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Original Research Communication

Formation of 8-Hydroxy-2′-Deoxyguanosine and 4-Hydroxy-2-Nonenal-Modified Proteins in Rat Liver after Ischemia-Reperfusion: Distinct Localization of the Two Oxidatively Modified Products

KAZUHIKO YAMAGAMI,[1,2] YUZO YAMAMOTO,[1] MAKOTO KUME,[1] YASUHIDE ISHIKAWA,[1] YOSHIO YAMAOKA,[1] HIROSHI HIAI,[2] and SHINYA TOYOKUNI[2]

ABSTRACT

Ischemia-reperfusion (IR) injury is an intractable process associated not only with therapeutic recanalization of vessels, but also with partial resection or transplantation of solid organs including liver. To develop methods for predicting the degree of hepatic IR injury and further to identify injured cells, we studied the formation of 8-hydroxy-2′-deoxy-guanosine (8-OHdG) and 4-hydroxy-2-nonenal (HNE)-modified proteins in the normothermic hepatic IR model of rats using immunohistochemistry, high-performance liquid chromatography (HPLC) determination and Western blot. The Pringle maneuver for either 15 or 30 min duration produced reversible or lethal damage, respectively. The levels of both products were significantly increased in proportion to ischemia duration 40 min after reperfusion, suggesting the involvement of hydroxyl radicals. Increased immunoreactivity of 8-OHdG was observed not only in the nuclei of hepatocytes but also in those of bile canalicular and endothelial cells. However, immunoreactivity of HNE-modified proteins was detected in the cytoplasm of hepatocytes, which was confirmed by Western blot, and in addition, in the nuclei of hepatocytes after severe injury. Thus, localization of the two oxidatively modified products was not identical. Our data suggest that these two products could be used for the assessment of hepatic IR injury in tissue, but that the biological significance of the two products might be different. Antiox. Redox Signal. 2, 127–136.

INTRODUCTION

REPERFUSION INJURY following interruption of arterial or portal blood flow is one of the major causes of perioperative complications in surgical procedures, including hepatectomy (Pachter *et al.*, 1983; Delva *et al.*, 1989; Huguet *et al.*, 1992) and liver transplantation (Starzl *et al.*, 1989; Blankenstenjn and Terpstra, 1991; Clavien *et al.*, 1992). Ischemia-reperfusion (IR) injury consists of ischemia or hypoxia, postischemic reperfusion, and reoxygenation. Generally, if a dead or dying tissue is reperfused *in vivo*, potentially toxic agents, including xanthine oxidase and catalytic transition metals, are generated and released, resulting in dam-

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8397, Japan.
[2]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.

age to cells, nearby structures, or other body tissues via systemic circulation (Halliwell and Gutteridge, 1999).

A number of reports prior to the last decade support a role for uncontrolled reactive oxygen species (ROS) in the IR injury of liver (Nordstorum *et al.*, 1985; Marubayashi *et al.*, 1986; Ohhori *et al.*, 1989). Involvement of neutrophils in the process was thereafter suggested (Komatsu *et al.*, 1992; Vollmar *et al.*, 1994). Furthermore, recent evidence indicates the involvement of adhesion molecules such as P-selectin (Scalia *et al.*, 1999; Yadav *et al.*, 1999), L-selectin, and intercellular adhesion molecule-1 (ICAM-1) (Yadav *et al.*, 1998), as well as cytokine networks including tumor necrosis factor-α (TNF-α) (Colletti *et al.*, 1990, 1996) and NF-κB (Hur *et al.*, 1999) in the IR injury of liver. However, there has been a paucity of data on the early events of IR injury and its localization *in vivo*. In the present study, we attempted to develop methods to predict the degree of hepatic IR injury in tissue, and further to identify the injured cells with immunohistochemical methods using specific antibodies.

8-Hydroxy-2'-deoxyguanosine (8-OHdG), one of the major DNA base-modified products, is induced either by hydroxyl radical, singlet oxygen, or photodynamic action (Kasai and Nishimura, 1984; Kasai, 1997), and is known to be mutagenic by pairing with adenine as well as cytosine, leading to a possible G:C to T:A transversion during DNA replication (Shibutani *et al.*, 1991). 4-Hydroxy-2-nonenal (HNE), one of the major final products of lipid peroxidation with established cytotoxicity and mutagenicity, reacts with histidyl, lysyl, or cysteinyl residues of proteins and forms stable Michael adducts (Uchida and Stadtman, 1992; Uchida *et al.*, 1993). It is considered as the most reliable marker of lipid peroxidation (Esterbauer *et al.*, 1991). Previously, we established specific immunohistochemical methods by raising specific antibodies against 8-OHdG (N45.1) (Toyokuni *et al.*, 1997, 1999; Toyokuni, 1999) and HNE-modified proteins (Uchida *et al.*, 1993; Toyokuni *et al.*, 1994b, 1995; Tanaka *et al.*, 1997).

We observed a significant increase in levels of the two different oxidatively modified products, and, more importantly, the localization of these two molecules was different in the hepatic IR injury.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) weighing 230–260 grams (10–11 weeks of age) were housed in an air-conditioned room (24 ± 1°C) with alternating 12-hr light and dark cycles, and fed a standard rat chow (F-2, Funabashi, Chiba) and tap water. A total of 79 rats were used; two groups of 16 animals each were used for the survival experiments, and 47 animals were used for the serum and immunohistochemical study. All of the experiments were performed according to the animal protection guidelines of Kyoto University.

Ischemia-reperfusion injury

Operative procedures were performed in a temperature-controlled operating room for animals (24 ± 1°C) under general anesthesia by administration of 50 μg of atropine sulfate intramuscularly (i.m.) and 40 mg/kg sodium pentobarbital intraperitoneally (i.p.). The ligaments were all removed from the liver following upper median laparotomy. Hepatic warm ischemia (Group 1, 15 min; Group 2, 30 min) was induced by cross-clamping the hepatoduodenal ligaments with a microvascular clamp (Pringle's maneuver). After unclamping, the liver was spontaneously reperfused and each rat carefully observed until sampling of hepatic tissue (at the end of Pringle's maneuver, or 40 min after reperfusion; $n = 10$ for each experiment). For determination of survival rate, rat abdomens were closed by suture immediately after unclamping. Control samples were obtained from sham-operated rats ($n = 7$).

Levels of liver-associated enzymes and survival rate

Blood samples were collected from aorta at the end of each ischemia, or 40 min after reperfusion, and serum was obtained after centrifugation. The serum concentration of aspartate

aminotransferase (AST), alanine aminotransferase (ALT), and lactic dehydrogenase (LDH) was measured in a bioclinical laboratory. The survival rate was determined on postoperative day 7.

DNA extraction and analysis of 8-OHdG

Hepatic tissue sample was immediately frozen in liquid nitrogen. DNA was extracted from approximately 100 mg of frozen samples by the chaotropic NaI method with minor modification (Nakae *et al.*, 1995) (DNA extractor WB kit, Wako, Osaka, Japan). The DNA was digested with nuclease P_1 (Sigma, St. Louis, MO) and alkaline phosphatase (Boehringer Mannheim, Tokyo). The digested product was then analyzed by high-performance liquid chromatography (HPLC) and electrochemical detector (Coulochem II, ESA, Bedford, MA) as previously described (Toyokuni *et al.*, 1997) using an 8-OHdG standard (Wako, Osaka).

Immunohistochemical analysis

Samples were fixed with Bouin's solution (Luna, 1968) followed by histological examination of hematoxylin and eosin-stained specimens. Immunohistochemical analyses of 8-OHdG and HNE-modified proteins were performed as previously described using the ABC method with alkaline phosphatase. Black substrate (Vector Laboratories, Burlingame, CA) was used for high sensitivity (Toyokuni *et al.*, 1994b, 1997; Toyokuni, 1999). No nuclear counterstaining was performed for detection of nuclear staining. At least five samples were evaluated for each group.

Western blot analysis

Approximately 40 mg of frozen samples were homogenized in 10 m*M* sodium phosphate buffer (pH 7.4) containing 1% Nonidet P-40, 1 m*M* phenylmethyl sulfonyl fluoride, 0.1 mg of tosyl arginine methyl ester per ml, and 5 m*M* ethylenediaminetetraacetic acid (wt/vol = 1:10). The suspensions were then centrifuged at 105,000 × *g* at 4°C for 60 min; the protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL). After treatment with Laemmli sample buffer at 100°C for 5 min, samples were run in duplicate on 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). One gel was stained with Coomassie brilliant blue to confirm loading of the equal amount of proteins, and the other was used for Western blot analysis as previously described (Kondo *et al.*, 1999a). The final reaction was carried out by enhanced chemiluminescence detection reagents (Amersham) and the results were visualized on X-ray film (Hyper film ECL, Amersham). Densitometric analysis was performed with a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Tokyo).

Statistical analysis

Data were expressed as means ± SD. One-way analysis of variance (ANOVA) and an unpaired *t*-test were used to analyze the differ-

TABLE 1. CHANGES IN AST, ALT, AND LDH IN THE SERUM

	Untreated control	*End of ischemia*	*Reperfusion (40 min)*
AST (IU/liter)			
Group 1	95 ± 14	105 ± 8.6	459 ± 166[a]
Group 2		103 ± 9.9	1259 ± 263[a,b]
ALT (IU/liter)			
Group 1	63 ± 9.4	68 ± 15	411 ± 76[a]
Group 2		76 ± 12	1421 ± 309[a,b]
LDH (IU/liter)			
Group 1	1012 ± 100	1105 ± 139	7104 ± 940[a]
Group 2		1127 ± 112	14835 ± 3842[a,b]

Group 1, 15-min ischemia; Group 2, 30-min ischemia.
Data are expressed as means ± SD; $n = 10$ except for the control ($n = 7$).
[a] $p < 0.0001$ vs. untreated control.
[b] $p < 0.01$ vs. Group 1.

TABLE 2. THE AMOUNT OF 8-OHDG AFTER WARM ISCHEMIA AND REPERFUSION

Treatment	*8-OHdG/10^5 × dG*
Untreated control	0.70 ± 0.32
End of ischemia	
Group 1	1.34 ± 0.37[a]
Group 2	1.68 ± 0.27[b,c]
Reperfusion (40 min)	
Group 1	2.91 ± 0.75[c]
Group 2	4.24 ± 0.90[c,d]

Group 1, 15-min ischemia; Group 2, 30-min ischemia. Data are expressed as means ± SD; $n = 10$ except for the control ($n = 7$).
[a] $p < 0.005$ vs. untreated control.
[b] $p < 0.05$ vs. Group 1.
[c] $p < 0.0001$ vs. untreated control.
[d] $p < 0.01$ vs. Group 1.

ence in parameters, and the chi-square test was used for survival rate. Any p value less than 0.05 was considered as significant.

RESULTS

Survival rate and liver-associated enzymes

While all the animals in Group 1 (15-min ischemia) survived the IR injury (16/16), only 50% (8/16) survived in Group 2 (30-min ischemia; $p < 0.01$). Changes in serum levels of AST, ALT, and LDH are summarized in Table 1. At the end of ischemia, each liver-associated enzyme in the serum remained at low levels and there was no significant difference in the two groups. However, after reperfusion all of the enzyme levels were significantly increased in the two groups ($p < 0.01$). Furthermore, those in Group 2 were significantly higher than Group 1 ($p < 0.01$).

8-OHdG level in liver after ischemia-reperfusion injury

8-OHdG levels are summarized in Table 2. 8-OHdG levels were increased after ischemia, which was further enhanced by reperfusion. The 8-OHdG levels of Group 2 were consistently higher than those of Group 1.

HNE-modified proteins in liver after ischemia-reperfusion injury

Western blot analysis revealed several kinds of proteins modified by HNE (Fig. 1A). Major bands were located at 42 and 31 kDa. By densitometric analysis, the intensity of the band

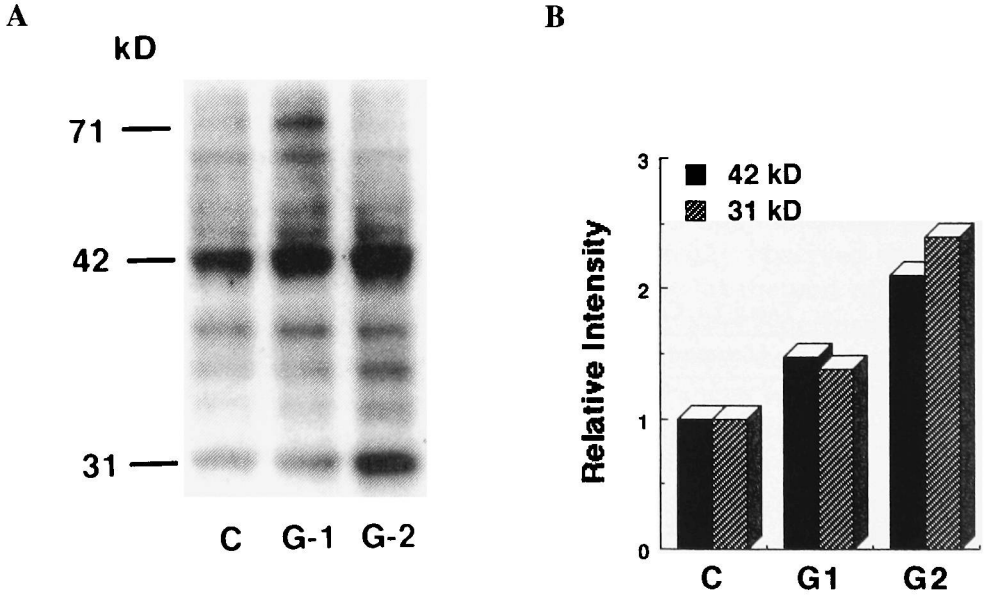


FIG. 1. Western blot analysis of liver after ischemia-reperfusion injury. Aliquots of cytoplasmic fraction of liver samples were run on a 12.5% SDS-PAGE, transferred to PVDF membrane, and immunoreacted with an antibody against HNE-modified proteins as described in Materials and Methods. **A**. Western blot. **B**. Densitometric analysis of 42- and 31-kD proteins. C, Untreated control; G-1, 15-min ischemia and reperfusion group; G-2, 30-min ischemia and reperfusion Group.

significantly increased after reperfusion, which was in proportion to the ischemia duration (Fig. 1B).

Histology

Figure 2F illustrates the histological findings of liver 40 min after reperfusion. Severe sinusoidal congestion, cytoplasmic degeneration, and necrosis with nuclear pyknosis were observed in the liver in Group 2. On the other hand, there were significantly less histological changes in Group 1 (data not shown).

Immunohistochemical analysis

Faint immunostaining of 8-OHdG and HNE-modified proteins was observed in the control liver (Fig. 2G, H). After IR, most of the hepatocytes, except necrotic areas and some sinusoidal cells, revealed strong nuclear immunostaining of 8-OHdG that increased in proportion to ischemia period (Fig. 2A, B). In Group 2, nuclei of bile canalicular cells and endothelial cells were intensely stained (Fig. 2B).

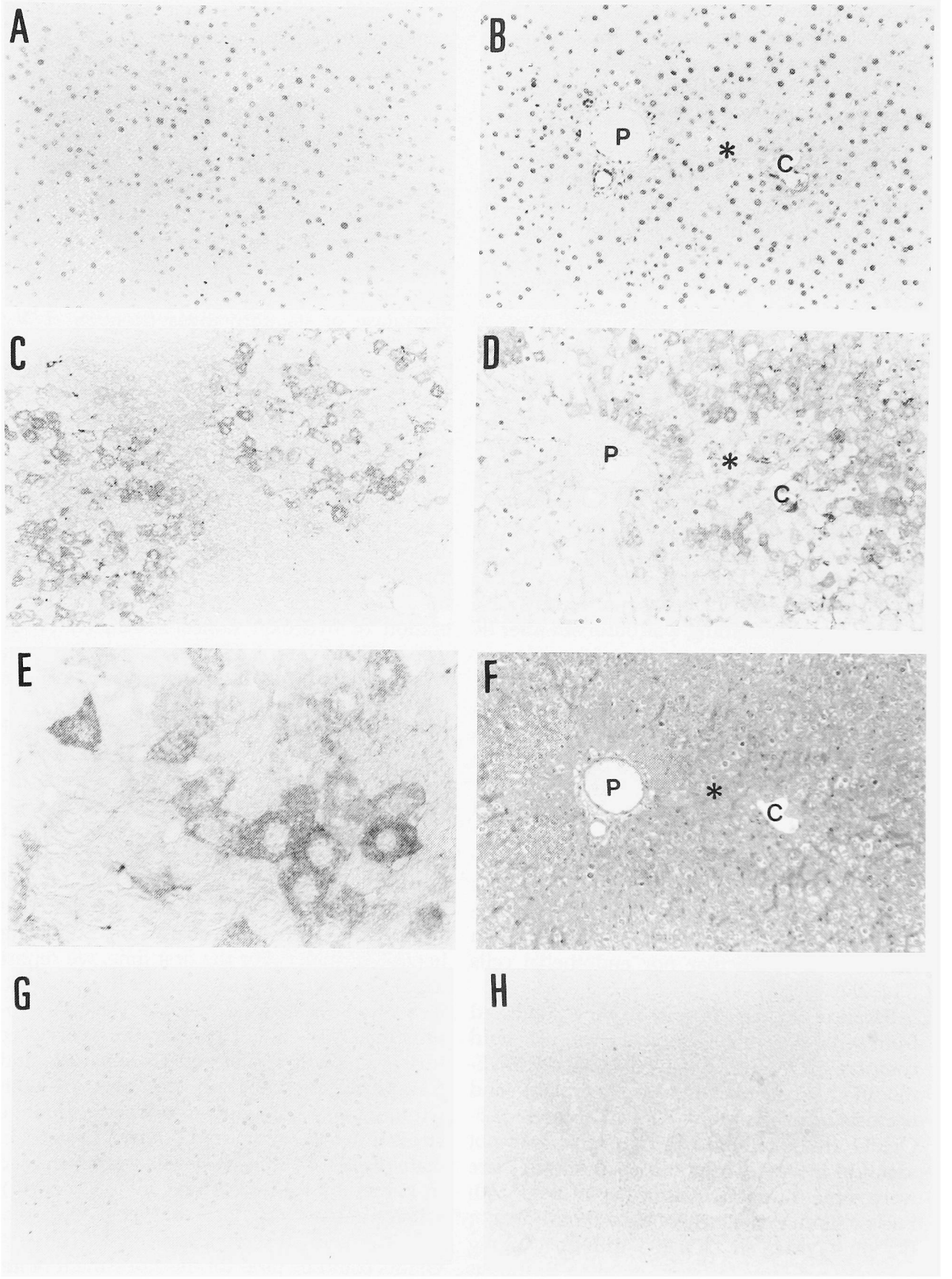
HNE immunostaining was observed after IR in hepatocyte cytoplasm of the midzonal area, which increased in proportion to ischemia period (Fig. 2C, D). Staining of sinusoidal red blood cells was sometimes detected, and in Group 2 some hepatocytes surrounding the necrotic area showed nuclear staining. Necrotized hepatocytes judged by hematoxylin and eosin staining were not stained. At higher magnification, cytoplasmic staining was rather granular, and stained or unstained hepatocytes were easily distinguished (Fig. 2E). Furthermore, HNE-modified proteins were found in neither bile canalicular nor endothelial cells (Fig. 2D).

Because Fig. 2B, D, and F were produced from serial paraffin sections of liver, we could compare the localization of 8-OHdG and HNE-modified proteins with degeneration and necrosis of hepatocytes. The distribution of 8-OHdG and HNE-modified proteins was not identical in the present study. Although there were some common areas of hepatocytes with intense immunostaining of both products after IR, an increase in 8-OHdG immunostaining was observed in a larger population as well as in most of the viable hepatocytes. In necrotized areas, neither 8-OHdG- nor HNE-modified proteins were immunostained.

DISCUSSION

Generation of uncontrolled ROS has been hypothesized to play an important role in the pathogenesis of hepatic IR injury (McCord, 1985; Homer-Vanniasinkam *et al.*, 1997). To date, several studies have indirectly shown that antioxidants and enzymes such as superoxide dismutase or its derivatives (Romani *et al.*, 1988; Mizoe *et al.*, 1997; Zwacka *et al.*, 1998; Kondo *et al.*, 1999b), catalase (Nauta *et al.*, 1990; Yabe *et al.*, 1999) and α-tocopherol (Marubayashi *et al.*, 1986) prevented IR injury. Thus, increased production of ROS as well as decreased antioxidant activity are believed to be important factors in IR injury. Activated neutrophils, resident macrophages, and Kupffer cells are also postulated to play a role in IR injury (Shappell *et al.*, 1990; Patel *et al.*, 1991). In the present study, we have focused on the generation of hydroxyl radical-associated products, 8-OHdG and HNE-modified proteins (Toyokuni, 1999), and their location in the liver immediately after IR injury.

Thirty-minute hepatic warm ischemia and reperfusion (Group 2) was lethal for male Wistar rats, whereas 15-min ischemia and reperfusion could be survived (Group 1). This was consistent with the data of liver-associated enzymes in the serum (Table 1). Although 8-OHdG has been one of the most commonly used markers for the evaluation of oxidative stress, no data have become available for the hepatic IR injury. For the first time, we report increased levels of 8-OHdG in the liver after IR. Ischemia alone increased 8-OHdG levels by approximately twofold. However, the levels were further increased after reperfusion (4.2- and 6.1-fold, respectively) in the liver of both Groups 1 and 2. In another model of oxidative stress-induced cell injury by ferric nitrilotriacetate (Fe-NTA), 8-OHdG levels were increased in kidney by 5.6-fold (Toyokuni *et al.*, 1994a), where massive necrosis of renal proximal tubules were observed thereafter. Thus, 8-OHdG could be used for the assessment of hepatic injury by IR before the actual necrosis of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hepatocytes. This might be useful especially in the clinical situations of liver transplantation. Indeed, Loft *et al.* (1995) reported that urinary excretion of 8-OHdG was increased by 2.9-fold in the first 1–3 hr after liver and small intestine transplantation in pigs, whereas how 8-OHdG is excreted to urine is not clear at present.

In a previous study, we demonstrated the presence of HNE-modified proteins in the liver after IR (Yamagami *et al.*, 1998). In the present study, we attempted to determine whether specific proteins are modified in the hepatic IR injury. We have identified two major proteins with molecular weights of 42 kD and 31 kD. Extensive studies are now in progress to identify and further characterize these proteins. Because, in general, HNE significantly decreases enzymatic activity by modification (Esterbauer *et al.*, 1991; Uchida and Stadtman, 1993), these two proteins might be targets for IR injury of hepatocytes. If so, therapeutic intervention targeted at these proteins might be possible.

Immunohistochemical analysis of 8-OHdG was for the first time performed on the IR injury model. In general, immunostaining for both 8-OHdG- and HNE-modified proteins increased after IR equivalent to the ischemia duration. Positive 8-OHdG immunostaining was observed after IR in the nuclei of most liver cells. Nuclei of bile canalicular and endothelial cells were intensely stained, especially in Group 2. Because HNE-modified proteins were not immunostained in bile canalicular and endothelial cells, metabolism of ROS or its reaction with cytoplasmic proteins might be different in these cells. Completely necrotized cells showed no immunostaining for both of the epitopes.

HNE-modified proteins were found predominantly in the cytoplasm of hepatocytes. Moreover, the immunostaining was distinct from cell to cell (Fig. 2E), which implies that hepatic IR injury is possibly dependent on the metabolic state of individual cells. This appears different from the model of Fe-NTA-induced renal injury, where Fenton-like reaction occurs in the lumina of proximal tubules. In this model, no such pattern of immunostaining was observed, whereas a continuous group of renal proximal tubular cells were immunostained (Toyokuni *et al.*, 1994b). Cytoplasmic staining was in a granular pattern at higher magnification (Fig. 2E), implying that not only the cytosol but also cytoplasmic organelles such as mitochondria are immunostained, as was determined in the recent Fe-NTA study (Zainal *et al.*, 1999). Our results clearly demonstrate that lipid peroxidation occurs in hepatocytes after IR, at least at an early stage in contradiction to a previous study (Walsh *et al.*, 1990). Furthermore, nuclear immunostaining of hepatocytes in severe IR injury was prominent. This could represent HNE modification of chromatin, and might be used as a marker of severe IR injury. Further study is necessary to elucidate the exact location of epitopes in the nuclei.

Previously, the production of superoxide anion in ischemic and postischemic tissue had been stressed (McCord, 1985). The linear response of 8-OHdG- and HNE-modified proteins in hepatic IR injury reveals that the chemical reaction indeed proceeded further than superoxide, leading to the generation of hydroxyl radicals or its equivalents.

In conclusion, we have shown that (i) 8-OHdG is increased in hepatic IR injury, (ii) cells immunoreactive for 8-OHdG- and HNE-modified proteins are not the same, but major immunoreactive cells are hepatocytes, (iii) intranuclear immunoreactivity for HNE-modified proteins were observed in severe hepatic

FIG. 2. Histology and immunohistochemistry of liver after IR injury. Immunostaining of 8-OHdG after 15-min ischemia and reperfusion (**A**), after 30-min ischemia and reperfusion (**B**), and untreated control (**G**) (×120). Immunostaining of HNE-modified proteins after 15-min ischemia and reperfusion (**C**), after 30-min ischemia and reperfusion (**D**), and untreated control (**H**) (×120); (**E**) is a higher magnification of (**C**) (×480). **F.** Histology of liver after 30 min of ischemia and reperfusion (hematoxylin and eosin, ×120). Parts **B**, **D**, and **F** are from serial sections. P, portal vein; C, central vein; asterisk necrotized area. All of the immunostainings are without nuclear counterstaining. Refer to Materials and Methods section and results for more details.

IR injury, and, finally, (iv) there are two major target proteins in hepatic IR injury.

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ABBREVIATIONS

ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; Fe-NTA, ferric nitrilotriacetate; HNE, 4-hydroxy-2-nonenal; HPLC, high-performance liquid chromatography; ICAM, intercellular adhesion molecule; IR, ischemia-reperfusion; LDH, lactic dehydrogenase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF, tumor necrosis factor.

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Address reprint requests to:
Dr. Shinya Toyokuni
Department of Pathology and Biology of Diseases
Graduate School of Medicine
Kyoto University
Yoshida-Konoe-cho, Sakyo-ku
Kyoto 606-8501, Japan

E-mail: toyokuni@path1.med.kyoto-u.ac.jp

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