Cytochrome c release into cytosol with subsequent caspase activation during warm ischemia in rat liver

JUNPEI SOEDA,1 SHINICHI MIYAGAWA,1 KENJI SANO,2 JUNYA MASUMOTO,3 SHUN'ICHIRO TANIGUCHI,3 AND SEIJI KAWASAKI1
1First Department of Surgery, 2Department of Laboratory Medicine, and 3Department of Molecular Oncology and Angiology, Research Center on Aging and Adaptation, Shinshu University School of Medicine, Nagano 390-8621, Japan
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Soeda, Junpei, Shinichi Miyagawa, Kenji Sano, Junya Masumoto, Shun’ichiro Taniguchi, and Seiji Kawasaki. Cytochrome c release into cytosol with subsequent caspase activation during warm ischemia in rat liver. Am J Physiol Gastrointest Liver Physiol 281: G1115–G1123, 2001.—Apoptosis plays an important role in liver ischemia and reperfusion (I/R) injury. However, the molecular basis of apoptosis in I/R injury is poorly understood. The aims of this study were to ascertain when and how apoptotic signal transduction occurs in I/R injury. The apoptotic pathway in rats undergoing 90 min of warm ischemia with reperfusion was compared with that of rats undergoing prolonged ischemia alone. During ischemia, mitochondrial cytochrome c was released into the cytosol in a time-dependent manner in hepatocytes and sinusoidal endothelial cells, and caspase-3 and an inhibitor of caspase-activated DNase were cleaved. However, apoptotic manifestation and DNA fragmentation were not observed. After reperfusion, nuclear condensation, cells positive for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling, and DNA fragmentation were observed and caspase-8 and Bid cleavage occurred. In contrast, prolonged ischemia alone induced necrosis rather than apoptosis. In summary, our results show that release of mitochondrial cytochrome c and caspase activation proceed during ischemia, although apoptosis is manifested after reperfusion.

apoptosis; mitochondria; inhibitor of caspase-activated deoxyribonuclease

ISCHEMIA AND REPERFUSION (I/R) injury in the liver is of clinical importance in humans after hemorrhagic and cardiogenic shock, liver surgery, or liver transplantation. It is increasingly recognized that apoptosis occurs in I/R injury models of the liver (20, 32). At least two main pathways execute apoptosis. Both share activation of effector caspases, specifically, caspase-3 (35, 36). Activated caspase-3 cleaves caspase substrates, such as an inhibitor of caspase-activated DNase (ICAD), during the execution phase of apoptosis (10, 30). ICAD exists as a complex with a caspase-activated DNase (CAD) that promotes apoptotic DNA fragmentation, and cleavage of ICAD releases the active CAD (10, 30).

The first pathway involves the mitochondria (mitochondrial pathway). Cytotoxic reagents, radiation, growth factor deprivation, and hypoxia activate it (1, 3, 4). These stimuli cause the release of cytochrome c from the mitochondria into the cytosol. The released cytochrome c activates caspase-9, in concert with the cytosolic factor dATP (or ATP) and apoptotic protease-activating factor-1, and subsequently activates caspase-3 via proteolytic processing (24, 25, 44). The second pathway is stimulated by cell surface death receptors such as tumor necrosis factor (TNF) receptor 1 and Fas. Ligation of their ligands to the receptor leads to caspase-8 activation, with subsequent activation of caspase-3 (death receptor pathway) (35).

Recently, it has been reported that Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, is cleaved and activated by caspase-8. In turn, the cleaved Bid induces the release of cytochrome c from mitochondria (23, 26).
This study was conducted to determine when and how the apoptotic cascade is initiated and the pathway through which apoptosis proceeds. We examined chronological changes in mitochondrial cytochrome c release, activation of caspase-8 and -3, and cleavage of Bid and ICAD in a well-documented rat model of warm I/R liver injury. General caspase inhibitor was also used to confirm our results.

MATERIALS AND METHODS

Antibodies. The following primary antibodies were obtained: anti-cytochrome c monoclonal antibody (clone 7H8.2C12, PharMingen, San Diego, CA and clone 6H2.B4, Promega, Madison, WI); anti-p11 subunit of caspase-3 (K19), Bid, and ICAD (Santa Cruz Biotechnology, Santa Cruz, CA); anti-caspase-8 (Chemicon, Temecula, CA); anti-cytochrome oxidase subunit IV (Molecular Probes, Eugene, OR); anti-TNF-α (R&D Systems, Minneapolis, MN); and anti-actin (Sigma, St. Louis, MO).

Rat model of liver I/R injury. Forty male Wistar rats (Japan SEC, Hamamatsu, Japan) weighing 250–300 g were used. All animals were maintained under standard conditions and fed water and rodent chow ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Shinshu. The animals were fasted for 12 h before surgery but were allowed access to water. Rats were anesthetized by inhalation of halothane (Takeda Chemical Industries, Osaka, Japan). A complete midline incision was made. All structures (hepatic artery, portal vein, and bile duct) leading to the left and median liver lobes (~70% of liver mass) were occluded with a microvascular clamp for 90 min. This method of partial hepatic ischemia allows for portal decompression through the right and caudate lobes and thus prevents mesenteric venous congestion. Reperfusion was initiated by removal of the clamp. The abdomen was closed in two layers, and the animals were returned to their cages. Rats were killed either 90 min (n = 5) and 270 min (n = 5) without reperfusion. Samples of liver tissue were obtained and preserved for the experiments described below.

Inhibition of caspase activity. To inhibit caspase activity, 0.3 mg of Z-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD-fmk; Enzyme System Products, Livermore, CA) in 1% DMSO was injected intravenously via the dorsal penile vein 2 min before induction of ischemia. Rats were killed either 90 min after the start of the ischemia and at 1 (n = 5) and 3 (n = 5) h after reperfusion. To examine the roles of reperfusion, ischemia was prolonged for up to 180 (n = 5) and 270 min (n = 5) without reperfusion. Samples of liver tissue were obtained and preserved for the experiments described below.

Measurement of serum alanine aminotransferase and TNF-α. Blood samples were collected from the abdominal aorta. The serum samples were centrifuged, and supernatants were stored at −80°C until used. After completion of the experiment, serum alanine aminotransferase (ALT) levels were measured with an automated serum analyzer (Olympus, Tokyo, Japan). Plasma TNF-α levels were measured with a commercially available rat TNF-α ELISA kit (R&D Systems) according to the manufacturer’s instructions.

TUNEL staining. To detect cells undergoing apoptosis, the tissue sections were stained via the TUNEL procedure (13), with some modifications. Briefly, the tissue was immediately fixed in 8% paraformaldehyde at 4°C for 20–22 h and embedded in paraffin. The tissue was sectioned at 4 μm, dewaxed, rehydrated, and digested with 20 μg/ml of proteinase K (Sigma). Endogenous peroxidase was blocked by treatment in 0.3% hydrogen peroxidase. The sections were then rinsed in water and incubated with 50 μl of terminal deoxynucleotidyl transferase (TdT) buffer (30 mmol/l Tris-HCl, pH 7.2, 140 mmol/l sodium cacodylate, and 1 mmol/l cobalt chloride) containing 8.3 U of TdT (Boehringer Mannheim, Mannheim, Germany) and 0.83 nmol biotinylated 16-dUTP (Boehringer Mannheim) in a moist chamber at 37°C for 60 min. The sections were then rinsed and incubated with horseradish peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark), diluted 1:500 in 0.01 mol/l Tris-HCl (pH 7.5) plus 150 mmol/l NaCl (TBS) containing 1% BSA (Sigma), for 30 min at room temperature. They were then rinsed in TBS and stained with diaminobenzidine.

DNA fragmentation. DNA fragmentation was assayed by agarose gel electrophoresis (33) with some modifications. Frozen liver samples were minced, and 1 μl of sample was lysed with 20 μl of lysis buffer (50 mmol/l Tris-HCl, pH 7.8, 10 mmol/l EDTA, and 0.5% SDS). Lysates were treated with 10 mg/ml of proteinase K at 60°C for 90 min and then incubated with 10 mg/ml of RNase (Sigma) for 30 min. After brief centrifugation of the lysates, the supernatants were separated by electrophoresis on 1.5% agarose gels with 0.5 μg/ml of ethidium bromide. Hinc II-digested X174 (TOYOBO, Tokyo, Japan) was used as a molecular weight standard. The

**Fig. 1. Cytochrome c as analyzed by Western blotting and ELISA.** Cytosolic and mitochondrial fractions during ischemia were separated as described in MATERIALS AND METHODS, and 25 μg of the cytosolic or 12 μg of the mitochondrial fraction were loaded onto 14% SDS-polyacrylamide gels. A: in the cytosolic fraction, 15-kDa cytochrome c was barely detected in control (con) tissue but was detected after 15 min of ischemia, increasing in a time-dependent manner. The same membrane was reprobed with an anti-actin antibody. B: cytochrome c in the mitochondrial fraction decreased over time. Same membrane was reprobed with anti-cytochrome oxidase subunit IV (COX IV) antibody. Results represent 5 independent studies. C: cytochrome c in the cytosolic fraction during ischemia and reperfusion was quantified by ELISA. *P < 0.05; **P < 0.001.
DNA fragmentation pattern was examined on photographs taken under ultraviolet illumination.

**Histological study.** Formalin-fixed, paraffin-embedded tissue was sectioned at 4 μm and stained with hematoxylin and eosin, and the morphological aspects of cell death were evaluated.

**Isolation of the mitochondrial and cytosolic fractions.** For Western blot analysis and ELISA, protein from both the mitochondrial and cytosolic fractions was extracted as follows. Samples were gently homogenized at 800 rpm with a speed-controlled mechanical skill drill (B-100; Tokyo Rikakikai, Tokyo, Japan) by douncing five times in a Teflon-glass Potter-Elvehjem homogenizer (Sanshyo, Tokyo, Japan) in 9 volumes of buffer A (20 mmol/l HEPES-KOH, pH 7.5, 250 mmol/l sucrose, 10 mmol/l KCl, 1.5 mmol/l MgCl₂, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 5 μg/ml of pepstatin). The homogenates were centrifuged at 8,000 g for 10 min at 4°C, and the supernatants were collected. Protein concentration was measured with a bicinchoninic acid protein assay (BCA protein assay kit; Pierce, Rockford, IL). The same amounts of protein from liver homogenates, the cytosolic fraction, and the mitochondrial fraction were dissolved in sample buffer (25 mmol/l Tris-HCl, pH 8.0, 2% SDS, 0.02% bromphenol blue, and 0.05% 2-mercaptoethanol), loaded on 12.5 or 14% polyacrylamide gels, and electrophoresed. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electroblotting. Membranes were blocked for 1 h at room temperature with 5% nonfat dried milk and 0.1% BSA in TBS containing 0.1% (vol/vol) Tween 20 (TBS-T) and then were incubated for 1 h with primary antibodies diluted in TBS-T containing 5% fetal bovine serum (FBS). After being washed in TBS-T three times, the membranes were incubated for 1 h with peroxidase-conjugated sheep anti-mouse antibody (Amersham...
Pharmacia Biotech), sheep anti-rabbit antibody (Amersham), or donkey anti-goat antibody (Chemicon) diluted in TBS-T containing 5% PBS. After another wash in TBS-T, the blots were developed by enhanced chemiluminescence (ECL; Amersham) and exposed to X-ray film (RX-U; Fuji, Kawasaki, Japan).

Quantification of cytochrome c in liver tissue. To quantify the cytochrome c released from mitochondria, the same amount of protein from the cytosolic fraction of the samples mentioned in Western blotting was measured with a commercially available cytochrome c ELISA kit (MBL, Nagoya, Japan) according to the manufacturer’s instructions.

Immunohistochemistry. Liver samples were placed in freezing medium (OCT compound; Sakura, Tokyo, Japan). This was snap-frozen in an acetone bath cooled in liquid nitrogen. The specimens were stored at -80°C until they were sectioned. Frozen sections were cut at 5 µm and placed on glass slides coated with poly-L-lysine. Sections were air-dried for 30 min and fixed for 15 min in cold acetone. The fixed sections were washed in TBS, blocked with 1% BSA, and incubated with an anti-cytochrome c or anti-cytochrome oxidase subunit IV antibody, at a dilution of 1:50 in 1% BSA overnight at 4°C, and with biotinylated anti-mouse antibody (DAKO) and fluorescein isothiocyanate-conjugated streptavidin (DAKO). The sections were then studied under a fluorescence microscope (BX50; Olympus).

Measurement of caspase activity in liver tissue. Caspase-3 or -8 activity was measured with commercially available caspase-3 or -8 fluorometric protease assay kits (MBL) according to the manufacturer’s instructions. Briefly, liver tissue was homogenized in a lysis buffer. The homogenates were centrifuged at 12,000 g for 10 min at 4°C, and the supernatants were collected. The same amounts of protein from the liver homogenates were dissolved in the lysis buffer. The samples were incubated at 37°C with N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) or N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Ac-IEDT-AFC) in the presence or absence of the specific caspase-3 or -8 inhibitor (Ac-DEVD-CHO or Ac-IEDT-CHO). The amount of 7-amino-4-trifluoromethylcoumarin released from mitochondria, the same amount of protein from the cytosolic fraction of the samples measured in Western blotting, was measured with a commercially available cytochrome c ELISA kit (MBL, Nagoya, Japan).

The samples were incubated at 37°C with N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) or N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Ac-IEDT-AFC) in the presence or absence of the specific caspase-3 or -8 inhibitor (Ac-DEVD-CHO or Ac-IEDT-CHO). The amount of 7-amino-4-trifluoromethylcoumarin released was measured by fluorometry (Fluoroskan Ascent, Dainippon Pharmaceutical, Osaka, Japan) with 400-nm excitation and 505-nm emission filters. Data are expressed as change in fluorescence (ΔF)·min⁻¹·mg protein⁻¹.

Statistics. The differences between two dependent groups were evaluated with the unpaired Student’s t-test. In the cases where a nonparametric test was required, data were analyzed with the Mann-Whitney U-test. Comparison among multiple groups was performed with one-way ANOVA followed by Fisher’s protected least significant difference test. The results are presented as means ± SE and were considered significant when P < 0.05.

RESULTS

Early release of mitochondrial cytochrome c during ischemia. Cytochrome c was detectable in the cytosolic fraction 15 min after clamping. It reached a maximum level 90 min after the start of ischemia (Fig. 1A). In the control liver samples, cytochrome c was barely detected. The same membrane was reprobed with a monoclonal antibody against cytochrome oxidase subunit IV. The absence of cytochrome oxidase subunit IV in the membrane confirmed that there was no contamination of mitochondria in the cytosolic fraction (data not shown). Cytochrome c in the corresponding mitochondrial fraction decreased in a time-dependent manner during ischemia (Fig. 1B). Reprobing the same membrane with anti-cytochrome oxidase subunit IV antibody revealed that an equal amount of mitochondrial cytochrome oxidase subunit IV levels among time points. Figure 1C shows the sequential changes of cytochrome c concentration in the cytosol. Cytochrome c in the cytosol increased significantly after 15 min of ischemia compared with levels in controls (P = 0.047) and continued to increase during the ischemic period. After 3 h of reperfusion, cytosolic cytochrome c increased ~1.5-fold compared with the concentration 1 h after reperfusion (P < 0.001; Fig. 1C).

![Western blotting of caspase-3 and inhibitor of caspase-activated DNase (ICAD) and measurement of caspase-3 activities.](http://ajpgi.physiology.org/)
Cytochrome c immunohistochemistry. To evaluate the types and extent of the cells that release cytochrome c from mitochondria, cytochrome c immunostaining was performed. In the control liver samples, anti-cytochrome c antibody showed punctate localization as was also the case for the anti-cytochrome oxidase subunit IV antibody (Fig. 2, A and B). In the liver subjected to 90 min of ischemia, hepatocytes and sinusoidal lining cells (SLCs) lost their cytochrome c immunoreactivity, reflecting either the release and degradation or a conformational change of cytochrome c (2, 17). Loss of immunoreactivity of cytochrome c was localized mainly in zones 2 and 3 (Fig. 2C). In contrast, the immunoreactivity and localization of cytochrome oxidase subunit IV did not change during ischemia (Fig. 2D).

Activation of caspase-3 and cleavage of caspase substrate during ischemia. To assess the presence or absence of proteolysis of caspase-3, liver homogenates were immunoblotted with anti-caspase-3 antibody. As shown in Fig. 3, the cleaved fragment of caspase-3 appeared 30 min after clamping. This fragment was most evident 3 h after reperfusion. To confirm the functional significance of caspase-3 proteolysis in ischemia and how far apoptotic signal transduction proceeds, the samples were also immunoblotted with anti-ICAD antibodies, which recognize the NH2 terminus of ICAD. A 12-kDa fragment of ICAD was observed at 30 min of ischemia, and cleaved ICAD was most apparent 3 h after reperfusion (Fig. 3). When compared with controls, caspase-3 activity increased significantly after 90 min of ischemia (P = 0.02), after 1 h of reperfusion (P < 0.01), and after 3 h of reperfusion (P < 0.01). Also, caspase-3 activity 3 h after reperfusion was significantly higher than that 1 h of reperfusion (P = 0.04).

Apoptosis assay. To determine whether apoptosis was manifested during ischemia or after reperfusion,

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Fig. 4. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining of tissue from liver subjected to 90 min of ischemia (A), 1 (B) and 3 (C) h of reperfusion after 90 min of ischemia, and 180 min of ischemia alone (D). Original magnification, ×200. E: TUNEL-positive hepatocytes and SLCs. Numbers are %apoptotic cells of the total number of cells in 20 randomly chosen high-power (original magnification, ×400) fields. *P < 0.05; **P < 0.01.
apoptosis was assessed with hematoxylin and eosin staining, TUNEL staining, and DNA fragmentation. When TUNEL-positive hepatocytes and SLCs were counted for each sample in 20 randomly chosen areas under high magnification (×400), the number of TUNEL-positive cells in the liver subjected to 90 min of ischemia (hepatocytes 0.3 ± 0.1%, SLCs 0.6 ± 0.2%; means ± SE) was not different from that in the control liver (hepatocytes 0.3 ± 0.1%, SLCs 0.9 ± 0.4%; Fig. 4, A and E). The number of TUNEL-positive cells significantly increased 1 h after reperfusion (hepatocytes 3.4 ± 1.0%, SLCs 4.5 ± 0.5%) compared with that after 90 min of ischemia (P = 0.04 and P = 0.03, respectively) and markedly increased in the liver 3 h after reperfusion (hepatocytes 18.6 ± 1.9%, SLCs 18.3 ± 2.4%; P < 0.001 and P < 0.001, respectively; Fig. 4, B, C, and E). Cells with morphological features of apoptosis (condensed chromatin, nuclear fragmentation, and aggregation of chromatin at the nuclear membrane) were also observed in hematoxylin- and eosin-stained sections 3 h after reperfusion (Fig. 5A). Furthermore, DNA laddering was observed 1 and 3 h after reperfusion (Fig. 6). On the other hand, there was no significant increase in TUNEL-positive cells in the liver samples subjected to 180 min of ischemia (hepatocytes 0.38 ± 0.12%, SECs 0.73 ± 0.38%; Fig. 4, D and E). When ischemia was prolonged up to 270 min, the cells with apoptotic morphological changes were scarcely visible, and most of the cells exhibited necrotic morphology (Fig. 5B).

Western blotting of caspase-8 and Bid and measurement of caspase-8 activity. To assess the activation of the death-receptor pathway, we performed Western blotting of caspase-8 and Bid and a caspase-8 activity assay. Proteolysis of procaspase-8 and Bid occurred simultaneously after reperfusion (Fig. 7A) but not in ischemia. Caspase-8 activity increased significantly after reperfusion (Fig. 7B).

TNF-α in serum and tissue. TNF-α expression was not detected in liver tissue during ischemia with the use of Western blotting analysis (data not shown), and there was no significant TNF-α elevation in serum during ischemia. After reperfusion, serum TNF-α levels increased significantly (Fig. 8).

Inhibition of caspase activity. The general caspase inhibitor ZVAD-fmk significantly reduced I/R injury as indicated by serum ALT- and TUNEL-positive hepatocytes and SLCs 3 h after reperfusion compared with levels in the nontreated group (Table 1). During the ischemia, the caspase inhibitor did not block the release of cytochrome c from mitochondria, although it blocked caspase-3 and -8 activities completely (P < 0.01 and P < 0.01, respectively). In contrast, 3 h after reperfusion, the general caspase inhibitor significantly suppressed cytochrome c levels in the cytosolic fraction as well as caspase-3 and -8 activities (P = 0.03, P < 0.001, and P < 0.01, respectively; Table 1).

Fig. 5. Histological appearance of liver stained with hematoxylin and eosin. A: in the liver subjected to 90 min of ischemia with 3 h of reperfusion, nuclear condensation, aggregation of chromatin at the nuclear membrane, and fragmented nuclei can be observed (arrowheads). B: in the liver subjected to 270 min of ischemia without reperfusion, most of the cells manifested necrotic morphology. Original magnification, ×400.

Fig. 6. Agarose gel electrophoresis of DNA from liver samples. No DNA laddering is seen in the livers subjected to 90 min of ischemia (I90), 180 min of ischemia (I180), or the con livers. DNA laddering was observed at 1 (R1) and 3 (R3) h of reperfusion. MW, molecular weight standard.
DISCUSSION

Recent studies have shown that apoptosis plays an important role in the pathogenesis of I/R injury in liver (7, 19). Indeed, our study also showed that caspase inhibitors block apoptosis of hepatocytes and SLCs and reduce serum ALT levels. Hepatic I/R injury has been reported to occur after reperfusion and to be mediated by various factors, including reactive oxygen species and TNF-α (5, 31), which are thought to be activated by reperfusion. However, little information exists about the type of signaling pathway and the initiation point of apoptosis in these models.

Previous studies have shown that cytochrome c accumulates in the cytosol in response to multiple apoptotic stimuli (18, 41) and that its release precedes morphological signs of apoptosis (3). Although mitochondrial cytochrome c release was reported in focal cerebral ischemia models (11, 12), no report has referred to changes in mitochondrial cytochrome c during liver ischemia. Our study presents the first evidence that mitochondria release cytochrome c into the cytosol during warm ischemia in the rat liver. Immunostaining of cytochrome c showed that both the hepatocytes and SLCs released cytochrome c into the cytosol and that they were localized mainly in zones 2 and 3. This might be related to the vulnerability of zones 2 and 3 to ischemic stress.

Cytochrome c with the apoptotic protease-activating factor-1-caspase-9 complex proteolytically processed and activated caspase-3 to induce apoptosis in a cell-free system (24). Our results also showed a possible involvement of mitochondria in an apoptotic pathway that is initiated during ischemia. The mitochondrial cytochrome c was released as early as 15 min after the start of ischemia and was followed at 30 min by caspase-3 and ICAD cleavage. Caspase-3 activity also significantly increased after 90 min of ischemia compared with that in controls. These results indicate that the released cytochrome c induced subsequent caspase activation and ICAD cleavage, which is further supported by the findings that the general caspase inhibitor did not inhibit the mitochondrial cytochrome c release, although it completely blocked caspase activa-

Table 1. Effect of caspase inhibition on ischemia and reperfusion injury

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<th>90 min of Ischemia</th>
<th>3 h After Reperfusion</th>
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<tr>
<td></td>
<td>ZVAD-fmk (-)</td>
<td>ZVAD-fmk (+)</td>
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<tr>
<td>ALT, IU/l</td>
<td>158 ± 15</td>
<td>187 ± 33</td>
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<td>TUNEL-positive hepatocytes, %</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>TUNEL-positive SLCs, %</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
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<td>Cytochrome c, ng/mg</td>
<td>84.2 ± 2.0</td>
<td>82.9 ± 2.9</td>
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<tr>
<td>Caspase-3 activity, ΔF·mg⁻¹·min⁻¹</td>
<td>275 ± 39</td>
<td>26 ± 11†</td>
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<tr>
<td>Caspase-8 activity, ΔF·mg⁻¹·min⁻¹</td>
<td>7.7 ± 0.9</td>
<td>3.1 ± 0.5†</td>
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Data are means ± SE. The effects of caspase inhibition were studied by comparing Z-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD-fmk, a caspase inhibitor) = treated liver samples with nontreated samples either 90 min after the onset of ischemia or 3 h postreperfusion (n = 3–5 samples/group).

ALT, alanine aminotransferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; SLCs, sinusoidal lining cells. *P < 0.05; †P < 0.01; ‡P < 0.001.

Fig. 7. A: time course of caspase-8 and Bid proteolysis. Proteolyzed fragments of caspase-8 (top) and Bid (bottom) were seen at 1 and 3 h of reperfusion. p18, 18-kDa-cleaved fragment of caspase 8. B: sequential change of caspase-8 activity. Caspase-8 activity increased significantly after 1 h of reperfusion (P < 0.05). *P < 0.05; **P < 0.01.
tion during ischemia. Numerous apoptotic proteases promote ICAD cleavage. Calpain cleaved ICAD into ~19- and 25-kDa fragments, and high concentrations of caspase-7 also cleaved ICAD. In physiological concentrations, only caspase-3 can cleave ICAD at the NH$_2$-terminal caspase site to produce the 12-kDa fragment (38). Our result, the prominent 12-kDa fragment of ICAD, suggests that ICAD was cleaved by caspase-3.

Recently, Li and colleagues (23) have reported that activated caspase-8, through death-receptor stimulation, cleaves Bid and that translocation of the truncated Bid from cytosol to mitochondria then mediates the release of cytochrome c. The present study revealed that there was no significant increase in caspase-8 activity during ischemia, and caspase-8 and Bid were first cleaved after reperfusion. Caspase-8 activation and the cleavage of Bid after reperfusion indicate that the death-receptor pathway was activated after reperfusion. That, together with the fact that TNF-α was detected after reperfusion, leads us to believe that TNF-α might activate the death-receptor pathway in liver I/R injury. Furthermore, caspase-3 was activated more after reperfusion than during ischemia, and the general caspase inhibitor did not block cytochrome c release during ischemia but did inhibit its release after reperfusion. These findings suggest that the death-receptor pathway may contribute to further increases in both mitochondrial cytochrome c release and caspase-3 activity after reperfusion.

The activation of caspase-3 usually leads to cleavage of cytoplasmic substrates for the manifestation of apoptotic morphological changes (6, 21, 28, 37). In caspase-3-null cells, DNA fragmentation was delayed or absent (16, 34, 39, 43). ICAD cleavage at NH$_2$-terminal caspase sites is both necessary and sufficient for CAD activation, which promotes apoptotic intermembrane DNA fragmentation (30, 38). In this study, we did not observe apoptotic manifestations during ischemia despite caspase-3 activation and the cleavage of ICAD at NH$_2$-terminal sites. In the 180-min ischemia model, there were neither TUNEL-positive cells nor cells with morphological manifestations of apoptosis (including nuclear shrinkage and fragmentation). In the liver samples subjected to 270 min of ischemia, most of the cells showed necrotic morphological changes. After reperfusion, however, we observed TUNEL-positive cells, DNA fragmentation, and cells with morphological manifestations of apoptosis. Although caspase-3 activation and cleavage of ICAD had already occurred during ischemia, they failed to induce biochemical or morphological changes characteristic of apoptosis during the ischemic period. This finding is further supported by a previous report that in the hypoxic, perfused liver, cell death occurred as necrosis, although caspase-3 was activated (40). Several reports have shown that apoptosis is composed of several ATP-dependent steps and that the availability of intracellular ATP determines whether cells undergo apoptosis or necrosis (8, 22). In the mitochondrial pathway, caspase-3 activation requires ATP or dATP (9). An active nuclear transport mechanism that requires ATP hydrolysis has been shown to be involved in apoptotic changes of the nuclei (42). During ischemia, intracellular ATP is rapidly exhausted as a result of insufficient oxygen supply and rapid consumption of glucose so that ischemia reduces the ATP content of liver tissue (14, 15). A study in cultured kidney cells showed that incubation of previously hypoxic cells in glucose-free medium led to cell death with necrotic morphology despite activation of the mitochondrial apoptotic cascade. In contrast, cells reoxygenated in the presence of glucose showed apoptotic morphology (29). These studies indicate that apoptosis consumes ATP.

The present results, showing that the morphological changes characteristic of apoptosis were observed only after reperfusion, indicate that whatever was generated during reoxygenation (probably ATP) might be required in order to manifest apoptosis. However, it remains unclear how a cell can produce ATP during reperfusion, because mitochondrial electron transport might be impaired due to loss of cytochrome c. One possibility is that anaerobic glycolysis may produce ATP to execute apoptosis. Another possible explanation is that, as Martinou and Green (27) recently proposed, a “mild death signal” might alter only a subpopulation of the mitochondria, and the spared mitochondria would be able to produce enough ATP to activate caspase, allowing cells to undergo apoptosis. Further studies are required to confirm the connection between ATP and apoptotic manifestation.

In summary, this study reveals that although apoptosis is manifested after reperfusion, mitochondrial cytochrome c release and caspase activation proceed during ischemia.

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