Hepatocyte apoptosis is enhanced after ischemia/reperfusion in the steatotic liver

Takeshi Suzuki, Hiroyuki Yoshidome,* Fumio Kimura, Hiroaki Shimizu, Masayuki Ohtsuka, Dan Takeuchi, Atsushi Kato, Katsunori Furukawa, Hideyuki Yoshitomi, Ayako Iida, Takehiko Dochi and Masaru Miyazaki

Department of General Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-0856, Japan

(Received 29 June, 2010; Accepted 13 July, 2010; Published online 26 February, 2011)

Liver steatosis is associated with organ dysfunction after hepatic resection and transplantation which may be caused by hepatic ischemia/reperfusion injury. The aim of the current study was to determine the precise mechanism leading to hepatocyte apoptosis after steatotic liver ischemia/reperfusion. Using a murine model of partial hepatic ischemia for 90 min, we examined the levels and pathway of apoptosis, and the peroxynitrite expression, serum alanine aminotransferase levels, and liver histology 1 and 4 h after reperfusion. In the steatotic liver, the peroxynitrite expression increased after ischemia/reperfusion. Significant hepatocyte apoptosis in the steatotic liver was seen after reperfusion, caused by upregulation of cleaved caspases 9 and 3, but not caspase 8. Serum alanine aminotransferase levels were elevated and histological examination revealed severe liver injury in the steatotic liver 4 h after reperfusion. In mice treated with aminoguanidine, ischemia/reperfusion-induced increases in serum alanine aminotransferase levels and apoptosis were significantly reduced in steatotic liver compared with mice treated with phosphate buffered saline. Survival of mice with steatotic livers significantly improved by treatment with aminoguanidine. Our data suggested that the steatotic liver is vulnerable to hepatic ischemia/reperfusion, leading to significant hepatocyte apoptosis by the mitochondrial permeability transition, and thereby resulting in organ dysfunction.

Key Words: steatosis, apoptosis, peroxynitrite, hepatic resection

Recently, the number of patients with hepatic steatosis has increased due to alcohol abuse and non-alcoholic fatty liver disease (NAFLD). There are several metabolic disorders involved in the steatotic liver, including oxidative stress, susceptibility to apoptosis, and dysfunction of the mitochondria. Steatosis in the liver is associated with postoperative complications after hepatic resection and primary graft nonfunction after liver transplantation. Furthermore, the current shortage of organ donors leads to an increase in use of steatotic livers for liver transplantation. Steatohepatitis after neoadjuvant chemotherapy for hepatic malignancies is another clinical concern for liver damage and insufficiency of liver regeneration after hepatic resection. For these reasons, further investigation of the cause of liver dysfunction in the steatotic liver after hepatic resection and transplantation is necessary.

One of the major causes of liver dysfunction/damage after hepatic surgery for a steatotic liver is hepatic ischemia/reperfusion injury. In experimental studies, hepatic ischemia/reperfusion injury is caused by the ischemic stress itself, the production of reactive oxygen species, inflammatory responses induced by proinflammatory mediators, neutrophil-mediated proteases, microcirculatory disturbance, and apoptosis of hepatocytes.

Nitric oxide (NO) is the metabolic product by NO synthase (NOS). Three isoforms of NOS have been identified; endothelial NOS (eNOS), neural NOS, and inducible NOS (iNOS). NO derived from eNOS has protective effects through maintenance of microcirculation, but NO derived from iNOS is harmful due to production of hazardous reactive oxygen species, such as peroxynitrite. Such hazardous reactive oxygen species may lead to hepatocyte apoptosis which is considered to be an important factor in hepatic ischemia/reperfusion injury. There are two main pathways leading to apoptosis. The type 1 (extrinsic) signaling pathway results from a death signal caused by the expression of tumor necrosis factor-alpha and Fas ligand. Tumor necrosis factor-alpha and Fas associated death domains promote the binding of procaspase 8, and the subsequent proteolytic activation of catalytic caspase 8. Caspase 8 is able to activate procaspase 3, leading to apoptosis. Conversely, the type 2 (intrinsic) signaling pathway is induced by the mitochondrial permeability transition, leading to cytochrome c release. The cytochrome c complex activates caspase 9 followed by the cleavage and subsequent activation of caspase 3. Apoptosis requires adenosine triphosphate (ATP), and a switch from apoptosis to necrosis occurs when cells are devoid of ATP. Although much is known regarding hepatic ischemia/reperfusion injury in the normal liver, the cause of steatotic liver ischemia/reperfusion injury has not been thoroughly elucidated. We hypothesized that excessive peroxynitrite production associated with iNOS expression leads to hepatocyte apoptosis, thus resulting in hepatic ischemia/reperfusion injury in the steatotic liver. The aim of the current study was to determine whether hepatocyte apoptosis is augmented in the steatotic liver after ischemia/reperfusion and to examine the production of peroxynitrite and the possible involvement of apoptosis after hepatic ischemia/reperfusion.

Materials and Methods

Male BKS.Cg-m+/Leprdb/db mice, genotype Leprdb/Leprdb (db/db mouse, JAX MICE Tsukuba Charles River Laboratories Japan, Inc., Tsukuba, Japan) weighing 37 to 42 g were used as the fatty liver (FL) group and lean littermates weighing 22 to 25 g were used as the wild type (WT) group. The mice were housed in a controlled environment, type 12-h light/dark cycle, and provided with commercial chow and water ad libitum. This project was approved by the Chiba University Animal Care and Use Committee and was in compliance with National Institutes of Health guidelines.

Hepatic ischemia/reperfusion model and reagents. The model of partial hepatic ischemia and reperfusion employed was

©2011 JCBN

doi: 10.3164/jcbn.10-74
prepared as described previously. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg administered intraperitoneally). A midline laparotomy was performed and an atraumatic clip was used to interrupt the arterial and the portal venous blood supply to the left lateral and median lobes of the liver for 90 min. Sham control mice underwent the same procedure but without vascular occlusion. The mice were sacrificed prior to surgery (basal), 4 h and 4 h after reperfusion, and liver tissue and blood samples were taken for analysis (n = 6, respectively). In the iNOS inhibition experiment, prior to partial ischemia, mice received either 25 mg/kg of aminoguanidine (AG) in sterile phosphate buffered saline (0.1 ml) or just the phosphate buffered saline (0.1 ml) (Sigma Aldrich, St. Louis, MO). The doses were adopted based on the study by Cottart et al. Survival was examined up to 8 h after reperfusion (n = 6, respectively).

Analysis of apoptosis and histological examination.
Liver tissue specimens were obtained, and sections of formalin-fixed paraffin-embedded liver samples were stained with hematoxylin-eosin to assess the degree of liver injury. In order to quantify the apoptotic hepatocytes, we used the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) biotin nick end-labeling (TUNEL) method, which labels the DNA double-strand breaks characteristics of apoptosis. The assay was carried out by utilizing an in situ apoptosis detection kit (Wako Pure Chemical, Co. Ltd., Osaka, Japan) according to the manufacture’s instructions. For quantitative evaluation for apoptotic index, percent ratios of TUNEL-positive hepatocyte to the total number of hepatocytes were calculated for each sample in five random fields (400×).

Western blot analysis. Liver tissue specimens were obtained and immediately frozen in liquid nitrogen. Frozen liver tissues were homogenized in lysis buffer containing 10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.6% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml apro tin, 10 μg/ml soybean trypsin inhibitor, and 1 μg/ml pepstatin on ice. The homogenates were sonicated and centrifuged at 5,000 rpm to remove cellular debris. The protein concentration was determined using the BCA Protein Assay kit (Pierce Chemical Co. Rockford, IL). Liver lysate protein (20 μg) was subjected to the XV PANTERA system (DRC, Tokyo, Japan). Samples were electrophoresed in a precast 7.5–15% gradient gel. Liver tissue specimens were obtained and frozen immediately, and extracts of liver tissue were prepared as described previously.

Electrophoretic mobility shift assay (EMSA). The nuclear extracts of liver tissue were prepared as described previously. The protein concentrations were determined by a bicinchoninic acid assay with trichloroacetic acid precipitation, using BSA as a reference standard. Double-stranded NF-κB consensus oligonucleotide (Promega, Madison, WI) was end-labeled with [γ-32P]ATP (3,000 Ci/mmol, Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of protein (20 μg) and 35 fmols (~50,000 cpm, Cherenkov counting) of oligonucleotide were performed for 30 min in binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, pH 8.0, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris, pH 7.6, 50 μg/ml poly (dI-dC); Pharmacia, Piscataway, NJ). The reaction volumes were held constant at 15 μl. The reaction products were separated on a 4% polyacrylamide gel and analyzed by autoradiography.

Blood and ELISA quantification of tissue proteins. Blood was obtained by cardiac puncture at the time of sacrifice. Serum samples were analyzed for alanine aminotransferase (ALT) as an index of hepatocellular injury, using a diagnostic kit (Wako Pure Chemical, Japan). Hepatic nitrotyrosine production in the liver was quantitatively assessed by methods described elsewhere. Briefly, specimens were obtained and frozen immediately, and then were homogenized in 10 volumes of homogenization buffer (10 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml soybean trypsin inhibitor, 1.0 mg/ml bovine serum albumin, 0.02% sodium azide, and 0.2 μl/ml protease inhibitor cocktail [1,000 × stock; 1 mg/ml leupeptin, 1 mg/ml apro tin, and 1 mg/ml pepstatin]). After incubating for 2 h at 4°C, the homogenate was centrifuged at 12,500 g for 10 min. Supernatant was removed and centrifuged again to obtain clear lysate. Total protein concentration of each sample was measured by using a bicinchoninic assay kit, and samples were dispensed for nitrotyrosine EIA kit (OXIS International Inc., Foster City, CA).
Protein concentration was calculated per mg total protein.

**Statistical analysis.** All data were analyzed for statistical significance using the Mann-Whitney test or were analyzed with a one-way analysis of variance and individual group means were then compared with a Student-Newman-Keuls test. All data were expressed as the mean ± SEM. Overall survival was calculated using the Kaplan-Meier method, and comparisons were evaluated using the log rank test. The data were analyzed by using the SigmaStat 3.0 or SPSS 11.5 software program. A value of *p* < 0.05 was considered to be statistically significant.

**Results**

**Liver apoptosis during ischemia/reperfusion.** TUNEL staining was performed to investigate whether hepatocyte apoptosis was induced after reperfusion. The number of TUNEL-positive apoptotic hepatocytes quantified by calculating the apoptotic index in FL mice increased significantly compared with that in sham mice 1 h after reperfusion (*n* = 6, respectively). This increase was not observed in WT mice. A significant increase in TUNEL-positive apoptotic cells in FL mice was seen relative to WT mice 1 and 4 h after reperfusion (Fig. 1). To determine the precise apoptosis pathway activated during ischemia/reperfusion, we performed western blot analysis of precursor and cleaved caspases-3, -8, and -9. In FL mice, cleavage of caspase-3 and -9 was increased more than that in WT mice 1 and 4 h after reperfusion. However, the expression of cleaved caspase-8 did not differ between the FL and WT mice (Fig. 2).

**Expression of peroxynitrite during ischemia/reperfusion.** Since NOS-mediated NO generates the peroxynitrite oxidant, we examined nitrotyrosine expression by ELISA. Hepatic nitrotyrosine expression was significantly increased in FL mice 1 h after reperfusion, as compared to WT mice (*n* = 6, respectively; FL, 16.9 ± 9.3, WT 5.2 ± 2.6 pmol/mg, *p* < 0.05).

**Activation of NF-κB during ischemia/reperfusion.** Since NF-κB is known to play a role in both inflammatory responses and anti-apoptosis effects, we performed EMSA to determine whether NF-κB activation was induced by hepatic ischemia/reperfusion. After reperfusion, nuclear translocation of NF-κB in WT mice increased 1 and 4 h after reperfusion, but little NF-κB activation was observed in FL mice up to 4 h after reperfusion (Fig. 3).

**Hepatocellular injury after hepatic ischemia/reperfusion.** To assess the extent of hepatic injury during ischemia/reperfusion, serum levels of ALT were examined (*n* = 6, respectively). The serum ALT levels in the FL mice were significantly increased relative to the WT mice 1 and 4 h after reperfusion (Fig. 4A). The histological findings showed that focal hepatic necrosis was observed in the FL mice 4 h after reperfusion (Fig. 4B–E).

**Role of iNOS in apoptosis and liver damage during ischemia/reperfusion.** To determine whether iNOS was involved in liver apoptosis during hepatic ischemia/reperfusion, we performed TUNEL staining. The number of TUNEL positive cells, quantified by calculating the apoptotic index, was significantly upregulated 4 h after reperfusion in WT and FL mice relative to sham-operated controls (*n* = 6, respectively; Fig. 5). There was significant difference in apoptotic index of WT mice during ischemia/reperfusion.
T. Suzuki et al.

NF-κB →

Fig. 3. NF-κB activation in whole liver homogenates during hepatic ischemia/reperfusion. Nuclear extracts from liver tissue were subjected to EMSA. The results are representative of two separate time-course experiments. Both WT and FL mice underwent either a sham operation or ischemia for 90 min (I/R).

administered AG as an iNOS specific inhibitor. Treatment with AG significantly reduced the number of apoptotic cells in FL mice relative to treatment with PBS (Fig. 5). To determine whether iNOS was involved in hepatic ischemia/reperfusion injury, we administered AG as an iNOS specific inhibitor. Treatment with AG significantly reduced the number of apoptotic cells in FL mice relative to treatment with PBS (Fig. 5). To determine whether iNOS was involved in hepatic ischemia/reperfusion injury, we examined the serum alanine aminotransferase (ALT) levels following the administration of AG. In sham-operated controls, there were no differences in serum ALT levels among WT and FL mice treated with PBS or AG. One hour after reperfusion, serum ALT levels were significantly decreased by administration of AG (56% decrease, p = 0.002) in the WT mice. In the FL mice,

Fig. 4. (A) Hepatocellular injury as indicated by serum levels of alanine aminotransferase (ALT). Samples were obtained from mice undergoing the sham operation or ischemia for 90 min and reperfusion (I/R). Values represent the mean ± SEM with n = 6 per group. *p<0.05, compared to the WT group. Histological findings of the liver assessed by hematoxylin and eosin staining. (B) Sham operated WT group. (C) Sham operated FL group. (D) WT group 4 h after reperfusion. (E) FL group 4 h after reperfusion. Original magnification ×100.

Fig. 5. Hepatocyte apoptosis induced by ischemia/reperfusion assessed by the TUNEL method. To assess the effect of the iNOS inhibitor on hepatocyte apoptosis, samples from mice treated with PBS or amino-guanidine (AG) prior to ischemia for 90 min were examined. Samples were obtained from (A) sham operated WT, (B) sham operated FL, and (C) FL mice undergoing 90 min ischemia and 4 h reperfusion (I/R) with PBS treatment, (D) WT mice undergoing I/R with PBS treatment, (E) FL mice undergoing I/R with AG treatment. (F) WT mice undergoing I/R with AG treatment. Original magnification ×200. Arrows indicate hepatocyte apoptosis. The apoptotic index was also calculated in mice 4 h after reperfusion (G). The data represent the mean ± SEM with n = 6 per group. *p<0.05 compared to mice treated with PBS.
administration of AG also attenuated serum ALT levels (20% decrease, \( p = 0.025 \)) compared with the FL mice treated with PBS 1 and 4 h after reperfusion (Fig. 6).

**Survival after ischemia/reperfusion.** To determine whether apoptosis and subsequent hepatic ischemia/reperfusion injury may be related to death, we examined the survival of mice after administration of AG. In sham-operated controls, neither WT nor FL mice died after treatment with PBS or AG (Fig. 7). None of WT mice undergoing ischemia for 90 min died during 8 h after reperfusion. On the other hand, the survival rate in FL mice undergoing ischemia for 90 min after reperfusion was significantly worse. However, treatment with AG significantly ameliorated survival after reperfusion in the FL mice (Fig. 7).

**Discussion**

Liver surgery for steatotic liver poses risks of post-operative morbidity, mortality, and primary graft nonfunction.\(^7\,\,8\) One of the possible causes of this event is hepatic ischemia/reperfusion injury. We herein provide evidence that the steatotic liver is vulnerable to hepatic ischemia/reperfusion.

We have also demonstrated that the production of reactive oxygen species is related to this type of injury. In fact, NO derived from iNOS is harmful because of production of hazardous reactive oxygen species, such as peroxynitrite.\(^1\,\,4\) Peroxynitrite concentrations were significantly upregulated in FL mice compared with WT mice in the current study, suggesting that upregulation of
reactive oxygen species produced by iNOS may initiate hepatocyte apoptosis in the steatotic liver after ischemia/reperfusion. This was confirmed by the fact that AG administration reduced the hepatocyte apoptosis, suggesting that the induction of apoptosis is, at least in part, attributable to upregulation of iNOS.

The activation of cleaved caspase 8 did not differ between the WT and FL mice, suggesting that death signaling is unlikely to play a role in augmenting apoptosis in the steatotic liver. However, the activation of cleaved caspase 9 was prominent in the initial phase of reperfusion, and cleaved caspase 3 was activated in the steatotic liver, thus suggesting that the mitochondrial permeability transition plays an important role in apoptosis associated with steatotic liver ischemia/reperfusion injury. This pathway has been known to occur in hepatocytes, and to induce apoptosis more rapidly than type 1 pathway. Immunohistochemistry revealed iNOS expression in hepatocytes, especially in the periportal area (personal observation). It has also been demonstrated that perportal hepatocytes are vulnerable to hypoxia-reoxygenation and that Kupffer cells in periportal areas engulf apoptotic cells, thus resulting in the release of reactive oxygen species. Taken together, the early induction of hepatocyte apoptosis was significantly observed after reperfusion in the steatotic liver.

In general, the role of NF-κB during hepatic ischemia/reperfusion injury has been considered proinflammatory. Previous studies have shown that the therapeutic modalities which reduce inflammatory injury after ischemia/reperfusion, also reduce NF-κB activation. On the other hand, data from other models of liver injury have suggested that hepatocyte activation of NF-κB is associated with its anti-apoptotic effects. In the current study, NF-κB activation in whole livers (which is largely representative of hepatocytes) was decreased in steatotic livers after hepatic ischemia/reperfusion, as demonstrated by EMSA. The steatotic liver after reperfusion was vulnerable to hepatocyte apoptosis, which may be associated with decreased NF-κB-related anti-apoptotic properties.

The steatotic liver is known to have low ATP levels after hepatic ischemia/reperfusion. Our previous studies have demonstrated that cellular apoptosis consumes a large amount of nicotinamide adenine dinucleotide (NAD+), and to resynthesize NAD+, resulting in a decrease in ATP levels. Combination with hepatocyte apoptosis and ATP depletion changes the cellular response to secondary oncotic necrosis. These findings suggest that hepatocyte apoptosis in conjunction with a decrease in ATP levels causes secondary oncotic necrosis, leading to significant liver injury. This is consistent with our observation that the serum ALT levels were greatest in the steatotic liver, which resulted in severe liver damage and lower survival in the late phase of reperfusion in the steatotic liver. Treatment with AG improved the survival of FL mice after reperfusion, suggesting that iNOS-related hepatocyte apoptosis seems to initiate hepatic ischemia/reperfusion injury in the steatotic liver. However, the current study demonstrated that the reduction in liver injury as defined by serum ALT levels under the blockade of iNOS is modest, thus suggesting that other mechanism might be related to this type of injury.

In conclusion, our data suggest that the steatotic liver is vulnerable to ischemia/reperfusion injury due to hepatocyte apoptosis associated with the early upregulation of iNOS-related peroxinitrite, which thus leads to subsequent oncotic necrosis. The present data are considered to expand our knowledge of steatosis and therefore clinically applicable for patients with ischemia/reperfusion injury during liver surgery for steatotic liver.

Acknowledgment

This work was supported in part by grants from the Japan Society for the Promotion of Science (#20591601, 22591500).

Abbreviations

| FL | fatty liver |
| NF-κB | nuclear factor-kappa B |
| NAFLD | non-alcoholic fatty liver disease |
| ALT | alanine aminotransferase |
| TUNEL | terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling |
| EMSA | electrophoretic mobility shift assay |
| ELISA | enzyme-linked immunosorbent assay |
| NOS | nitric oxide synthase |
| AG | aminoguanidine |

References

1 Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; 37: 1202–1219.
2 Roskams T, Yang SQ, Koteish A, and *et al*. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol* 2003; 163: 1301–1311.
3 Feldstein AE, Canbay A, Angulo P, and *et al*. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003; 125: 437–443.
4 Vendemiale G, Grattagliano I, Caraceni P, and *et al*. Mitochondrial oxidative injury and energy metabolism alteration in rat fatty liver: effect of the nutritional status. *Hepatology* 2001; 33: 808–815.
5 Todo S, Demetris AJ, Makowka L, and *et al*. Primary nonfunction of hepatic allografts with preexisting faking liver. *Transplantation* 1989; 47: 903–905.
6 Strasberg SM, Howard TK, Molmenti EP, Hertl M. Selecting the donor liver: risk factors for poor function after orthotopic liver transplantation. *Hepatology* 1994; 20: 829–833.
7 Kooby DA, Fong Y, Surawicz MA, and *et al*. Impact of Steatosis on perioperative outcome following hepatic resection. *J Gastrointest Surg* 2003; 7: 1034–1044.
8 Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001; 21: 105–113.
9 Marsman WA, Wiesner RH, Rodriguez L, and *et al*. Use of fatty donor liver is associated with diminished early patient and graft survival. *Transplantation* 1996; 62: 1246–1251.
10 Vauthay JN, Pavlik TM, Ribero D, and *et al*. Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. *J Clin Oncol* 2006; 24: 2065–2072.
11 Yoshidome H, Kato A, Edwards MJ, Lentsch AB. Interleukin-10 suppresses hepatic ischemia/reperfusion injury in mice: implications of a central role for nuclear factor Kappa B. *Hepatology* 1999; 30: 203–208.
12 Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991; 260: G355–G362.
13 Rüdiger HA, Clavien PA. Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver. *Gastroenterology* 2002; 122: 202–210.
14 Laroux FS, Pavlik KP, Hines IN, and *et al*. Role of nitric oxide in inflammation of donor livers from transplanted patients. *Acta Physiol Scand* 2001; 173: 113–118.
15 Song SW, Tolba RH, Yonezawa K, Manekeller S, Minor T. Exogenous superoxide dismutase prevents peroxynitrite-induced apoptosis in non-heart-beating donor livers. *Eur Surg Res* 2008; 41: 353–361.
16 Peter ME, Brammer PH. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr Opin Immunol* 1998; 10: 545–551.
17 Soeda J, Miyagawa S, Sano K, Masumoto J, Taniguchi S, Kawasaki G. Cytochrome c release into cytosol with subsequent caspase activation during warm ischemia in rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001; 281: G1115–G1123.
18 Murin D, Pires F, Plin C, Tillement JP. Role of the permeability transition pore in cytochrome c release from mitochondria during ischemia-reperfusion in rat liver. *Biochem Pharmacol* 2004; 68: 2065–2073.
19 Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;
20 Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology 2003; 125: 1246–1257.

21 Cottart CH, Do L, Blanc MC, and et al. Hepatoprotective effect of endogenous nitric oxide during ischemia-reperfusion in the rat. Hepatology 1999; 29: 809–813.

22 Gavrieli Y, Sherman Y, Ben-Sasson S.A. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493–501.

23 Takeuchi D, Yoshidome H, Kato A, and et al. Interleukin 18 causes hepatic ischemia/reperfusion injury by suppressing anti-inflammatory cytokine expression in mice. Hepatology 2004; 39: 699–710.

24 Iimuro Y, Nishiura T, Hellerbrand C, and et al. NFκB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998; 101: 802–811.

25 Taniai H, Hines IN, Bharwani S, and et al. Susceptibility of murine periportal hepatocytes to hypoxia-reoxygenation: role for NO and Kupffer cell-derived oxidants. Hepatology 2004; 39: 1544–1552.

26 Cursio R, Gugenheim J, Ricci JE, and et al. A caspase inhibitor fully protects rats against lethal normothermic liver ischemia by inhibition of liver apoptosis. FASEB J 1999; 13: 253–261.

27 Iida A, Yoshidome H, Shida T, and et al. Hepatocyte nuclear factor-kappa beta (NF-kappaB) activation is protective but is decreased in the cholestatic liver with endotoxemia. Surgery 2010; 148: 477–489. DOI: 10.1016/j.surg.2010.01.014.

28 Selzner N, Selzner M, Jochum W, Clavien PA. Ischemic preconditioning protects the steatotic mouse liver against reperfusion injury: an ATP dependent mechanism. J Hepatol 2003; 39: 55–61.

29 Sakuragawa T, Hishiki T, Ueno Y, and et al. Hypotaurine is an energy-saving hepatoprotective compound against ischemia-reperfusion injury of the rat liver. J Clin Biochem Nutr 2010; 46: 126–134.

30 Iida A, Yoshidome H, Shida T, and et al. Does prolonged biliary obstructive jaundice sensitize the liver to endotoxemia? Shock 2009; 31: 397–403.

31 Jaeschke H, Gujral JS, Bajt ML. Apoptosis and necrosis in liver disease. Liver Int 2004; 24: 85–89.