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Heat Shock Preconditioning Reduces the Formation of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal Modified Proteins in Ischemia-reperfused Liver of Rats

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Heat shock preconditioning (HSPC) is a promising strategy for providing ischemic tolerance. The objective of this study is to investigate the effectiveness of HSPC in preventing oxidative damage of cellular proteins and DNA during ischemia–reperfusion of the liver. Male Wistar rats were divided into a heat shock group (group HS) and control (group C). Forty-eight hours prior to ischemia, rats in group HS received HSPC at 42°C for 15 min. All rats received hepatic warm ischemia for 30 min and subsequent reperfusion. The formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), 4-hydroxy-2-nonenal (HNE) modified proteins in liver tissue, survival rate of the animals, and changes in biochemical and histological parameters were compared between groups. Heat shock protein 72 was produced only in group HS. The 7-day survival of rats was significantly better in group HS (10/10) than in group C (5/10) ($p < 0.01$). **The serum release of alanine aminotransferase** ($n = 10$, $p < 0.01$) **and the concentration of adenosine triphosphate in liver tissue** ($n = 10$, $p < 0.01$) **40 min after reperfusion was significantly better in group HS than in group C. The formation of 8-OHdG in liver tissue measured by high-performance liquid chromatography was suppressed in group HS** ($p < 0.01$). **The production of HNE-modified proteins as determined by Western-blot analysis was also decreased in group HS. These results were also confirmed by immunohistochemical analysis. As determined by levels of 8-OHdG and HNE-modified proteins produced during ischemia–reperfusion of the liver, HSPC reduced the oxidative injury of cellular proteins and DNA in the liver tissue.**

Keywords: Heat shock protein 72; Ischemia–reperfusion injury; Liver; Reactive oxygen species; 8-Hydroxy-2′-deoxyguanosine; 4-Hydroxy-2-nonenal modified proteins

INTRODUCTION

Conditions resulting from ischemia–reperfusion (IR) occur during such surgical interventions as temporary total hepatic inflow clamping (Pringle's maneuver) or during the reconstruction of large vessels in liver surgery and liver transplantation. IR injury greatly impairs postischemic liver function and easily leads patients into postoperative liver failure. Although the exact mechanism of IR injury is still unclear, numerous reports in the last decade have shown that the uncontrolled production of reactive oxygen species (ROS) plays a significant role in the pathogenesis of IR injury of the liver.[1–3] The involvement of neutrophils in this process has been suggested.[4,5] To regulate intracellular ROS, cells develop various enzymatic and non-enzymatic defense systems. However, a certain fraction of ROS escapes cellular defenses and causes either permanent or transient damage to constitutive proteins, lipids, and nucleic acids,[6,7] resulting in cellular death.

Antioxidants and enzymes such as superoxide dismutase,[8–11] catalase,[12] and alpha-tocophenol[13] were reported to be protective against ROS injury during IR, however, it would be, difficult to utilize these antioxidants or enzymes clinically in their present form.

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We reported the efficacy of heat shock preconditioning (HSPC), a new powerful strategy to evoke an endogenous protection response, which induced abundant heat shock protein 72 (HSP72) in normal, fibrotic, and steatotic rat livers.[14–16] Heat shock protein 72, a molecular chaperone, prevents denaturation and aggregation of proteins during stress and is recognized as a key protein in conferring stress tolerance.[17] ROS-induced DNA damage includes oxidative modification of bases as well as single and double strand breaks.[18] Among the modification of DNA bases, the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the most commonly used markers for evaluating oxidative DNA damage. In our previous study, the level of 8-OHdG was quite useful for assessing DNA damage during IR injury in rat livers.[19] 4-Hydroxy-2-nonenal (HNE) is an alpha, beta-unsaturated aldehyde and one of the major components of lipid peroxidation products. It reacts with proteins to form HNE-modified proteins.[20,21] Since HNE-modified proteins are relatively stable, they can be used as a marker of ROS-mediated protein damage. We have shown that the formation of HNE-modified proteins is closely correlated with liver damage due to IR injury,[16,19,22] and suggests that two major proteins with molecular weights of 31 and 42 kDa are key targets for IR injury in rat livers.[19]

In this study, we investigated the effect of HSPC in light of oxidative DNA damage and cellular protein denaturation during IR of the liver by evaluating the formation of 8-OHdG and HNE-modified proteins, respectively.

MATERIALS AND METHODS

Animals

This study was conducted in compliance with the Animal Protection Guidelines of Kyoto University and all rats were treated humanely. Specific pathogen-free male Wistar rats (Shizuoka Laboratory Animal Center: Shizuoka, Japan), weighing between 230 and 260 g and between 10 and 11 weeks of age, were housed in an air conditioned room (24 ± 1°C) with alternating 12 h light and dark cycles, and were fed with a standard animal diet.

Study Protocol

A total of 76 rats were used. All surgical procedures were performed under general anesthesia with intraperitoneal injection of sodium pentobarbital (40 mg/kg) in an operating room temperature-controlled for these animals (24 ± 1°C). The animals were divided into two groups: A heat shock preconditioning group (group HS) and a control group (group C). Rats in group HS were placed in a temperature-controlled water bath to keep their rectal temperature at 42°C for 15 min. Rats in group C were temperature-controlled at 37°C instead of 42°C. Forty-eight hours after this pretreatment, the livers of rats in both groups were exposed to a 30 min warm ischemic insult by clamping inflow vessels. This was followed by a 40 min *in situ* reperfusion.

Using a steel tongue pre-cooled with liquid nitrogen, shock-frozen liver samples from the left lateral lobes were obtained before ischemia ($n = 7$), after ischemia ($n = 10$) and 40 min after reperfusion ($n = 10$). At these same time intervals, liver samples from the right lobes were also obtained for histological examination. Aortic blood samples were collected 40 min after reperfusion ($n = 10$).

Measurement of Liver Related Enzymes, Energy Metabolism, and Survival Rate

Alanine aminotransferase (ALT) was measured in a biochemical laboratory. Concentration of adenine nucleotides in liver tissues was assayed enzymatically.[23,24] Survival rate was determined on post-operative day 7.

Analysis of 8-OHdG

DNA was extracted from the frozen samples by the NaI method with minor modifications (DNA extractor WB kit: Wako, Osaka, Japan).[25] DNA was digested with nuclease P1 (Sigma: St. Louis, MO, USA) and alkaline phosphatase (Boehringer Mannheim: Tokyo, Japan). As previously described,[26] the production of 8-OHdG was then analyzed from the DNA extracts using high performance liquid chromatography (HPLC) and an electrochemical detector (Coulochem II, ESA: Bedfold, MA, USA).

Immunohistochemical Analysis

After samples were fixed with Bouin's solution,[27] immunohistochemical staining of HSP72 (anti-HSP72 antibody: SPA-810; StressGen Biotechnologies, Victoria, British Columbia, Canada), 8-OHdG,[26] and HNE-modified proteins[28] was performed using the ABC method in conjunction with alkaline phosphatase. In order to achieve high sensitivity, black substrate (Vector Laboratories: Burlingame, CA, USA) was used as the final detection reagent.

Western Blotting Analysis

Frozen samples (weighing approximately 40 mg) were homogenized in a lysis buffer. The suspensions for HSP72 and HNE-modified proteins were centrifuged at 15,000*g* for 15 min and 105,000*g* for

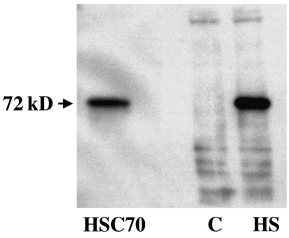

FIGURE 1 Western blotting analysis of HSP72 production in livers 48 h after reperfusion C: control; HS: group HS. Protein concentrations are 3 μg/lane in the samples and 0.2 μg/lane in the positive control (HSC70).

60 min, respectively, at 4°C. Protein concentration was determined using a BCA protein assay kit (Pierce: Rockford, IL, USA). Samples were run onto a 10% (HSP72) or a 12.5% (HNE-modified proteins) polyacrylamide gel with 0.1% sodium dodecyl sulfate and were used for Western blot analysis as previously described.[19,29] Finally, they were reacted with enhanced chemiluminescence detection reagent (Amersham International: Buckinghamshire, England) and the results were visualized on X-ray (Hyper film ECL: Amersham). Densitometric analysis was performed with a GS-700 Imaging Densitometer (Bio-Rad Laboratories: CA, USA).

Statistical Analysis

Data are shown as means ± SD. One way analysis of variance and an unpaired *t*-test were used to analyze the changes in metabolic parameters. A chi-square test was used for survival rate. *p*-Values less than 0.05 were considered statistically significant.

RESULTS

Detection of HSP72

Figure 1 shows the amount of HSP72 in liver tissue 48 h after HSPC. Heat shock protein 72 was strongly expressed in group HS, while a band of HSP72 was not detected in group C. Figure 2 shows immunohistochemical staining of HSP72 in liver tissue. The control liver was not stained, but HSP72-positive hepatocytes were distributed in pericentral and periportal liver tissue after HSPC. The nuclei as well as the cytoplasm of hepatocytes were stained with a specific anti-HSP72 antibody.

Animal Survival, Liver Related Enzymes and Energy Metabolism

Seven-day survival of the rats, as well as their serum levels of ALT and their ATP concentrations in liver tissue after warm ischemic insult is presented in Table I. While all the animals survived (10/10) in group HS, only 50% (5/10) survived in group C ($p < 0.01$). Forty minutes after reperfusion, levels of ALT in group HS were significantly lower than levels in group C ($p < 0.01$). In addition, during the 40 min after reperfusion, there was a better recovery of ATP concentrations in liver tissue in group HS than in group C ($p < 0.01$).

8-OHdG Level in the Liver Tissue After IR Injury

Tissue levels of 8-OHdG are summarized on Table II. In group C, there was a 2.4-fold increase in 8-OHdG levels during ischemia and a further increase to 6.1-fold during reperfusion. In group HS, the increase in 8-OHdG during ischemia was not different from the increase seen in group C, but the increase during reperfusion was significantly less than the levels in group C ($p < 0.01$).

HNE-modified Proteins in the Liver After IR Injury

Several bands of HNE-modified proteins were detected by Western blotting analysis (Fig. 3A). Two major bands were located at 42 and 31 kDa

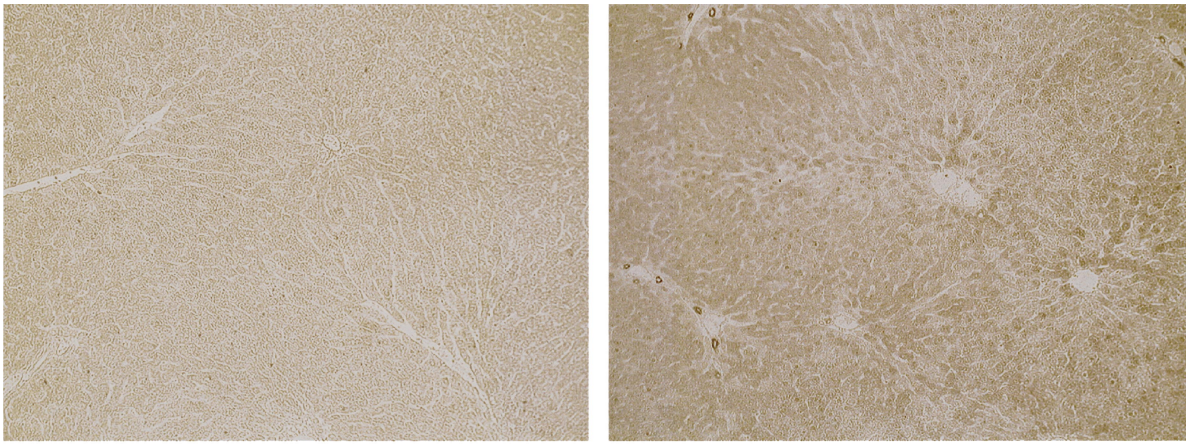

FIGURE 2 Immunohistochemical analysis of HSP72. (A) Control rat livers. (B) Rat livers 48 h after heat shock preconditioning (Original magnification × 10).

TABLE I

Survival rate on POD 7	
Group C	5/10 (50%)
Group HS	10/10 (100%)*
Serum ALT levels 40 min after reperfusion	
Group C	1349 ± 240
Group HS	699 ± 160*
ATP concentration in liver tissue 40 min after reperfusion	
Group C	0.759 ± 0.277
Group HS	1.680 ± 0.244*

C: control; HS: heat shock; POD: postoperative day.
Data are expressed as mean ± SD, $n = 10$, $*p < 0.01$ vs. control.

after reperfusion in both groups. Although these two bands were visible even in untreated group reflecting the baseline lipid peroxidation in the normal liver tissue, they were prominently strong in group C and depressed in group HS. Densitometric analysis demonstrated that the intensity of these two major bands was lower in group HS than in group C (Fig. 3B).

Immunohistochemical Staining of 8-OHdG and HNE-modified Proteins

Figure 4 illustrates hematoxylin and eosin staining of the liver tissue after reperfusion. In group C, severe congestion as well as massive necrotic areas were noted, but these damages were markedly less in group HS. Both 8-OHdG and HNE-modified proteins were faintly stained in livers before IR in group C and in group HS. After IR, 8-OHdG

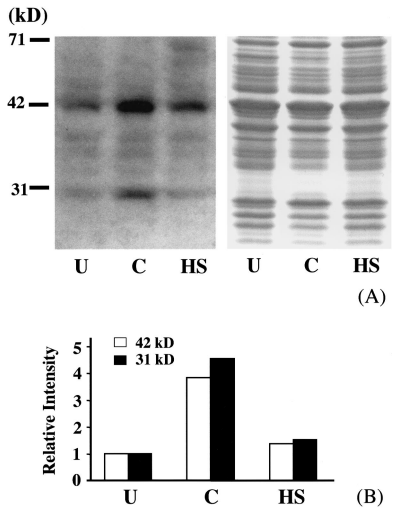

FIGURE 3 HNE-modified proteins in livers after ischemia–reperfusion injury. (A) Western blotting analysis (Protein concentrations are 20 μg/lane in the sample). Two major bands were located at 42 and 31 kDa. The intensity of these two major bands was lower in group HS than in group C. Coomassie brilliant blue staining of the gel shows the equal amount of proteins applied on each lane. (B) Densitometoric analysis of 42 and 31 kDa proteins. U: untreated liver; C: control; HS: group HS.

TABLE II 8-OHdG levels after warm ischemia and reperfusion

Treatment	8-OHdG/10^5 × dG
Before clamping ($n = 7$)	
Control	0.70 ± 0.32
HS	0.83 ± 0.24
End of ischemia ($n = 10$)	
Control	1.68 ± 0.27
HS	1.50 ± 0.34
Reperfusion (40 min) ($n = 10$)	
Control	4.24 ± 0.90
HS	2.95 ± 0.60*

HS: heat shock.
Data are expressed as mean ± SD, $*p < 0.01$ vs. control.

immunostaining was intense in the nuclei of cells in group C. However, the nuclear staining in group HS was significantly less modified than in group C (Fig. 5). In a similar way, HNE-modified proteins were strongly immunostained after IR in the cytoplasm of hepatocytes in group C except in necrotic areas. On the other hand, staining in group HS was significantly suppressed (Fig. 6).

DISCUSSION

Our prior experiments have demonstrated that HSPC or concomitantly induced HSP72 provided livers with tolerance against IR injury.[14–16] The beneficial effects of HSP72 on IR injury were also reported in other organs including the heart and lungs.[30,31] But the cytoprotective mechanism of HSPC against IR injury has not been clearly defined. Our present data show that the formation of oxidative-damage products (8-OHdG and HNE-modified proteins) was significantly decreased in group HS. That is, HSPC reduced the oxidative damage of DNA in nuclei and of proteins in cytosol. Decreased formation of these unfavorable products in hepatocytes after lethal ischemia in group HS is consistent with better animal survival, less release of liver-related enzymes and more rapid recovery of hepatic energy metabolism.

Membrane lipids are the first targets of ROS attacks. The final products of lipid peroxidation are mostly aldehydes. HNE, one of these aldehydes, has high reactivity with cytosolic proteins. We have demonstrated that the formation of HNE-modified proteins is a sensitive marker for evaluating levels of lipid peroxidation and oxidative denaturation of cytosolic proteins.[32] Identification of two major proteins—42 and 31 kDa—was effective for assessing the IR injury of rat livers.[19] Concerning these two proteins, further characterization is of great importance to clarify the detailed mechanism, but the molecular weights do not provide enough information to estimate the candidate proteins. Proteins having histidine-, cystein- or lysine-residue, which easily reacts with HNE, on their outer surface, will be

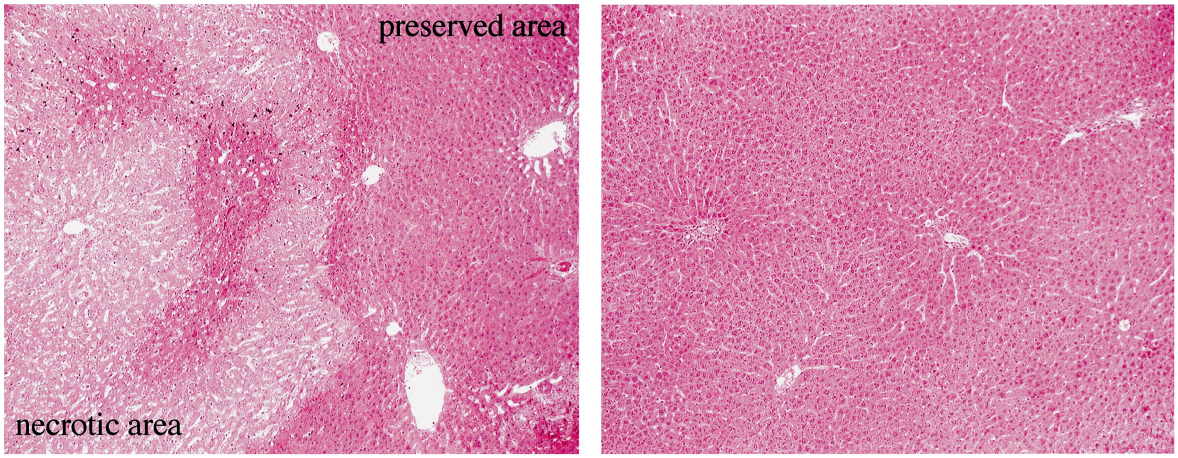

FIGURE 4 Hematoxylin and eosin staining of liver tissue. (A) After reperfusion in group C. Congestion and necrosis were noted. (B) After reperfusion in group HS. Almost normal histology was preserved. (Original magnification × 10).

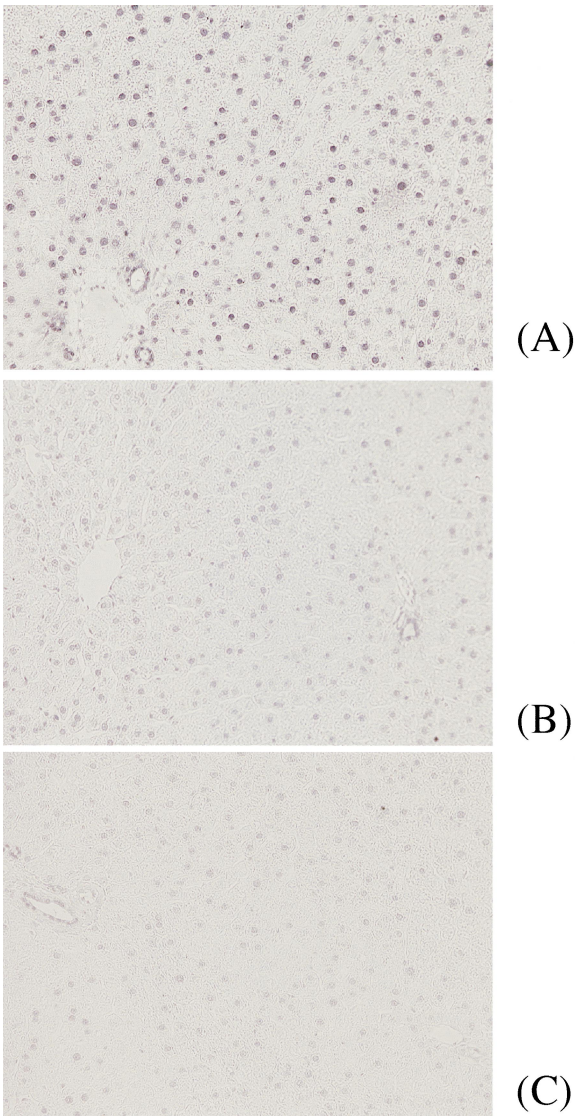

FIGURE 5 Immunohistochemical staining of 8-OHdG. (A) After a 30 min ischemia and reperfusion in group C. (B) After a 30 min ischemia and reperfusion in group HS. (C) Untreated livers. (Original magnification × 20).

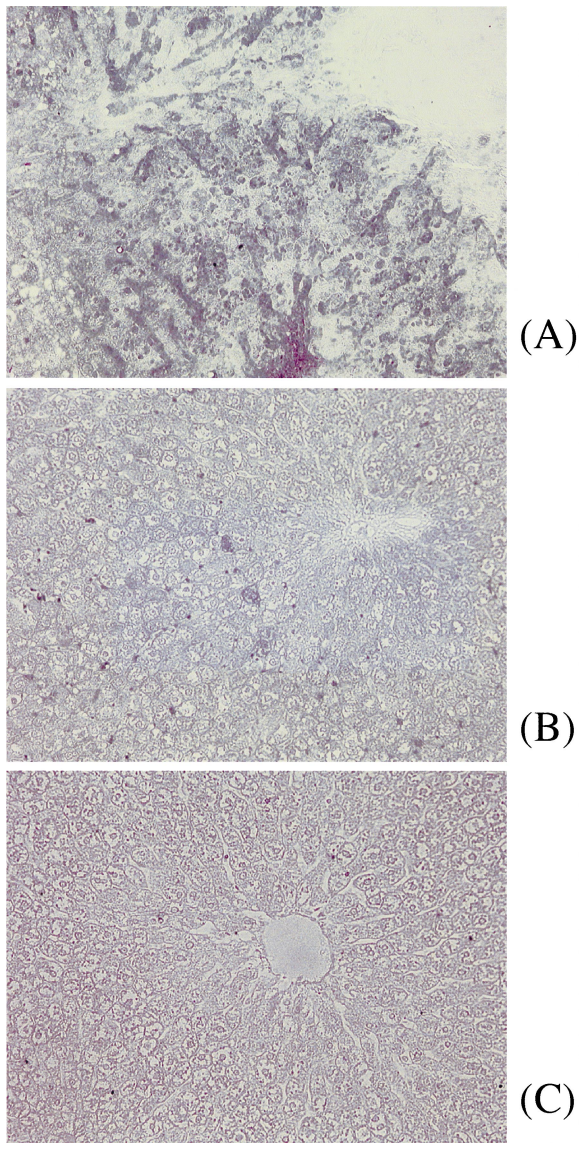

FIGURE 6 Immunohistochemical staining of HNE-modified proteins. (A) After a 30 min ischemia and reperfusion in group C (B) After a 30 min ischemia and reperfusion in group HS. (C) Untreated livers. (Original magnification × 20).

candidates. However, it is still difficult to nominate the proper proteins at present because the liver is a special organ producing numerous kinds of proteins. In spite of the difficulty in characterizing the nature of proteins, the present study demonstrates that these two key proteins were produced significantly less in group HS. As molecular chaperones, HSP72 may bind to denatured proteins and prevent their irreversible denaturation.[17] In light of the known functions of HSP72, other possible mechanisms behind reduction of HNE-modified proteins are: (1) suppression of the initiation and/or the propagation of ROS synthesis;[33] (2) suppression of the lipid peroxidation cascade;[34] and (3) degradation of HNE-modified proteins by proteasome.[35] Further studies are necessary to clarify the effect of HSPC on the decreased production of HNE-modified proteins.

Reperfusion of ischemic tissue causes an immediate increase in DNA damage, that includes base modifications and strand breaks. DNA is an important target for ROS attacks in cells. More than 100 different oxidative modifications have been observed in DNA after ROS attacks.[36,37] However, to date, only a few base modifications have been established as biomarkers. Among them, oxidative C-8 adduct of guanine has been the one most frequently studied. A sensitive and easy procedure for measurement using HPLC and electrochemical detector has been established;[38] and therefore, 8-OHdG has become the most popular indicator of oxidative DNA damage *in vivo* and *in vitro*.[39,40]

Our previous data have demonstrated that the production of 8-OHdG after reperfusion clearly reflects the intensity of an ischemic load to the liver and is a good marker in hepatic IR injury.[19] In this study, it was shown that a substantial level of 8-OHdG was produced in the nuclei even in untreated livers, and HSPC significantly suppressed the production of 8-OHdG during IR of the liver (Table II). Concerning the detection of 8-OHdG in untreated livers, artificial oxidation of DNA during sample preparation may be considered. However, it is very likely that to a certain extent normal metabolic pathways do generate ROS, resulting in oxidative DNA damage.

Immunohistochemical staining of liver tissue with HSP72 antibody revealed nuclear accumulation of HSP72 in hepatocytes (Fig. 2). However, it has not been determined what signals regulate this nuclear accumulation of HSP72, or why HSP72 moves to nuclei in response to a wide variety of stressors. Abe *et al.* suggested that HSP72 protein protected DNA from further damage or facilitated the repair of DNA through some unknown mechanism in nuclei.[41] In addition, HSP72 may act indirectly in reducing oxidative DNA damage. Actually, some current studies suggest that HSP72 may have the ability to decrease ROS injury by facilitating the translocation of nuclear factor-kappa B (NF-κB),[42] inhibiting the binding activity of NF-κB to DNA,[43] and attenuating the leukocyte-endothelial cell interaction.[44]

Our data do not allow us to ascribe the benefit of HSPC only to the induction of HSP72 because HSPC induces several other HSPs and free radical scavengers like superoxide dismutase[45] as well. However, other studies provide direct evidence for a significant role of HSP72 in cytoprotection. For example, Musche *et al.* demonstrated that the role of HSP72 in cellular protection using transfection of full-length complementary DNA of HSP72.[46] In addition, Feinstein *et al.* reported that antisense oligonucleotides against HSP72 blunted heat shock inhibition of nitric oxide synthase-2 activity in astroglial cells.[47]

In conclusion, the present study demonstrated that HSPC attenuated the formation of 8-OHdG in DNA, suppressed the production of HNE-modified proteins in cytosol, and improved liver-related enzyme release, energy metabolisms and survival rates after IR in rat livers.

Acknowledgments

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