BCL-2 UPREGULATION AFTER 3-NITROPROPIONIC ACID PRECONDITIONING IN WARM RAT LIVER ISCHEMIA

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Received 4 Sep 2007; first review completed 21 Sep 2007; accepted in final form 19 Feb 2008

ABSTRACT—We aimed to determine whether 3-nitropropionic acid (3-NPA) preconditioning protects rat livers against warm ischemia/reperfusion injury. We hypothesized that 3-NPA mediates its protective effects by Bcl-2 upregulation. Brown-Norway rats (200 g) were injected with 3-NPA (10 mg/kg intraperitoneally) 24 h before 90 min of selective warm in situ ischemia. In additional experiments, 30-day survival was studied after 90 min of warm liver ischemia and resection of nonischemic liver tissue. We demonstrated increased mRNA and protein levels of Bcl-2 by real-time polymerase chain reaction, immunohistochemistry, and Western blot analysis in 3-NPA-pretreated rats. All treated animals survived, whereas all untreated rats died within 3 days after selective ischemia and resection of the nonischemic tissue. This corresponded well with a significant decrease of caspases 3 and 9 activity at 1 h of reperfusion after preconditioning with 3-NPA as compared with untreated rats. The histological sections showed protection of liver tissue after 3-NPA by reduction of apoptotic and oncotic tissue damage. Lipid peroxidation in liver tissue was reduced after 3-NPA preconditioning. We show that subtoxic doses of the mitochondrial toxin 3-NPA induces tolerance to warm liver ischemia in rats associated by synthesis of Bcl-2. Bcl-2 upregulation might protect against the postischemic burst of reactive oxygen species and therefore reduces apoptotic- and oncotic-related cell death.

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

INTRODUCTION

Several experimental methods have been introduced to reduce liver injury resulting from ischemia/reperfusion. Among those, tolerance induction or ischemic preconditioning, defined as sublethal stress before ischemia, can protect organs (1) by the use of endogenous adaptive mechanisms for protection (2). In addition to ischemic preconditioning, pharmacological compounds can induce preconditioning-like effects (3): partial inhibition of the respiratory chain by 3-nitropropionic acid (3-NPA), which blocks succinate dehydrogenase, has been shown to induce tolerance to ischemia in heart (4, 5). In a recent study, we were able to demonstrate that neuronal protection by a subtoxic dose of 3-NPA is accompanied by an upregulation of the antiapoptotic protein Bcl-2 and its mRNA (6).

The Bcl-2 family proteins regulate the mitochondria-dependent apoptotic pathway of hepatocytes. Whereas the prodeath family members, such as Bid, Bax, or Bak can promote mitochondrial release of cytochrome C, the antideath members of the family, such as Bcl-2 or Bcl-xL, can suppress this process (7). Cytochrome C in turn binds to Apaf-1 to induce its oligomerization, which activates initiator caspase 9, which then activates the downstream effector caspases (8).

In the current study, we were interested to analyze whether subtoxic 3-NPA preconditioning (24 h) can upregulate liver Bcl-2 before liver ischemia and can induce tolerance to 90 min of warm liver ischemia in vivo. Because of the strong metabolic activity of the liver related to a high density of mitochondria in this tissue, we hypothesized that this approach might be especially suitable for this organ. Indeed, 3-NPA was shown to induce lipid peroxidation (LPO) in liver tissue (9).

In the first part of the study, we investigated early reperfusion injury after selective liver ischemia with and without pretreatment of 3-NPA. Next, we analyzed survival in a long-term study after resection of the nonischemic tissue. The results show effective protection of a single dose of 3-NPA against ischemia/reperfusion injury (IRI).

MATERIALS AND METHODS

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); 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 by the National Institutes of Health (NIH publication 86-23, revised 1985). The study was approved by the local institutional animal care and use committee.

Liver ischemia

The animals were anesthetized by inhalation of ether. After a median laparotomy, ischemia was induced selectively in the left lobe of the liver (in short-term experiments) or the median lobe (in case of long-term experiments) by clamping the left branch of the portal vein, the hepatic artery, and the bile duct.
proximal to its origin. This method prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Ischemia was maintained over a period of 90 min for all experiments. The body temperature was monitored and maintained at 37°C ± 0.4°C by a heating lamp.

Animal survival was investigated by resecting the nonischemic lobe at the time of reperfusion. After infiltration anesthesia with ether, the abdominal cavity was accessed through a midline incision. In-fovo incision 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. Immediate color changes of the right, the caudate, and the left liver lobes were resected. The animals were monitored for 30 days.

Three experimental groups were studied: (a) liver resection without ischemia/reperfusion (n = 5), (b) 90 min of liver ischemia followed by resection of nonischemic liver tissue (n = 7), and (c) pretreatment with 3-NPA (10 mg/kg body weight) 24 h before ischemia and resection of nonischemic liver tissue (n = 7).

In another model, for acute experiments, 90-min ischemia of the left liver lobe was performed. 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 biochemical assays and histological investigations. Again, three experimental groups were studied: (a) sham-operated animals (n = 5), (b) 90-min ischemia/1-h reperfusion without pretreatment (n = 5), and (c) 90-min ischemia/1-h reperfusion after 24-h pretreatment with 3-NPA (10 mg/kg body weight i.p.; n = 5).

Another group of animals was killed 24 h after pretreatment with 3-NPA (10 mg/kg body weight i.p.; n = 3) for histology, immunohistochemistry, and Western blot analysis.

Liver homogenization
The liver tissue was weighed before homogenization, then dilated 1:5 with 0.01 M phosphate-buffered saline (PBS). Homogenization was then carried out with a Potter tissue homogenizer. A homogeneous mixture was achieved by vortexing at 800 rpm. Next, the mixture was centrifuged at 1,000 × g for 10 min, and 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.

Protein concentration
The protein concentration was determined by spectrophotometry with a standardized test (Protocol Assay ESL; Roche Diagnostics, Mannheim, Germany).

Enzyme release
Lactate dehydrogenase (LDH) in plasma was measured by spectrophotometry according to a standardized method (10) in plasma samples obtained 1 h after onset of reperfusion.

α-Glutathione–S-transferase (α-GST) in plasma 4 h after the onset of reperfusion was measured by enzyme-linked immunosorbent assay in accordance to the manufacturer’s instructions (Biotrin,pes, Schenken-Reichen, Germany) using a microplate reader (Bio-Rad, Munich, Germany).

Measurement of LPO in liver tissue
Lipid peroxidation was determined with a standardized test (Cayman Chemical, Grünberg, Germany). Absorption measurements were done at 500 nm using a microplate reader. The test measures lipid hydroperoxide based on the calibration curve, utilizing redox reactions with ferrous ions. The resulting ferric ions are detected, using thiocyanate ion as the chromogen. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a sensitive and reliable assay for LPO.

Activity of caspases
Caspases 3, 8, and 9 activity was measured using a colorimetric reaction (Caspase Colorimetric Assay; R and D Systems, 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 nonischemic sham liver tissue (11).

Polymerase chain reaction
The expression of TNF-α, Bax, and Bcl-2 was assessed by reverse transcriptase (RT)-polymerase chain reaction (PCR). The expression of 18S was used as the housekeeping gene. Total RNA was isolated using Qiagen RNeasy Mini-Kits (Qiagen). After photometric determination of the amount of RNA, first-strand cDNA was synthesized from 4 μg RNA from each liver, using the following procedure: the transcription mix contained 5 μL oligo(dT) (Gibco-BRL), 3.6 μL dNTP-Mix (10 mM) (Gibco-BRL), 8 μL first strand buffer, 4 μL dTT (0.1 M), 1.5 μL RT (MLV-T; Roche Diagnostics), and 1.5 μL RNA-free water (Qiagen). In controls, the procedure was performed without the addition of RT. 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 18S. Primers were designed specifically for rat sequences with the Primer 3 Software (Whitehead Institute, Boston, Mass) and synthesized by MWG Biotech, Germany. The following primers were used as described before (12–14): Bcl-2 neu forward: 5′-TGC AGT GAT TGC TTC CCA AGC GTC GTT GC-3′ (expected product, 200 base pairs [bp]); Bax forward: 5′-ACA GAT CAT GAA GAC AGG GG-3′, and reverse 5′-CAA AAT AGA AGG GGG CC-3′ (expected product, 203 bp); p22 Bid forward: 5′-GCC GGG ATA TCA TGG ACT CGT AGG TCA GCA CGG-3′, and reverse 5′-CCG CGG GAA TTC GGG GTT GTG AGC CCT CAG TC-3′ (expected product, 200 bp).

Real-time quantitative RT-PCR
Real-time quantitative RT-PCR analysis of transcripts for Bcl-2 and Gapd was performed with predesigned and optimized TaqMan Gene Expression Assays (Applied Biosystems) 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. Bcl-2 TaqMan Gene Expression Assay Rn 99999125-m1 was used.

Histology and Immunohistochemistry
Formalin-fixed tissue samples were embedded in paraffin, and 5-μm sections were cut. Sections were stained with hematoxylin-eosin for the evaluation of morphological features of oncosis/necrosis or stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (see below) for the evaluation of apoptosis.

Morphological features of oncosis were evaluated in serial sections stained with hematoxylin-eosin. Morphological criteria of oncosis 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 as described before.

The liver sections were fixed in freshly prepared neutral buffered formalin (PBS). Frozen sections (5 μm) of the fixed tissue were prepared and stained with the TUNEL method, using a commercial kit (ApopTag peroxidase in situ apoptosis detection kit; Chemicon International, Hampshire, UK). The number of TUNEL-positive hepatocytes was counted in 10 high-power (×400) fields.

All histological evaluations were done in a blinded fashion.

Immunohistochemical staining of Bcl-2 in liver tissue
For immunohistochemical detection of Bcl-2, a polyclonal rabbit anti-rat bcl2 antibody was used. After 20-min microwave pretreatment of dewaxed formalin-fixed paraffin sections in citrate buffer (pH 6.0), immunostaining was performed on an automated immunostainer (Techmate 500 plus, DAKO) using a DAB-peroxidase based detection system (K5001, DAKO) as described before (12).

Western blot
One hundred milligrams of rat livers was homogenized in 1 mL lysis buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl; 50 mM NaF; 1 mM EDTA; 1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride, and 1× Complete mini (Roche Applied Science, Mannheim, Germany). To remove cell debris, lysates were centrifuged at 14,000g for 15 min at 4°C. The supernatant was aliquoted, supplemented with 5% glycogen (vol/vol), and frozen until usage. Protein concentrations were determined by Bradford Assay (Bio-Rad) according to standard protocols.

Supernatants were mixed with one-third volume of 4× RotiLoad (Carl Roth, Karlsruhe, Germany) and boiled for 5 min at 95°C. From each sample, 20 μg of whole-cell protein was electrophoretically separated on 15% sodium dodecyl sulfate–polyacrylamide gels, transferred to Immobilon-FL PVDF membranes (Millipore GmbH, Schwabach/Ts, Germany), and subjected to immunoblotting.

For visualization of proteins with Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg/Velburg, Germany), membranes were blocked in PBS containing 0.1% Tween-20 (PBST) and 2.5% milk powder (Carl Roth). Primary antibodies (Bcl-2 antibody was from Cell Signalling Technology Inc, New England Biolabs, Frankfurt, Germany; β-actin antibody was from Biomol, Hamburg, Germany) were incubated overnight at 4°C in a 1:1,000 dilution in Odyssey blocking buffer (LI-COR Biosciences).

After washing, membranes were incubated with an IRdye680-conjugated goat anti-rabbit IgG secondary antibody (LI-COR Biosciences) in a 1:5,000 dilution in Odyssey blocking buffer (LI-COR Biosciences). After a subsequent washing step, proteins were detected and quantified with Odyssey Infrared Imaging System.

Statistics
All data are expressed as means ± SD. Statistical differences between experimental groups were calculated by GraphPad Prism using one-way analysis of variance followed by Mann-Whitney U test. Survival curves were calculated...
using the Kaplan-Meier method. Proportions were calculated using Fisher exact test. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

### Expression of Bcl-2, Bax, and Bid in liver tissue and immunohistochemical staining

In sham-operated animals, we saw no expression of Bcl-2 in liver tissue, whereas mRNA of Bax and Bid was detectable (Fig. 1, bottom). After preconditioning with 3-NPA, Bcl-2 expression was present, and expression of Bax and Bid remained unchanged. Correspondingly in real-time quantitative RT-PCR analysis, mRNA of Bcl-2 was upregulated to 2.3-fold ($\pm 0.1$) compared with sham-operated animals after pretreatment with 3-NPA ($P < 0.05$).

To determine whether 3-NPA-induced mRNA expression in fact results in increased protein expression, we performed immunohistochemical staining (Fig. 1, A and B). Livers pretreated with 3-NPA showed marked staining, whereas no Bcl-2 protein could be detected in untreated tissues. Western blot analyses confirmed increased Bcl-2 protein levels in NPA-pretreated organs (Fig. 1C): band intensity was 1.5-fold ($\pm 0.04$) compared with untreated controls ($P < 0.03$).

### Caspase activity and TUNEL staining

To prove these results, we determined caspase 3 activity in liver tissue which was 3-fold increased without treatment of 3-NPA. Accordingly, caspase 9 activity was also decreased by the treatment of 3-NPA, whereas caspase 8 activity remained unaffected (Fig. 2).

To analyze more specifically the apoptotic-related cell death, we performed first TUNEL staining which showed increased amount of TUNEL-positive hepatocytes without treatment of 3-NPA (Fig. 2).

### Enzyme release and reactive oxygen species

Next, we focused on ischemic tissue injury with and without treatment of 3-NPA. As indicator of tissue damage,
we analyzed cytosolic LDH and α-GST release after ischemia. Both parameters increased significantly in the nontreated group after 60 min of ischemia/reperfusion, whereas 3-NPA decreased LDH and α-GST release (Fig. 3).

Lipid peroxidation increased significantly without treatment of 3-NPA after 90 min of ischemia and 60 min of reperfusion (Fig. 3). 3-Nitropropionic acid decreased the burst of reactive oxygen species (ROS) after ischemia reperfusion.

Histology and TUNEL

Correspondingly, we found after 90 min of ischemia and 60 min of reperfusion several signs of oncosis and necrosis (swelling, vacuolization, blebbing, cell disruption, and shrinkage...
of hepatocytes; Fig. 4B) as well as increased TUNEL staining (Fig. 4E). Preischemic application of 3-NPA reduced significantly tissue injury (Fig. 4, C and F).

**Long-term survival**

Because of the above shown protective effects on early reperfusion damage, we were interested whether 3-NPA pretreatment could also reduce long-term damage and thus prevent lethal hepatic ischemia/reperfusion. Therefore, we performed in-flow occlusion of the median lobe and resected the nonischemic hepatic tissue before reperfusion. We compared the survival rates of animals from the 3-NPA–treated group and the nontreated group after 90 min of ischemia. One group of rats with liver resection served as control. All animals of the nontreated ischemia/reperfusion group died within the first 48 h (n = 7). In the 3-NPA group, all animals survived the 90 min of ischemia and resection of the nonischemic lobes of the liver up to 30 days after surgery (n = 7). Also, all animals with liver resection and without ischemia survived the 30-day monitoring period (n = 5). Doubling the concentration of 3-NPA resulted in the death of all animals within the first hour after application (n = 3). All animals survived reperfusion after 60 min of ischemia and resection of nonischemic liver tissue (n = 3; Fig. 5).

**DISCUSSION**

Our results demonstrate three important findings: first, an upregulation of Bcl-2 in liver tissue after a single dose of 3-NPA one day before selective hepatic ischemia; second, reduced reperfusion injury in livers pretreated with 3-NPA; and third, complete survival of animals preconditioned with 3-NPA, whereas all nontreated animals died.

Mitochondria are one of the major intracellular sources of ROS (15). Until now, the mechanism of action of ROS in IRI of the liver is still not completely clarified in detail. One of the most convincing hypotheses of ROS-induced cell injury is the destruction of cellular membranes through peroxidation of lipids (16). Antioxidants have therefore been proven a useful strategy to prevent IRI (12–14). In our present study, we could demonstrate apoptotic cell injury involving the activation of caspases 3 and 9 paralleled to oxidative stress and oncotic cell death after selective ischemia. Application of 3-NPA reversed all signs of injury which could be associated by the overexpression of Bcl-2. Bcl-2 overexpression has been demonstrated to protect cells against oxidative cell death by complete suppression of LPO (17) and reduced generation of ROS (18). Our results suggest that this mechanism of Bcl-2 expression inducing attenuated ROS-induced cell damage is induced by 3-NPA in the liver.

3-Nitropropionic acid is a potent mitochondrial toxin which blocks succinate dehydrogenase and, thereby, complex II of the electron transport chain.
the respiratory chain (4). In subtoxic doses, the blocking of succinate dehydrogenase is partial and can induce tolerance to ischemia as has previously been shown for brain and heart (5, 19). In accordance with these observations, we could demonstrate increased Bcl-2 mRNA and protein in liver tissue after 3-NPA preconditioning, suggesting a transcriptional process (6). Answering the question how 3-NPA upregulates Bcl-2 expression in detail, we assume that 3-NPA leads to the generation of sublethal oxidative stress by blocking succinate dehydrogenase (4, 9). Referring to previous results, we suggest that this mild burst of oxidative stress could trigger antiapoptotic mechanism (2).

3-Nitropropionic acid preconditioning significantly suppressed caspases 3 and 9 without interfering with caspase 8 activation after ischemia. Hence, apoptotic mechanisms are likely affected on the mitochondrial level. This is supported by the impressive reduction of apoptotic hepatocytes in the 3-NPA–treated group. This is most likely facilitated via the observed upregulation and synthesis of Bcl-2; Bcl-2 and other antiapoptotic Bcl-2 family members block cytochrome C release, either by blocking the mitochondrial permeability transition pore or by antagonism of Bax/Bak-dependent pore formation in the mitochondrial outer membrane (20). Similarly, in mice overexpressing Bcl-2 (21, 22), liver ischemia/reperfusion was better tolerated than in wild-type mice as far as survival and liver damage by apoptotic mechanisms were concerned. In addition, Zhu et al. (23) observed inhibition of caspase 3 activation and apoptosis in transient focal cerebral ischemia in rats by 3-NPA. Interestingly, other Bcl-2 family proteins such as Bax or Bid were not affected by 3-NPA preconditioning.

Our study represents a novel and simple strategy for the prevention of hepatic IRI. While hepatocytes do not express Bcl-2 under normal circumstances, until now induction of this protein has been mostly described in complex and time-consuming models (20, 21). We demonstrate Bcl-2 upregulation before the surgical procedure, inducing hepatoprotection in a model of warm liver ischemia. These findings support the impact of the induction of hepatic defense mechanism before the onset of IRI of the liver. Future experimental studies need to clarify hepatoprotective potentials of 3-NPA in liver transplantation, which might prove as a suitable clinical approach. However, these results show indeed the toxic potential of 3-NPA. This means that expanded dose-response studies would of course be necessary before clinical use could be recommended.

In summary, we showed reduction of oncotic- and apoptotic-related cell death in warm hepatic IRI by preconditioning animals with 3-NPA. Under these conditions, upregulation of Bcl-2 mRNA and protein was observed. To the knowledge of the authors, this is the first study which describes the effects and the mechanism of action of subtoxic doses of 3-NPA in IRI of the liver.

REFERENCES