

Role of UCP2 Expression after Hepatic Warm Ischemia-Reperfusion in the Rat

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Background/Aims: The role of uncoupling protein-2 (UCP2) in the liver is currently unclear. Emerging evidence suggests a relationship between UCP2 and oxidative stress. In the present study, we tested the hypothesis that UCP2 expression in the liver might change during warm ischemia-reperfusion (I/R) according to oxidative stress. **Methods:** Wistar rats were subjected to 40 (short ischemia) or 90 (long ischemia) minutes of partial lobar ischemia followed by 4 hours of reperfusion. UCP2 expression in the ischemic and nonischemic lobes was assessed using reverse transcription-polymerase chain reaction and immunohistochemistry. Malondialdehyde concentrations in the liver tissue were also compared. **Results:** Malondialdehyde concentrations in the ischemic lobes were significantly higher in the long ischemia group. In the ischemic lobes of the short ischemia group, UCP2 protein expression was induced in hepatocytes, which did not express the protein prior to treatment, and the expression levels were higher than in the long ischemia group. The intralobular distribution of UCP2 seemed to correlate inversely with that of the necrotic area. UCP2 expression was observed, even in nonischemic lobes with similar intralobular heterogeneity. **Conclusions:** UCP2 was induced in hepatocytes after warm I/R. Although the primitive role of UCP2 expression may be cytoprotective in nature, its actual protective effect in hepatic I/R may be minimal. (**Gut Liver 2011;5:486-492**)

Key Words: Oxidative stress; Ischemia-reperfusion; Uncoupling protein; Liver; Surgery

INTRODUCTION

The mechanism of tissue injury by hepatic ischemia-reperfusion (I/R) is thought to involve oxygen-derived free radicals.¹⁻³ The antioxidant status of the tissue affected by I/R is of great

importance for the primary endogenous defense against free radical-induced injury. Under normal physiological conditions, cells have developed multiple, complementary mechanisms to minimize oxidative damage, including the induction of oxidant detoxifying enzymes, such as superoxide dismutase, and the accumulation of oxidant buffers, such as reduced glutathione.^{4,5} A better understanding of these molecular pathophysiology of I/R injury in liver surgery will aid in the design of advanced therapeutic strategies.

The uncoupling protein 2 (UCP2) is integral membrane protein of the mitochondrial inner membrane, where they function as a proton channel or shuttle.^{6,7} The gene UCP2 belongs to a family of genes found in animals and plants, encoding a protein homologous to the brown fat uncoupling protein UCP1.⁸ The common characteristic of these proteins is to uncouple the respiratory chain from ATP synthesis by dissipating the proton electrochemical gradient. Accordingly, these proteins have been implicated mainly in the maintenance of metabolic rate and adaptational thermoregulation.^{6,9} However, the diversity of their possible biological roles is illustrated by the paper describing the enhanced ability of UCP2 knock-out mouse to destroy intracellular pathogens due to alterations in the formation of reactive oxygen species (ROS), suggesting that UCP2 is related to the regulation of ROS production.^{10,11} Furthermore, recent reports have revealed that an abundance of these proteins is modulated by several physiological conditions, suggesting that these proteins are involved in metabolic adaptations.^{10,12-14} In contrast to UCP1, which is considered specific to brown adipocytes, UCP2 has been detected in a large spectrum of tissues.^{7,15} In the healthy liver, UCP2 messenger RNA (mRNA) was identified in Kupffer cells, but not in hepatocytes.¹⁶ Chavin *et al.*¹⁷ reported that the expression of UCP2 mRNA in hepatocytes is increased in genetically obese mice and induction of UCP2 protein contribute to diminish the redox pressure on the mitochondrial

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electron transport chain, and provide an advantage by constraining mitochondrial ROS production.

Given the published evidence that UCP2 can modulate their abundance in response to a variety of stimuli and environmental conditions, we speculated that UCP2 expression in the liver might alter during warm I/R, in which mitochondrial oxidant production increases. The aim of the present study was to test this hypothesis and to evaluate the implications of altered UCP2 expression after hepatic I/R.

MATERIALS AND METHODS

1. Animals

Inbred male Wistar rats (250 to 280 g) were purchased from Kyudo (Fukuoka, Japan), housed in temperature- and light-controlled environmental conditions with a 12-hour light and dark cycle, and permitted *ad libitum* consumption of water and standard pellet chow. The Kyushu University Institutional Animal Care and Use Committee approved all animal protocols, according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (NIH).

2. Operative procedure

All animals were anesthetized with intraperitoneal pentobarbital 50 mg/kg. Laparotomy was carried out through a midline incision. Forty minutes (Short ischemia group; n=14) or 90 minutes (Long ischemia group; n=14) of warm hepatic ischemia was induced in the median and left lateral hepatic lobes by clamping the corresponding hepatic arterial and portal venous branches.¹⁸ The remaining lobes retained an intact portal and arterial blood supply, as well as venous drainage, preventing intestinal venous hypertension and bacterial translocation. When the assigned period of warm ischemia was completed, the microvascular clip was released and the abdomen was closed. Nonischemic lobes were not excised after reperfusion to allow comparison of the UCP2 expression on ischemic and nonischemic lobes separately. Sham animals (n=8) were treated in an identical fashion with the omission of vascular occlusion. The animals were sacrificed at 4 hours after reperfusion. Blood samples were drawn from abdominal aorta, and serum alanine aminotransferase (ALT) levels were assayed using standard enzymatic methods. Liver samples were excised before ischemia and 4 hours after reperfusion. The specimens were immersed in liquid nitrogen immediately after sampling. Another tissue samples were fixed in 10% neutral buffered formalin.

3. Determination of malondialdehyde levels in the liver tissue

The hepatic levels of malondialdehyde (MDA) after 4 hours of reperfusion were determined using a commercially available kit (Lipid Peroxidation Assay Kit; Calbiochem, San Diego, CA,

USA) as described previously.² Protein concentrations were determined using a Bio-Rad protein assay kit.

4. Histologic and immunohistochemical examinations

Formalin-fixed liver specimens were embedded in paraffin. Sections 3 μ m thick were stained with hematoxylin and eosin (H&E) for histologic evaluation by light microscopy. For immunohistochemical detection of UCP2, the avidin-biotin peroxidase complex method was used. The liver sections obtained at 4 hours after reperfusion were deparaffinized and incubated at 4°C overnight with primary goat antisera (1:50, v/v) to a conserved peptide sequence in murine and human UCP2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The secondary biotinylated rabbit anti-goat antibody and peroxidase-conjugated streptavidin were from a Histofine SAB-PO (G) kit (Nichirei, Tokyo, Japan). After staining with diaminobenzidine, the slides were counterstained with hematoxylin, dehydrated and mounted. As negative controls, sections exposed to nonimmune antisera were included in each assay to assure specificity.

Four hours after reperfusion, the extent of UCP2 immunoreactivity was semi-quantitatively assessed in 10 random high power fields of ischemic and nonischemic lobes as follows: none, 0; up to 30%, 1; up to 60%, 2; more than 60%, 3. These scores were averaged and compared. All sections were examined by the same investigator (T.T.), who did not know the assignment of specimens to a particular group of rats.

5. RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen liver tissue using commercially available kits (QIAGEN RNeasy Mini Kit; QIAGEN GmbH, Hilden, Germany). RNA was quantified by spectroscopy and its quality was evaluated by agarose gel electrophoresis and subsequent ethidium bromide staining. Synthesis of the first strand cDNA was done with 2.0 μ g of total RNA using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Amersham, UK) according to manufacturer's protocol.

The levels of UCP2 mRNA extracted from liver were analyzed by semi-quantitative RT-PCR method as described previously¹ using recombinant Taq DNA polymerase (Takara Taq; Takara Shuzo, Tokyo, Japan). UCP2 primers used were: 5'-GGCTGGTG-GTGGTCGGAGAT-3'; 5'-CCGAAGGCAGAAAGTGAAGTG-3'.¹⁹ PCR was conducted for 27 cycles for UCP2 and 25 cycles for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR products were analyzed by 1.2% agarose gel electrophoresis and stained with ethidium bromide. The PCR gel image was captured and analyzed using NIH image software. The data were expressed as a ratio of the measured calibrated mean pixel intensity of the UCP2 band to that of the corresponding GAPDH band.

6. Statistical analysis

All values were expressed as mean±standard error. Statistical analysis was performed using the Student's t-test. A p-value of less than 0.05 was considered significant.

RESULTS

1. Hepatic injury after I/R

At 4 hours after reperfusion, serum ALT levels in the short ischemia group were significantly lower than those in the long ischemia group ($p<0.05$, Table 1). Fig. 1 shows representative pictures of H&E staining of ischemic lobes of both groups. In the short ischemia group, a relatively small amount of necrotic area was observed preferentially around periportal regions. Such changes were more pronounced and extended to pericentral regions in the long ischemia group. Nonischemic lobes maintained almost normal appearance in both groups.

Table 1. Serum ALT and MDA Levels in Liver Tissue before and after Ischemia-Reperfusion

	Before ischemia (n=8)	Sham operation (n=8)	Short ischemia (n=14)	Long ischemia (n=14)
ALT*, IU/L	28±3	40±2	1,302±205 [‡]	2,729±256 ^{‡,§}
MDA [†] , nmol/mg protein				
Ischemic lobe			19.3±3.2	43.6±5.1 ^{‡,§}
Nonischemic lobe	2.4±0.6	2.9±1.3	6.2±1.2	8.1±1.6

*ALT, serum alanine aminotransferase levels at 4 hours after reperfusion; [†]MDA, hepatic tissue levels of malondialdehyde at 4 hours after reperfusion; [‡] $p<0.01$ vs before ischemia; [§] $p<0.05$ vs short ischemia; ^{||} $p<0.05$ vs before ischemia.

2. Liver malondialdehyde concentrations after I/R

The hepatic MDA concentrations, a marker of hepatocellular lipid peroxidation, were measured before and after I/R. As shown in Table 1, MDA concentrations increased after I/R, and the increase in the ischemic lobes of the long ischemia group was significantly higher than in the short ischemia group ($p<0.05$). Although the differences were less pronounced, the increase was also observed in the nonischemic lobes of both groups, probably due to hyperperfusion during partial lobar clamping.^{20,21}

3. Determination of UCP2 by semi-quantitative RT-PCR

To explore whether hepatic warm I/R induces changes in UCP2 mRNA levels, and whether any differences may occur according to degree of I/R injury, mRNA samples were collected before ischemia and 4 hours after reperfusion and the expression of UCP2 mRNA levels were determined by semi-quantitative RT-PCR method. In the ischemic lobes, the expression of UCP2 mRNA after I/R in the long ischemia group was significantly lower than that in the short ischemia group ($p<0.01$). Interestingly, UCP2 mRNA levels were increased even in nonischemic lobes of both groups with similar extent (Fig. 2).

4. Immunohistochemical examinations for UCP2

The localization of cells expressing UCP2 protein was analyzed by immunohistochemistry. It is known that UCP2 is expressed by macrophages in healthy liver. Before the treatment, the expression of UCP2 protein was not detected in hepatocyte, and only a small number of nonparenchymal cells had positive UCP2 immunoreactivity (data not shown). However, after 40 minutes of ischemia followed by 4 hours of reperfusion, UCP2 immunoreactivity was readily detected in the cytoplasm of hepatocytes. Furthermore, abundance of UCP2 expression dif-

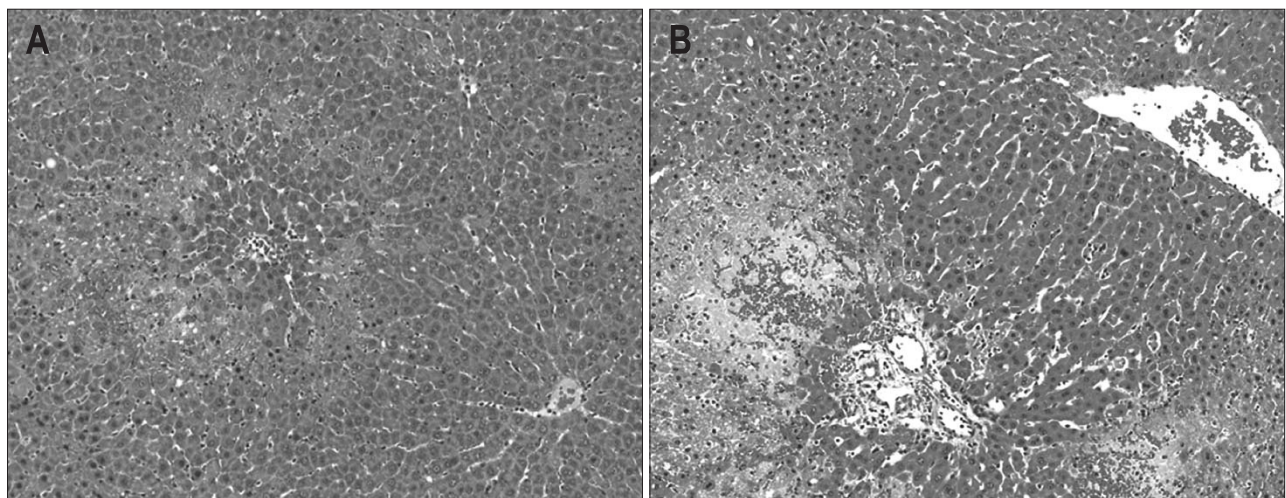


Fig. 1. Histopathologic findings at 4 hours after reperfusion (H&E stain, ×100). The livers of the long ischemia group showed multiple and extensive areas of hepatocyte necrosis distributed through almost the entire intralobular zone (B), whereas the necrotic area in the short ischemia group was localized preferentially around the periportal region (A).

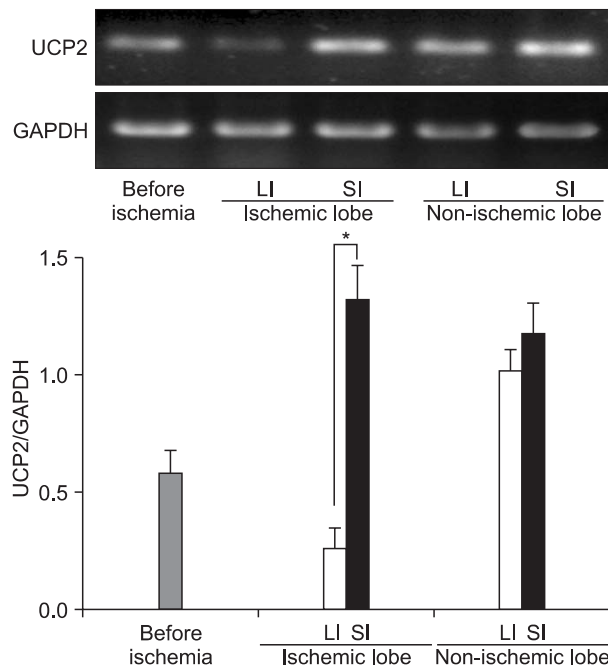


Fig. 2. The expression of uncoupling protein-2 (UCP2) mRNA was determined after 40 (SI; short ischemia) or 90 (LI; long ischemia) minutes of ischemia followed by 4 hours of reperfusion using a semi-quantitative reverse transcription-polymerase chain reaction method. (A) A representative photograph of UCP2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) bands is shown. (B) The graph summarizes the data from 8 to 14 rats per group. Values are expressed as relative intensity of the UCP2 band divided by the intensity of respective GAPDH band, as determined by densitometry. * $p < 0.01$.

ferred between the 2 groups. In short ischemia group, UCP2 was expressed almost the entire lobular area (Fig. 3A), whereas the liver of long ischemia group showed a small amount of UCP2 positive hepatocytes (Fig. 3B). Interestingly, UCP2 protein was also detected even in nonischemic lobes after I/R (Fig. 3C, D). No significant changes in UCP2 immunoreactivity were observed in hepatocytes of sham operated animals (data not shown). UCP2 immunoreactivity was scored as defined in Materials and Methods, and compared between the groups. In the ischemic lobes, the averaged score of the short ischemia group was significantly higher than that of the long ischemia group (2.2 ± 0.1 and 1.3 ± 0.1 , respectively; $p < 0.01$). The score in the nonischemic lobes was not significantly different (2.3 ± 0.1 and 2.4 ± 0.1 , respectively). The necrotic area and immunoreactive area were quantitatively estimated for each groups at each intralobular zone and summarized in Table 2. After I/R, UCP2 immunoreactivity was observed preferentially around the pericentral region (zone 3), while the necrotic area was more prominent around the periportal (Zone 1) to mid-zonal region (Zone 2). Thus, intralobular distribution of UCP2 positive hepatocytes seemed to correlate inversely with that of necrotic area.

DISCUSSION

ROS generation is widely accepted as an important event in the pathogenesis of hepatic warm I/R injury. Previous reports revealed that not only Kupffer cells but also hepatocytes constitute a major cellular component that displays oxidative changes after I/R.³ Although hepatocytes of healthy rats do not express UCP2, when given bacterial lipopolysaccharide, UCP2 appears in hepatocytes via tumor necrosis factor- α (TNF- α) dependent mechanism.²² In the present study, expression of UCP2 protein, which were defined to nonparenchymal cells at baseline, have been induced on cytoplasm of hepatocytes by warm I/R. It is not unreasonable to consider that UCP2 is induced by TNF- α , which is well known to be released into circulation after hepatic I/R.²³ Induction of UCP2 protein in the nonischemic lobes supports the hypothesis that UCP2 is induced by soluble mediators after I/R. However, intralobular heterogeneity of UCP2 immunoreactivity leads us to speculate the presence of another mechanism of inducing UCP2 after I/R. Yang *et al.*¹³ have reported that a mitochondrial redox cycling agent that increases mitochondrial ROS production up-regulates UCP2 mRNA in primary cultures of normal rat hepatocytes. Considering the presence of intralobular heterogeneity of oxidative stress,²⁴ it may be assumed that increases in oxidant production promoted the induction of UCP2 after I/R of the liver. As shown in Table 1, MDA concentrations increased after I/R. Even in the nonischemic lobes, MDA levels were increased after short and long ischemia and reperfusion, although the increase was less pronounced as compared to the ischemic lobes. These results suggest that low levels of ROS generation had certainly occurred in the nonischemic lobes after I/R. ROS generation in the nonischemic lobes is supposed to be due to hyperperfusion during partial lobar clamping.^{20,21}

Comparing the 2 groups, a greater amount of UCP2 protein were expressed in the short ischemia group, in which less liver injury occurred, as indicated by the lower ALT levels in the serum, and weaker histological damage. Furthermore, intralobular distribution of UCP2 positive hepatocytes tended to correlate inversely with that of necrotic area. After hepatic I/R, cellular damage is more prominent in Zone 1 as compared to Zone 3 because the extent of oxidative stress is stronger in Zone 1 than in Zone 3.²⁴ Two possible explanations would be raised for the interpretation of these results. First, UCP2 had been up-regulated due to oxidant production after I/R, and inverse correlation between UCP2 expression and necrotic area may be the result of cytoprotective role of UCP2. Another possible explanation is that, although oxidative stress after I/R prompted the production of UCP2, loss of cellular viability secondary to severe damage due to I/R injury resulted in less expression of UCP2 around the necrotic area.

Regarding the role of UCP2 in acute liver injury, Serviddio *et al.*²⁵ reported that, in NASH livers, UCP2 compromises the liver capacity to respond to acute energy demands after hepatic I/R

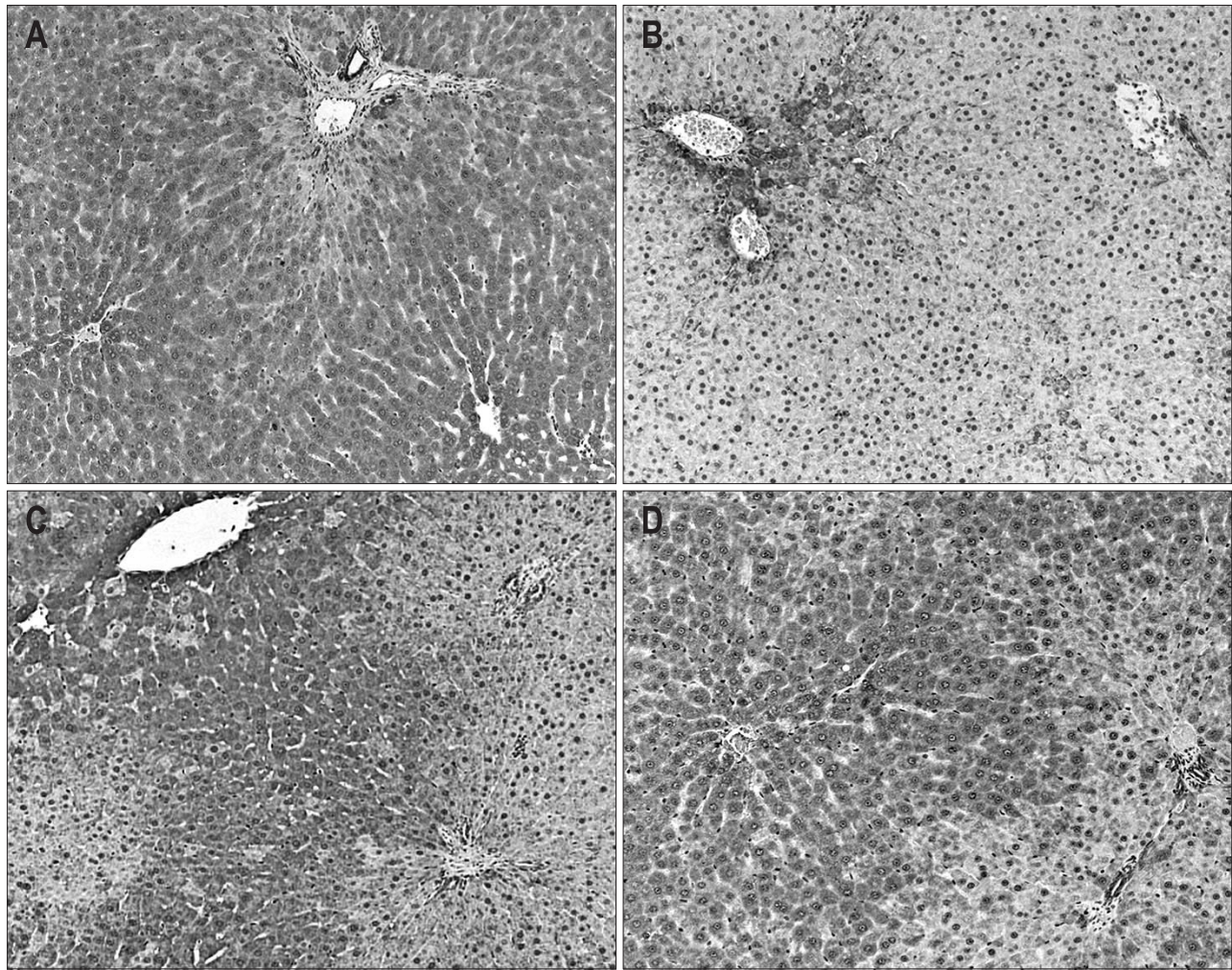


Fig. 3. Immunohistochemical evaluation of uncoupling protein-2 (UCP2) expression after 40 or 90 minutes of ischemia followed by 4 hours of reperfusion. The livers of the short ischemia group (A) accumulated much more UCP2 proteins than those of the long ischemia group (B). In the nonischemic lobes, both groups showed a similar degree of UCP2 expression levels after the treatment (C, short ischemia group; D, long ischemia group) (×100).

Table 2. Comparison of the Intralobular Distribution of UCP2-Positive Hepatocytes and Necrotic Lesions

	UCP2 immunoreactivity			Necrosis		
	Zone 1	Zone 2	Zone 3	Zone 1	Zone 2	Zone 3
Ischemic lobe						
Short ischemia	+	++	+++	+	+	-
Long ischemia	-	-	+	+	++	+
Nonischemic lobe						
Short ischemia	-	+	++	-	-	-
Long ischemia	-	+	++	-	-	-

-, 0-10%; +, 10-30%; ++, 30-60%; +++, >60%.

by depleting hepatic ATP content. On the other hand, Le Minh *et al.*²⁶ showed minimum relevance of UCP2 for the manifestation and extent of I/R injury in nondiseased livers. Thus, the role of UCP2 in hepatic I/R injury seems to differ according to underlying liver condition and ATP availability.

UCPs are thought to dissipate the proton gradient across the inner membrane of mitochondria, leading to production of heat without the phosphorylation of adenosine 5'-diphosphate. Additionally, emerging evidence suggests that the up-regulation of mitochondrial UCPs may provide another means of constraining

oxidant production during mitochondrial respiration.^{11,27-29} Li *et al.*³⁰ reported that overexpression of UCP2 is associated with protection from ROS-induced toxicity in pancreatic β -cells. Given published evidence that UCP2 may decrease mitochondrial oxidant production in some cells, the results from present study suggest that UCP2 may be part of a negative feedback mechanism by which mitochondrial ROS limit their own production. Moreover, considering the differences in UCP2 expression levels by the duration of ischemia and the presence of zone dependent UCP2 immunoreactivity, it is inferred that there may be a range in degree of oxidative stress in which UCP2 is induced. Namely, only relatively weak oxidative stress may be able to produce feedback signals to induce UCP2 on hepatocytes. The extent of oxidative stress in the periportal region is stronger than that of the pericentral region, and longer ischemic periods lead to a greater amount of oxidative stress.²⁴ These published evidences explain the intralobular heterogeneity and different levels of UCP2 expression between the 2 groups. Although a slight increase in oxidative stress in the centrilobular region led to UCP2 induction, stronger redox pressure around the periportal region overwhelmed the cellular protection system, and thus might have resulted in reduced levels of UCP2 expression. In the same way, shorter ischemic periods produced less oxidant stress with the consequence of much more UCP2 expression as compared to longer ischemia. Therefore, a moderate rather than a dramatic cytoprotective effect by UCP2 is speculated. UCP homologues form a subfamily that are evolutionarily related and can be identified even in plants, suggesting that UCPs emerged early during phylogenesis. Thus, UCPs might have played an important role in detoxifying relatively low levels of free radicals that can be generated in the normal physiological environment, such as infection or obesity,^{17,22} rather than severe I/R injury. Therefore, the modulation of hepatic UCP2 expression would not be expected to serve as an effective therapeutic method for mitigating the hepatic I/R injury.

In summary, UCP2 are induced on hepatocytes after warm I/R of rat liver. Although the primitive role of UCP2 expression after oxidative stress may be cytoprotective one, an actual protective effect in hepatic I/R injury may be minimum.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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