n = 3 different serial sections for sham group, n = 5 different serial sections for NT and LA group). $^{*}P < 0.05$ NT versus LA.

by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). The study was registered with the Institutional Animal Care and Use Committee.

In a model for acute experiments, 90 min of ischemia of the left liver lobe was performed by clamping the corresponding branch of the portal vein, the hepatic artery, and bile duct proximal to its origin. Reperfusion was initiated by removal of the clamp. Thereafter, the abdominal cavity was closed with running suture, and animals were allowed to awake. After 1 h of reperfusion, anesthetized animals were killed, and liver tissue and plasma were stored for further examination. Three experimental groups were studied: (*a*) sham-operated animals (n = 3); (*b*) 90 min of ischemia with 1 mL of normal saline *via* the abdominal vena cava 15 min before ischemia (n = 5, no treatment [NT]), and (*c*) 90 min of ischemia with 500 μmol of LA (120 μg) *via* the abdominal vena cava 15 min before ischemia (n = 5, LA pretreatment [LA]). The body temperature was monitored and maintained at 37 ± 0.4°C by heating lamp.

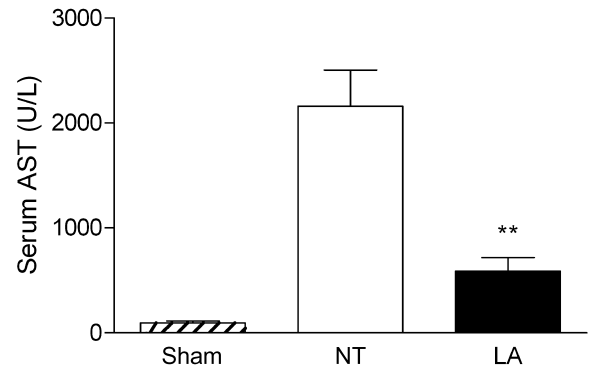

FIG. 2. **AST levels (U/L) in serum after 90 min of ischemia and 1 h of reperfusion with and without 500 μmol of LA 15 min before ischemia.** Values are given as means ± SE (n = 3 for sham group, n = 5 for NT and LA group). $^{**}P < 0.01$ NT versus LA. ALT levels in serum follow a similar pattern (data not shown).

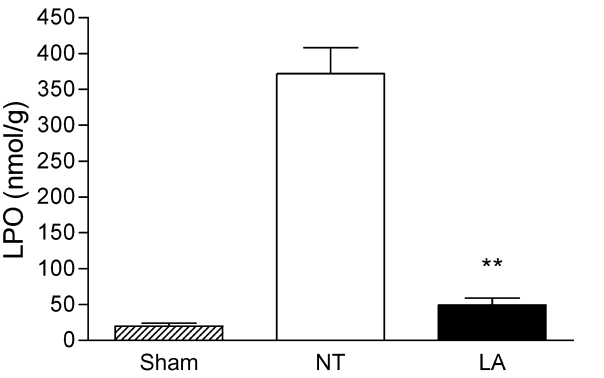

FIG. 3. **LPO (nmol/g) after 90 min of ischemia and 1 h of reperfusion with and without 500 μmol of LA 15 min before ischemia.** Values are given as means ± SE (n = 3 for sham group, n = 5 for NT and LA group). $^{**}P < 0.01$ NT versus LA.

Long-term outcome and animal survival were investigated by resecting nonischemic liver tissue at the time of reperfusion. After inhalation anesthesia with ether, the abdominal cavity was accessed through a midline incision. Inflow occlusion of the median lobe was performed by placing a microvascular clamp to the median branch of the portal vein, hepatic artery, and bile duct. Ischemia was maintained for 90 min. Immediately after ischemia, the nonischemic liver tissue (i.e., the caudate and the right and left lobes of the liver) was resected (70% liver resection). One group of animals was monitored for 30 days. Another group of animals was killed 32 h after operation for histology and immunohistochemistry.

Three experimental groups were studied: (*a*) animals that were only treated by liver resection of the caudate and the right and left lobes of the liver (70% liver resection) without ischemia and with 1 mL of normal saline *via* the infrahepatic vena cava 15 min after laparotomy (n = 3, resection only), (*b*) 90 min of ischemia of the median liver lobe with 1 mL of normal saline via the abdominal vena cava 15 min before ischemia and resection of the caudate and right and left lobes of the liver (70% liver resection, n = 5 NT), and (*c*) 90 min of ischemia with 500 μmol LA (120 μg) *via* the abdominal vena cava 15 min before ischemia and resection of the caudate and the right and left lobes of the liver (70% liver resection, n = 5, LA). Animal survival was calculated using the Kaplan-Meier method.

In our previous study, we could demonstrate reduction of IRI by 50 μmol of LA in our *in vitro* model (17). Therefore, we tried to show the influence of 50 μmol of LA in our described animal survival model; however, we observed that after pretreatment with 50 μmol of LA, no animals survived (n = 3, data not shown). Next, we used 500 μmol of LA to reduce IRI of the liver. Here, we could observe a massive reduction of IRI of the liver in the same model.

Homogenization of liver tissue

The liver tissue was weighed before homogenization and diluted 1:5 with 0.01 mol/L phosphate-buffered saline. Homogenization was then carried out with a

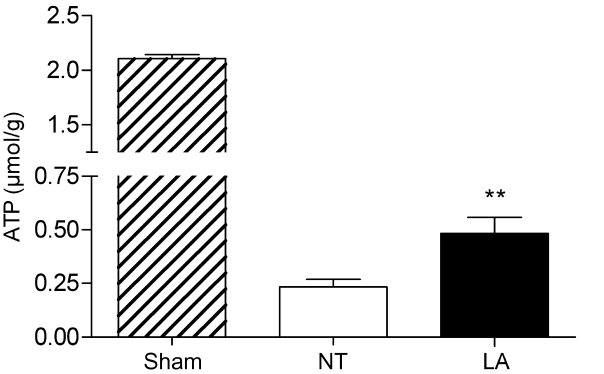

FIG. 4. **ATP (μmol/g wt/wt) levels in liver tissue after 90 min of ischemia, 1 h of reperfusion with and without 500 μmol of LA.** Values are given as means ± SE (n = 3 for sham, n = 5 for NT and LA group). $^{**}P < 0.01$ NT versus LA.

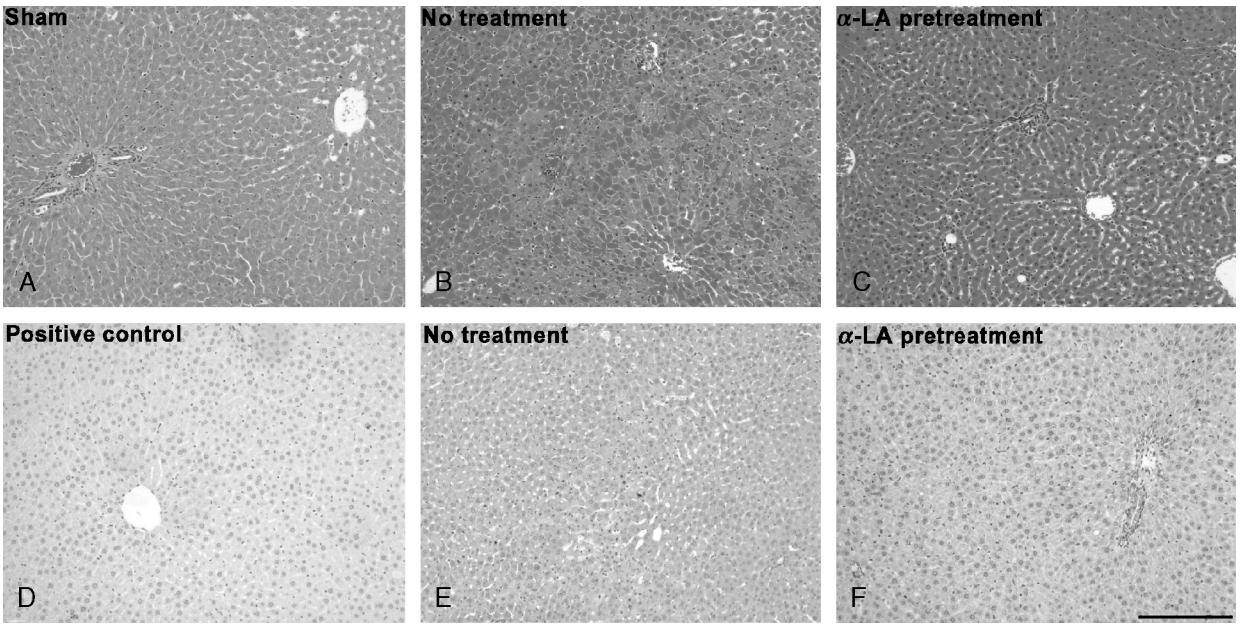

FIG. 5. **Representative liver sections stained with H&E and TUNEL after 90 min of ischemia and 1 h of reperfusion without treatment and with pretreatment by LA.** In sham-operated animals, we saw no TUNEL staining (data not shown) (all micrographs, ×400, bar = 200 μm). A, Representative liver sections stained with H&E of sham-operated animals. B, Representative liver sections stained with H&E of the untreated group: liver sections were characterized by areas of oncotic cell injury, such as eosinophilia, loss of architecture, and vacuolization. C, Representative liver sections stained with H&E after treatment with 500 μmol of LA: only a mild form of oncotic injury was observed in the LA-treated group. D, Representative TUNEL staining of positive control: positive control was prepared from liver-tissue samples by treating with DNase I in accordance to manufacturer's guidelines. E, Representative TUNEL staining of the untreated group: we observed TUNEL positive hepatocytes. F, Representative TUNEL staining after treatment with 500 μmol of LA: TUNEL-positive hepatocytes were rare.

Potter tissue homogenizer. A homogeneous mixture was achieved at 800 rpm. Next, the mixture was centrifuged at 1000*g* for 10 min. The supernatant was pipetted into an Eppendorf cup and centrifuged at 10,000*g* for 20 min. The clear supernatant was pipetted into a fresh Eppendorf cup and stored frozen at −20°C until further measurement.

Enzyme release

Blood samples for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assessment were obtained and analyzed using a serum analyzer (Olympus AU Connector 640; Olympus, Hamburg, Germany).

Determination of lipid peroxidation

Determination of lipid peroxidation (LPO) in homogenized liver tissue was carried out with a standardized test (Cayman Chemical, Grünberg, Germany) kit in accordance with the manufacturer's instructions.

Measurement of ATP

Measurement of ATP in liver tissue was carried out using a standardized method as described by Trautschold et al. (18). The liver slices were weighed before homogenization and diluted in 4% $HClO_4$ (1:10). After homogenization, the supernatant was pipetted into an Eppendorf cup and neutralized with 150 μL of 2 mol/L Tris, 150 μL of KOH, and phenol red. Then 5 mol/L of KOH was added until a blue-lilac complex was observed. The mixture was again centrifuged at 10,000 U/min, 5 min, and 4°C. The supernatant was measured immediately by UV photometry and hexokinase/glucose-6-phosphate dehydrogenase.

Histology and immunohistochemistry

Formalin-fixed tissue samples were embedded in paraffin, and 5-μm sections were cut. Replicate sections were stained with hematoxylin-eosin (H&E) for the evaluation of oncosis and liver regeneration, stained with the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay and stained for Ki-67 protein. All histological evaluations were done in a blinded fashion.

Oncotic cell injury was evaluated in serial sections stained with H&E. Morphological criteria of oncotic cell injury such as increased swelling, vacuolization, and blebbing were used. The percentage of oncosis was estimated by evaluating the number of microscopic fields with oncosis compared with the entire histological section.

To determine hepatocyte replication, mitotic figures were counted in 10 high power fields (HPF, 400-fold magnification) and given as mitotic index (number of mitotic figures per HPF).

The monoclonal antibody MIB-5 (DAKO, Hamburg, Germany) was used for immunohistochemical analysis of hepatocellular regeneration. After 20 min of microwave pretreatment of dewaxed formalin-fixed paraffin sections in citrate buffer (pH 6.0), immunostaining was performed on an automated immunostainer (Techmate 500plus; DAKO) using an DAB peroxidase–based detection system (K5001; DAKO). The proliferative activity was assessed by counting the number of labeled hepatocytes nuclei per HPF (400-fold magnification) and given as proliferative index (number of Ki-67–positive cells per HPF).

To determine apoptotic cell injury, we perform TUNEL staining: livers were fixed with freshly prepared 4% paraformaldehyde in neutral phosphate-buffered saline. Frozen sections (5 μm) of the fixed tissue were prepared and stained with the TUNEL method using a commercial kit (ApoTag peroxidase apoptosis detection kit; Chemicon International, Hampshire, UK). The number of TUNEL-positive hepatocytes was counted in 10 HPF (400-fold magnification).

Before cutting the liver into slices and shock freezing, a small liver sample obtained from each liver was fixed using 0.1 mmol/L glutaraldehyde (Sigma, Hamburg, Germany). Fixation of the tissue was carried out in preparation for electron microscopy: the tissue was subsequently stained using methylene blue. All other procedures were done according to standardized techniques.

Phosphorylation of Akt

Phosphorylation of Akt was determined by Western blot as previously described (17).

Caspase activity in liver tissue

Caspase 3, 8, and 9 activity was measured using a colorimetric reaction (Caspase Colorimetric Assay; R&D Systems Germany, Wiesbaden, Germany) at a wavelength of 405 nm. All samples were prepared in pairs, one pair was measured with and one without substrate, according to the manufacturer's instructions. The results are expressed as fold increase in caspase activity in postischemic versus sham liver tissue (19).

Determination of TNF-α in liver tissue and serum

TNF-α determination in homogenized liver tissue and serum was carried out using a commercial enzyme-linked immunosorbent assay (R&D Systems Germany) kit in accordance with the manufacturer's instructions.

Polymerase chain reaction

The expression of TNF-α, Bax, and Bcl-2 was assessed by reverse transcription–polymerase chain reaction (RT-PCR). The expression of 18 S was used as the housekeeping gene. Total RNA was isolated using Qiagen (Hilden, Germany) RNeasy Mini-Kits. After photometric determination of the amount of RNA, first-strand complementary DNA (cDNA) was synthesized from 4 μg of RNA from each liver, using the following procedure: the transcription mix contained 5 μL of oligo(dT) (Gibco-BRL, Paisley, UK), 3.6 μL dNTP mix (10 mmol/L) (Gibco-BRL), 8 μL first strain buffer, 4 μL DTT (0.1 mol/L), 1.5 μL reverse transcriptase (MLV-T, Roche Diagnostics), and 1.5 μL RNA free water (Qiagen). Controls were performed without the addition of reverse transcriptase. Cycle conditions were chosen as follows: 90 min at 37°C, 10 min at 94°C, and 30 s at 4°C. The cDNA was checked by amplification of 18 S. Primers were designed specifically for rats with the Primer 3 Software (Whitehead Institute, Boston, Mass), and synthesized by MWG Biotech (Eberberg, Germany). The following primers were used:

Bcl-2 neu
forward: 5′-TGC-AGA-GAT-GTC-CAG-TCA-GC-3′
reverse: 5′-CAT-CCA-CAG-AGC-GAT-GTT-GT-3′ (expected product 200 bp);
Bax
forward: 5′-ACA-GAT-CAT-GAA-GAC-AGG-GG-3′
reverse: 5′-CAA-AGT-AGA-AGA-GGG-CAA-CC-3′ (expected product 203 bp).

All sequences were compared with the complete rat GenBank library to ensure that each primer was unique for its intended target and that areas of sequence polymorphisms were avoided.

Equivalent levels of starting cDNA were used for amplification of PCR products. The master reagent mix consisted of 4 μL dNTP mix (1.25 mmol/L), 1 μL specific primer (50 pmol/μL), 1 μL *Taq* polymerase (5 U/μL; Boehringer, Ingelheim, Germany), and 10 μL PCR buffer (1.5 mmol/L) were heated to 93°C, for 1 min at 59°C, 1 min at 72°C, and then cooled to 4°C. The resulting PCR products were visualized by gel electrophoresis stained with ethidium bromide.

Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR analysis of transcripts for TNF-α and glyceraldehyde-3-phosphate dehydrogenase was performed with predesigned and optimized TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif) on an Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. Relative quantification was carried out using the delta-delta-CT method.

Statistics

All data are expressed as means ± SE. Statistical differences between experimental groups were calculated by GraphPad Prism (GraphPad Software, San Diego, Calif) using one-way analysis of variance followed by the Mann-Whitney *U* test. Survival curves were calculated using Kaplan-Meier method. Proportions were calculated using Fishers exact test. Differences were considered statistically significant at $P < 0.05$.

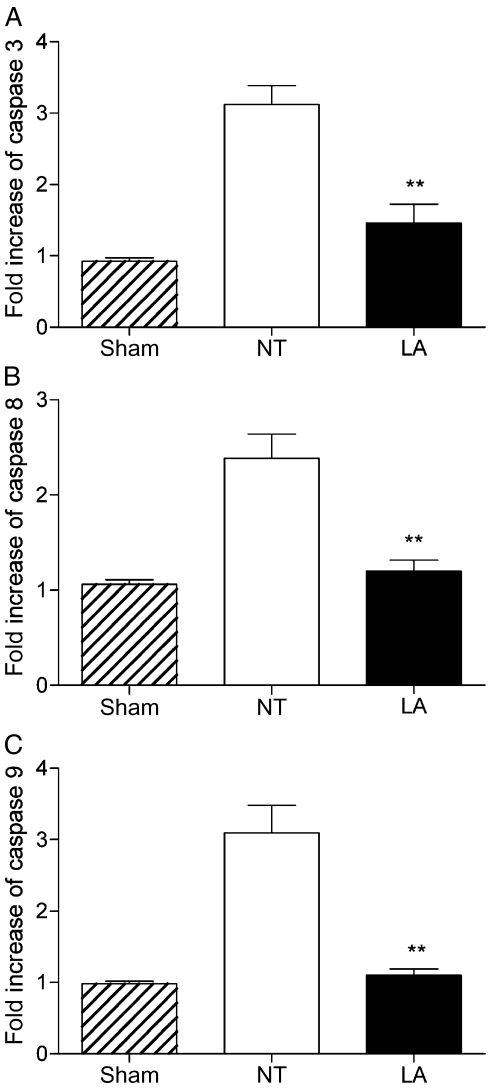

FIG. 6. **Caspase 3 (A), 8 (B), and 9 (C) activity as fold increase to sham-operated animals after 90 min of ischemia and 1 h of reperfusion with and without 500 μmol of LA 15 min before ischemia.** Values are given as means ± SE (n = 3 for sham group, n = 5 for NT and LA group). $^{**}P < 0.01$ NT versus LA.

RESULTS

TUNEL staining

No TUNEL staining was observed in the livers of the sham-operated animals (data not shown, Figs. 1A, 5). In the untreated group, the number of TUNEL-positive cells was increased after 90 min of ischemia and 1 h of reperfusion. The LA-treated group showed a significantly lower incidence of TUNEL-positive hepatocytes at the same time point.

Oncotic cell injury

After 90 min of ischemia and 1 h of reperfusion there was a marked increase in oncotic hepatocytes in the untreated group compared with that observed in the sham-operated animals (not detectable; Figs. 1B, 5). In the LA-pretreated group, we observed a massive decrease of oncotic hepatocytes after 1 h of reperfusion (Fig. 1B).

AST, LPO, and ATP

After 90 min of ischemia and 1 h of reperfusion, AST release increased markedly in the untreated group in comparison with the sham-operated group (Fig. 2), whereas AST increased only slightly after 1 h of reperfusion in the LA group. Serum ALT levels followed a similar pattern (data not shown).

LPO in liver tissue

LPO values obtained 1 h after reperfusion showed a substantial increase in the untreated livers compared with the sham-operated group. In the LA group, LPO was reduced by about 85% (Fig. 3).

ATP content in liver tissue

In comparison with the sham-operated animals, ATP levels in liver tissue after 90 min of ischemia and 1 h of reperfusion were decreased in the untreated group to about 10% of sham-treated animals. LA induced a marked increase in ATP levels (Fig. 4).

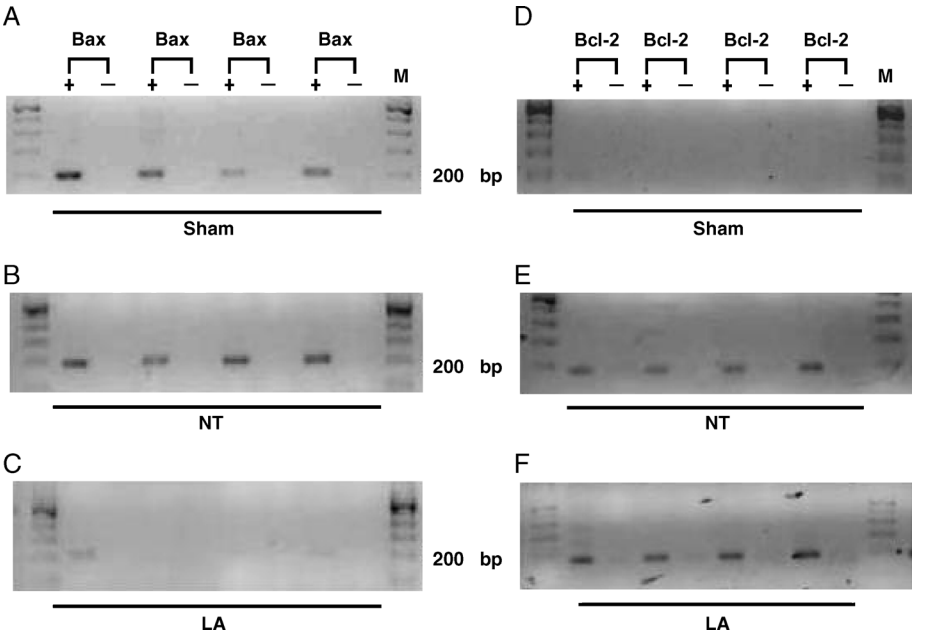

FIG. 7. **Visualization of PCR products of Bax and Bcl-2 in sham-operated animals (A and D) and animals after 90 min of ischemia and 1 h of reperfusion without treatment (B and E) and with LA pretreatment (C and F).** The amounts of total RNA assayed in the groups were equal. M–DNA marker; bp–base pairs; plus–sample with reverse transcriptase; minus–sample without reverse transcriptase. Each horizontal lane represents one different animal of the described groups (n = 4 each group).

Caspase activity

Significant differences were found for all of the described caspases after 1 h of reperfusion after 90 min of ischemia (Fig. 6). We observed a three-fold increase of caspase 3 activity in the untreated group in comparison with sham-operated animals (Fig. 6A). Caspase 8 activity in the untreated animals was doubled in comparison with that observed in the sham-operated animals (Fig. 6B), and a three-fold increase in caspase 9 activity was noted in the untreated group compared with sham-operated animals (Fig. 6C). In the LA group, caspase 3, 8, and 9 activities were significantly decreased in comparison with the untreated group (Fig. 6, A–C).

Messenger RNA expression of Bax and Bcl-2 in liver tissue

In sham-operated animals, we saw no expression of Bcl-2 in liver tissue, whereas messenger RNA (mRNA) of Bax was detectable (Fig. 7, A and D). In the untreated group, there was an expression of Bax and Bcl-2 after 90 min of ischemia and 1 h of reperfusion (Fig. 7, B and E). In the LA-pretreated rats, PCR product of Bcl-2 was detectable at the same time point (Fig. 7E). Interestingly, mRNA of Bax was absent in the LA group after 90 min of ischemia and 1 h of reperfusion (Fig. 7C). Phosphorylation of Akt was not influenced by ischemia/reperfusion or LA (data not shown).

TNF-α level in liver tissue and serum

TNF-α was measured in both serum and liver tissue. After 90 min of ischemia and 1 h of reperfusion, we could not detect any TNF-α in serum (data not shown).

After 90 min of ischemia and 1 h of reperfusion, we observed a substantial increase of the TNF-α content in liver tissue after LA pretreatment in comparison with the livers of the sham-operated and untreated group (Fig. 9). Accordingly, our real-time RT-PCR showed significant up-regulation of TNF-α in LA-pretreated livers in comparison with the untreated group (18.1 ± 3.9 vs. 2.6 ± 0.2; Fig. 9).

Electron micrographs

We performed electron micrograph investigations after 90 min of ischemia and resection of the nonischemic liver tissue and 32 h of reperfusion. We could observe mitochondrial swelling and rupture of the mitochondrial membrane in the untreated group (Fig. 10B), whereas most of the mitochondria of the LA group were without severe features of injury (Fig. 10C).

Liver regeneration

We determined the parameters of liver regeneration after 90 min of ischemia and resection of the nonischemic liver tissue 32 h after ischemia. We observed a mild increase of

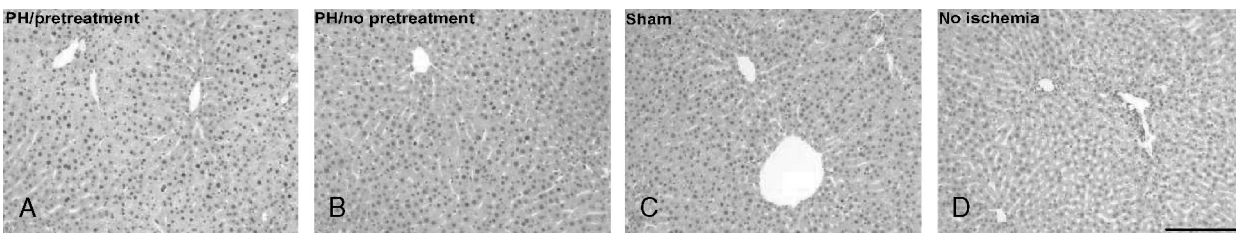

FIG. 8. **Representative liver sections stained for Ki-67 32 h after operation (all micrographs, ×400, bar = 200 μm).** A, Animals after 90 min of ischemia and resection of the nonischemic liver tissue 32 h after operation with LA treatment: there were a massive increase of Ki-67–positive hepatocytes in comparison with the untreated group. B, Animals after 90 min of ischemia and resection of the nonischemic liver tissue 32 h after operation without treatment: there was increase of Ki-67–positive hepatocytes. C, Sham-operated animal: no staining of Ki-67 was observed. D, Animals that were only treated by injection with 500 μmol of LA before examination and killed 32 h after injection: no staining of Ki-67 was observed.

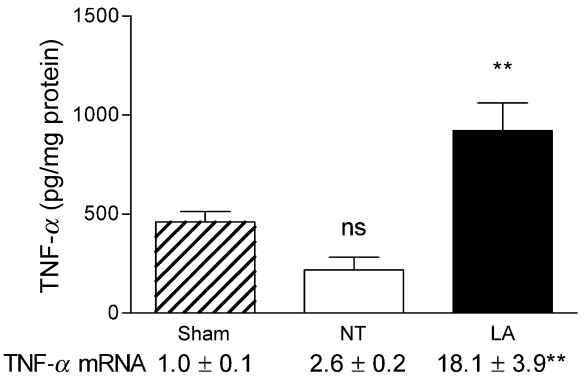

FIG. 9. **TNF-α in liver tissue (pg/mL) after 90 min of ischemia and 1 h of reperfusion in serum was not detectable in any operated group (data not shown).** Values are given as means ± SE (n = 3 for sham group, n = 5 for NT and LA group). $^{**}P < 0.01$ NT versus LA. NS–not significant sham versus NT. TNF-α mRNA–real-time RT-PCR of TNF-α in liver tissue; in each individual sample, the expression level of TNF-α was first normalized with glyceraldehyde-3-phosphate dehydrogenase and then the relative differences between groups were expressed as relative increases, setting sham-operated animals as 1.0. Values are given as means ± SE (n = 4 each group). $^{**}P < 0.01$ NT versus LA.

mitosis and Ki-67 staining in animals that were only treated by liver resection (Fig. 8, 11, A and B). After 90 min of ischemia, levels of mitotic index increased in the untreated group in comparison with animals that underwent liver resection without ischemia. In the LA-pretreated group, the mitotic index was almost doubled in comparison with the untreated group.

The proliferative index of Ki-67 staining was increased in the untreated group in comparison with animals who were only treated by liver resection. After pretreatment with LA, the proliferative index was strongly increased.

Animal Survival

We compared the survival rates of animals from the LA-treated group, the nontreated group, and the group undergoing liver resection as the only measure (Fig. 12). All animals of the untreated group died within the first 3 days (n = 5). In the 500-μmol-LA group, 4 of 5 animals survived the 90 min of ischemia and resection of the nonischemic lobes of the liver up to 30 days after surgery. All animals with liver resection and without ischemia survived the 30-day monitoring period (n = 3).

DISCUSSION

Results of the present study demonstrate a substantial reduction in hepatic IRI accompanied by increased liver regeneration and animal survival after preconditioning with α-LA.

LA is a naturally occurring compound present in the majority of prokaryotic and eukaryotic cells. For many years, LA has been used as a pharmacological agent against diabetic polyneuropathy (20). It has also gained considerable attention as a powerful lipophilic antioxidant (21, 22). After oral and intravenous application, LA is subjected to a high hepatic extraction (23). Moreover, studies have shown that the liver has a high capacity for uptake and accumulation of LA in carrier-mediated and diffusion-transport mechanisms. The detection of biliary excretion of LA metabolites confirms that LA is transported into and not only bound to the hepatocyte (24).

Interestingly, we found that both necrosis- and apoptosis-related cell deaths were decreased in the present model under preconditioning with LA. These data are in contrast to the previous study (17), where Akt phosphorylation but not protection from apoptotic cell death was observed under LA preconditioning in isolated buffer-perfused livers. This difference could be explained by the different models of liver ischemia: protective effects of Akt activation have been described in *ex vivo* and *in vivo* settings such as isolated perfused rat liver and liver transplantation (17, 25). Studies that describe Akt activation in a model of selective and warm liver ischemia with duration of 90 min are rare.

In the present model, the application of LA leads to an increase in the ATP level in liver tissue after ischemia and reperfusion. These findings corroborate the results obtained by our previous study, showing the identical capacity of LA in isolated perfused rat livers (17). The potency of LA to increase ATP levels in rat heart mitochondria has been reported by another group (26). This gives rise to the question

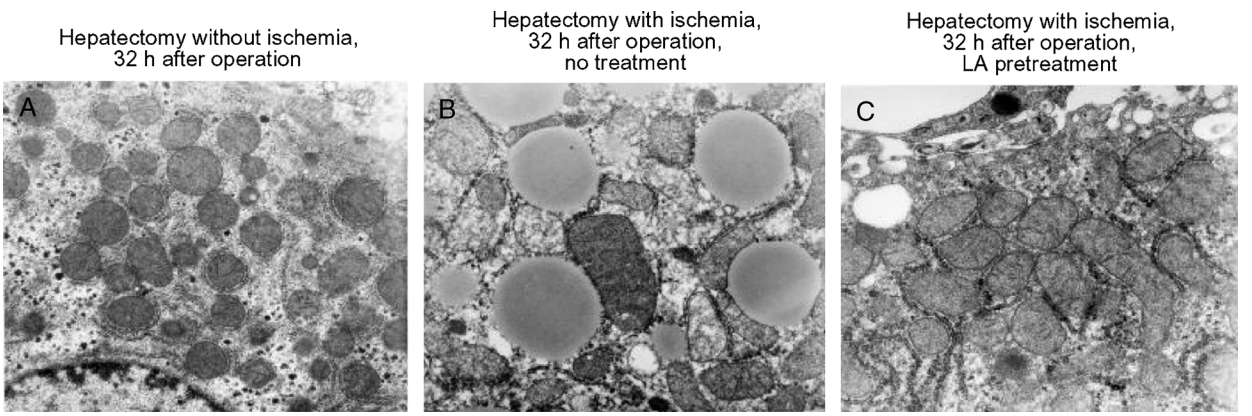

FIG. 10. **Representative electronic microscopic examination of regenerating livers.** A, Examination of animals after resection of the caudate and the right and left lobes of the liver without ischemia 32 h after operation: mitochondria were without features of injury. B, In the untreated group, we observe mitochondrial swelling and rupture of the mitochondrial membrane and increased fatty vacuoles after 90 min of ischemia and resection of the nonischemic liver tissue (the caudate and the right and left lobes of the liver) 32 h after operation. C, In the LA-treated animals, most of the mitochondria were without severe features of injury after 90 min of ischemia and resection of the nonischemic liver tissue (the caudate and the right and left lobes of the liver) 32 h after operation (3500-fold magnification).

as to how LA increases ATP levels in liver tissue after ischemia/reperfusion. One possible answer may be the known potential of LA to reform vicinal thiol groups in the ATP synthase. Dithiols have been shown to form disulfide bridges during ischemia/reperfusion. ATP synthesis occurs only if the dithiols between the F1 and the F_0- part of the enzyme remain functional (27). The lipophilic character of LA allows it to reach the inner membrane of the mitochondrion and to enhance mobility and integrity of the enzyme (28). ATP synthesis may therefore also occur under conditions of ischemia/reperfusion. Accordingly, our electron microscopic investigations show decreased injury of the mitochondrial membrane after LA preconditioning.

TNF-α plays a central role in inducing apoptosis in hepatocytes (29). A prevention of cytochrome c release, activation of caspase 3, and DNA fragmentation was observed in TNF receptor 1–deficient mice, as well as following inhibition of TNF-α release with pentoxifylline. A survival rate of 60% was noted in a model of 75-minute total hepatic ischemia in the murine liver after the application of pentoxifylline (29). In the current study, a survival rate of up to 80% was observed after preconditioning with 500 μmol of LA accompanied by reduction of necrotic and apoptotic cell death. Interestingly, increased levels of TNF-α in liver tissue paralleled the reduction of apoptosis and necrosis. The improved regeneration and animal survival in our model suggests that LA-induced TNF-α preferably acts as a growth factor and not so much as a mediator of cell death and inflammation. In contrast to the induction of TNF-α in our LA-treated animals, LA has been demonstrated to significantly attenuate the lipopolysaccharide-induced production of TNF-α in isolated Kupffer cells (30). It therefore seems that the mechanism of action of LA, either increased or decreased TNF-α expression, depends on the stimulus applied, and might also differ between isolated cells and *in vivo* conditions.

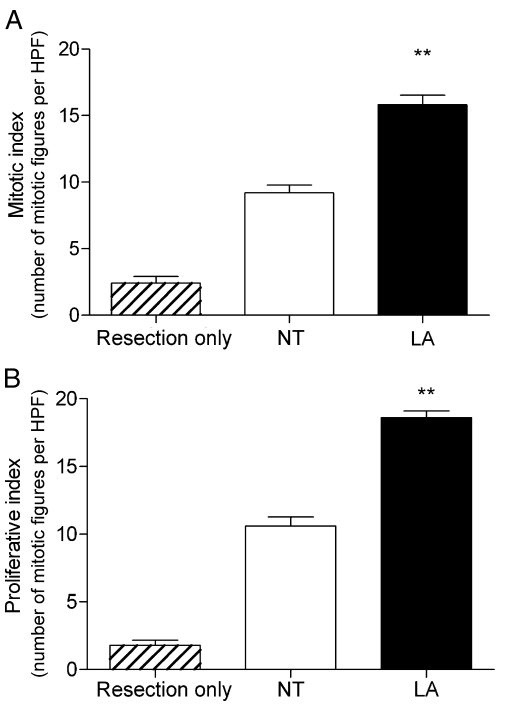

FIG. 11. **Mitotic index, number of mitotic figures per HPF (A), and proliferative index, number of Ki-67–positive cells per HPF (B) in regenerating livers: after resection of the caudate and the right and left lobes of the liver as the only treatment without ischemia (resection only) and after 90 min of ischemia and resection of the nonischemic liver tissue (the caudate and the right and left lobes of the liver) after ischemia, 32 h after operation with and without treatment.** Values are given as means ± SE (n = 10). $^{**}P < 0.01$ NT versus LA.

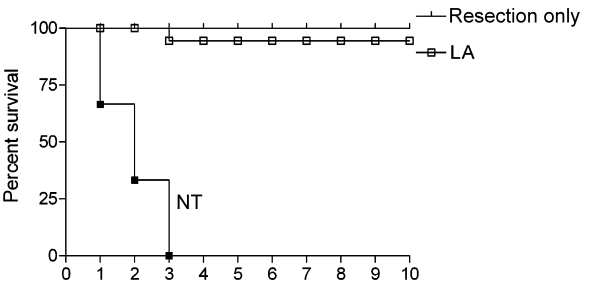

FIG. 12. **Survival curves using the method of Kaplan-Meier after 90 min of ischemia and resection of the nonischemic liver tissue after ischemia with and without treatment and after resection alone (n = 3 group with resection only, n = 5 NT and LA group).** Log-rank $P < 0.01$, comparing the survival curves of NT and LA.

After pretreatment with LA, we observed decreased content of mRNA of the proapoptotic protein Bax in postischemic liver tissue, whereas Bcl-2 expression increased in the untreated and LA groups. Decreased Bax by LA might represent a crucial event in attenuating IRI of the liver. Bax is localized in the cytosol of cells and translocates to mitochondria in response to death stimuli (31). Cleavage of Bax then stimulates cytochrome c release and apoptotic cell death in hepatocytes. It seems that LA regulates Bax and Bcl-2 balance of the rat liver. Because such modulation of proapoptotic and antiapoptotic proteins is described in rat brain to reduce IRI (32), our data strongly suggest that LA protects from apoptosis because of its action on proapoptotic and antiapoptotic proteins.

Generation of ROS could lead to the activation of caspase 8 and Bid (33–36). We could demonstrate decreased LPO and decreased activation of caspase 8 after LA pretreatment. Moreover, reduction of caspase 9 and 3 activation was observed at the same time point accompanied by reduced TUNEL staining after pretreatment with LA. Our data support the hypothesis that LA also reduces apoptosis by its antioxidative potential. This conclusion is further supported when comparing the hepatoprotection potential of LA, glycine, and IP; we observed that LA and IP decrease LPO in liver tissue; however, we also observed that LA is much more effective in reducing other parameters of hepatic injury in comparison with IP (in data preparation). As expected, glycine did not affect LPO in liver tissue and other parameters of warm hepatic injury and is therefore not as effective as LA in reducing IRI of the liver (37).

Previous studies have highlighted TNF-α as major and initial growth factor involved in liver regeneration (38). After LA pretreatment, we could demonstrate significantly higher levels of TNF-α in liver tissue compared with untreated animals. Our real-time RT-PCR results underline these

findings. Moreover, we could demonstrate increased features of liver regeneration by improvement of the mitotic index and Ki-67 staining, while animal survival was highly increased by LA pretreatment. These findings are completely new and underline the therapeutic potential of LA in pharmacological preconditioning. In comparison with established methods of liver preconditioning, such as IPC, this might be a powerful advantage of LA (14).

In summary, we could demonstrate impressive results about the influence of LA on IRI of the liver and liver regeneration *in vivo*. The potency of LA to protect from IRI and improve liver regeneration leading to dramatically improved animal survival makes it an attractive candidate for clinical application (39). In comparison with other drugs that were only used in experimental settings, this is an important difference and improvement of LA.

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REFERENCES

1. Wobbes T, Bemelmanns B, Kuypers JH, Beerthuizen GI, Theeuwes AG: Risk of postoperative septic complications after abdominal surgery treatment in relation to perioperative blood transfusion. *Surg Gynecol Obstet* 171:59–62, 1990.
2. Cowan RE, Jackson BT, Grainger SL, Thompson R: Effects of anesthetic agents and abdominal surgery on liver blood flow. *Hepatology* 14:1161–1166, 1991.
3. Huguet C, Gavelli A, Bona S: Hepatic resection with ischemia of the liver exceeding one hour. *J Am Coll Surg* 178:454–458, 1994.
4. Trump BF, Goldblatt PJ, Stowell RE: Studies of necrosis in vitro of mouse hepatic parenchymal cells. Ultrastructural alterations in endoplasmic reticulum, Golgi apparatus, plasma membrane and lipid droplets. *Lab Invest* 14: 2000–2028, 1965.
5. Lemasters JJ, Nieminen AL, Herman B: Blebbing, free Ca++ and mitochondrials membrane potential preceding cell death in hepatocytes. *Nature* 325: 78–81, 1987.
6. Jaeschke H, Bautista AP, Spolarics Z: Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after ischemia in rats. *J Leukoc Biol* 52:377–382, 1994.
7. Suzuki S, Toledo-Pereyra LH: Interleukin 1 and tumor necrosis factor production as the initial stimulants of liver ischemia and reperfusion injury. *J Surg Res* 57:253–258, 1994.
8. Scales WE, Campbell DA, Green ME, Remick DG: Hepatic ischemia/reperfusion injury: importance of oxidant/tumor necrosis factor interactions. *Am J Physiol Gastrointest Liver Physiol* 30:G1122–G1127, 1994.
9. Yamada Y, Kirillova I, Peschon JJ, Fausto N: Initiation of liver growth by tumor necrosis factor alpha: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc Natl Acad Sci USA* 94:1441–1446, 1997.
10. Giakoustidis D, Papageorgiou G, Iliadis S, Kontos N, Kostopoulou E, Papachrestou A: Intramuscular administration of very high dose of alpha-tocopherol protects liver from severe ischemia/reperfusion injury. *World J Surg* 26:872–877, 2002.
11. Bilzer M, Baron A, Schauer R, Steib C, Ebensberger S, Gerbes AL: Glutathione treatment protects the rat liver against injury after warm ischemia and Kupffer cell activation. *Digestion* 66:49–57, 2002.
12. Schemmer P, Bradford BU, Rose LM, Bunzendahl H, Raleigh JA, Lemasters JJ: Intravenous glycine improves survival in rat liver transplantation. *Am J Physiol Gastrointest Liver Physiol* 276:G924–G932, 1999.
13. Yadav SS, Sindram D, Perry DK, Clavien PA: Ischemic preconditioning protects the mouse liver by inhibition of apoptosis through a caspase-dependent pathway. *Hepatology* 30:1223–1231, 1999.
14. Eipel C, Glanemann M, Nuessler AK, Menger MD, Neuhaus P, Vollmar B: Ischemic preconditioning impairs liver regeneration in extended reduced-size livers. *Ann Surg* 241:477–484, 2005.
15. Arivazhagan P, Ramanathan K, Pannerselvam C: Effects of alpha-lipoic acid on glutathione metabolic enzymes in aged rats. *Exp Gerontol* 37:81–87, 2001.
16. Bastiaans H, Haenen G, Bast A: Interplay between ascorbic acid, alpha-tocopherol, glutathione and lipoic acid in the protection against microsomal lipid peroxidation. *Eur J Pharmacol* 183:2436–2437, 1990.
17. Müller C, Dünschede F, Koch E, Vollmar AM, Kiemer AK: Alpha lipoic acid preconditioning reduces ischemia-reperfusion injury of the rat liver via the PI3-kinase/Akt pathway. *Am J Physiol Gastrointest Liver Physiol* 285: G769–G778, 2003.
18. Trautschold I, Lamprecht W, Schweitzer G: ATP: UV-method with hexokinase and glucose-6-phosphate dehydrogenase. In Bergmeyer, (ed.): *Methods of Enzymatic Analysis. Vol. VII. 3rd ed*. Hoboken, NJ: John Wiley & Sons, pp 346–357, 1988.
19. Gurtu V, Kain SR, Zhang G: Fluorometric and colorimetric detection of caspase activity associated with apoptosis. *Anal Biochem* 251:98–102, 1997.
20. Ziegler D, Hanefeld M, Ruhnau J, Meissner HB, Lobisch M, Schutte K, Gries FA: Treatment of symptomatic diabetic peripheral neuropathy with the antioxidant alpha-lipoic acid. A 3-week multicentre randomized controlled trial (ALADIN Study). *Diabetologia* 38:1425–1433, 1995.
21. Suzuki JY, Tsuchiya M, Packer L: Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species. *Free Radic Res Commun* 15:255–263, 1991.
22. Packer L, Witt EH, Tritschler HJ: Alpha lipoic acid as a biological antioxidant. *Free Radic Biol Med* 19:227–250, 1995.
23. Hermann R: Human Pharmacokinetics of alpha-lipoic acid. In Fuchs J, Packer L, Zimmer G, (eds.): *Lipoic Acid in Health and Disease*. New York: Marcel-Dekker, pp 337–360, 1997.
24. Peinado J, Sies H, Akerboom TPM: Hepatic lipoate uptake. *Arch Biochem Biophys* 273:389–395, 1989.
25. Grützner U, Keller M, Bach M, Kiemer AK, Meissner H, Bilzer M, Zahler S, Gerbes AL, Vollmar AM: PI 3-kinase pathway is responsible for antiapoptotic effects of atrial natriuretic peptide in rat liver transplantation. *World J Gastroenterol* 12:1049–1055, 2006.
26. Zimmer G, Beikler TK, Schneider M, Ibel J, Tritschler H, Ulrich H: Dose/response curves of lipoic acid *R*- and *S*-forms in the working rat heart during reoxygenation: superiority of the *R*-enantiomer in enhancement of aortic flow. *J Mol Cell Cardiol* 27:1895–1903, 1995.
27. Gaballo A, Zanotti F, Solimeo A, Papa S: Topological and functional relationship of subunits F1-g and F0-I-PVP (b) in the mitochondrial H+ ATP-synthase. *Biochemistry* 37:17519–17526, 1998.
28. Dünschede F, Zwicker K, Ackermann H, Zimmer G: ADP- and oligomycin-sensitive redox behaviour of F0b thiol in ATP synthase depends on neighbored primary structure: investigation using 14-C–labeled alpha lipoic acid. *Biofactors* 19:19–32, 2003.
29. Rüdiger HA, Clavien PA: Tumor necrosis factor–alpha, but not Fas, mediates hepatocellular Apoptosis in the murine ischemic liver. *Gastroenterology* 122:202–210, 2002.
30. Kiemer AK, Vollmar AM: Inhibition of LPS-induced nitric oxide and TNFa production by a-lipoic acid in rat Kupffer cells and in RAW 264.7 murine macrophages. *Immunol Cell Biol* 80:550–557, 2002.
31. Jürgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC: Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci USA* 95:4997–5002, 1998.
32. Zhao Y, Li S, Childs EE, Kuharsky DK, Yin XM: Activation of pro-death Bcl-2 family and mitochondria apoptosis pathway in tumor necrosis factor alpha induced liver injury. *J Biol Chem* 276:27432–27440, 2001.
33. Brambrink MA, Schneider A, Noga H, Astheimer A, Götz B, Körner I, Heimann A, Welschof M, Kempski O: Tolerance-inducing dose of 3-nitropropionic acid modulates bcl-2 and bax balance in the rat brain: a potential mechanism of chemical preconditioning. *J Cereb Blood Flow Metab* 20:1425–1436, 2000.
34. Pessayre D, Fromenty B, Mansouri A: Mitochondrial injury in steatohepatitis. *Eur J Gastroenterol Hepatol* 16:1095–1105, 2004.
35. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiate an apoptotic protease cascade. *Cell* 91:479–489, 1997.
36. Bossy-Wetzel E, Green DR: Caspase induce cytochrome c release from mitochondria by activating cytosolic factors. *J Biol Chem* 274:17484–17490, 1999.
37. Duenschede F, Westermann S, Riegler N, Ewald P, Kircher A, Schaefer H, Schneider J, Schad A, Junginger T: Different protection mechanisms after pretreatment with glycine or alpha-lipoic acid in a rat model of warm hepatic ischemia. *Eur Surg Res.* 38:503–512, 2006.
38. Selzner N, Selzner M, Tian Y, Kadry Z, Clavien PA: Cold ischemia decreases liver regeneration after partial liver transplantation in the rat: a TNF-alpha/IL-6–dependent mechanism. *Hepatology* 36:812–818, 2002.
39. Dünschede F, Erbes K, Kircher A, Westermann S, Seifert J, Schad A, Kempski O, Kiemer AK, Junginger T: Reduction of ischemia reperfusion injury after liver resection and hepatic inflow occlusion by α-lipoic acid in humans. *World J Gastroenterol.* 12:6812–6817, 2006.