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Pharmacological preconditioning with doxorubicin: Implications of heme oxygenase-1 induction in doxorubicin-induced hepatic injury in rats

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Abstract

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme into biliverdin, carbon monoxide, and iron. HO-1, an inducible form, is thought to contribute to resistance to various types of oxidative stress. Doxorubicin (DOX) produces clinically useful responses in a variety of human cancers. We reported previously that prior administration of DOX ameliorated subsequent hepatic ischemia and reperfusion injury. The aim of this study was to examine whether this pharmacological preconditioning was useful for another type of hepatic injury induced by a non-surgical method. When a high dose of DOX (10 mg/kg body weight) was administered directly to rat liver via the portal vein, serum aspartate transaminase (AST) and alanine transaminase (ALT) levels increased markedly 24 hr after the injection. Under this condition, zinc-protoporphyrin IX, a specific inhibitor of HO-1, caused both serum AST and ALT levels to be elevated further. When a low dose of DOX (5 mg/kg body weight) was administered to rats via the tail vein as pharmacological preconditioning 3 days before the injection of a high dose of DOX via the portal vein, the levels of serum AST and ALT in rats clearly were improved as compared with rats without the preconditioning. Expression of HO-1 in the liver was confirmed 3 days after the administration of a low dose of DOX. In addition, prior administration of zinc-protoporphyrin IX abolished the effect of DOX preconditioning. Immunohistochemical analysis showed that the positive staining of HO-1 protein induced by a low dose of DOX was localized to histiocytes infiltrating periportal areas. These results strongly suggest that pharmacological preconditioning with DOX may generally help to attenuate subsequent oxidant-induced hepatic injury. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Heme oxygenase-1; Doxorubicin; Pharmacological preconditioning; Hepatic injury

1. Introduction

Cells primed by various types of stress are known to develop a tolerance to subsequent lethal injury. This biological adaptation is known as preconditioning. A short period of ischemia and reperfusion protects various tissues against subsequent long-term ischemic injury. This phenomenon, called ischemic preconditioning, has been studied mainly in the heart, intestine, and liver [1,2]. Peralta *et al.* [3] reported that ischemic preconditioning in rat liver was defined by the balance between the tissue levels of adenosine and xanthine. In addition, several groups suggested that NO might play a critical role in ischemic preconditioning [4,5]. The heat-shock stress protein HSP72 also seems to play an important role in protection against ischemia and reperfusion injury. Heat shock preconditioning as well as ischemic preconditioning induced the expression of HSP72, and the induced HSP72 ameliorated the succeeding severe ischemia and reperfusion injury in liver [6,7] and heart [8], respectively.

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Abbreviations: NO, nitric oxide; HSP, heat shock protein; HO, heme oxygenase; DOX, doxorubicin; AST, aspartate transaminase; ALT, alanine transaminase; GSH, glutathione; ROS, reactive oxygen species; and DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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DOX, an anthracyclin anti-neoplastic agent, produces clinically useful responses in a variety of human cancers. The potential usefulness of DOX is restricted because of its cardiotoxic side-effects [9–11]. The exact causal mechanism of DOX-induced cardiomyopathy remains unclear, but most of the evidence indicates that free radicals are involved [12–14]. We have reported recently that pharmacological preconditioning with DOX protects against hepatic ischemia and reperfusion injury via an HO-1-mediated mechanism [15]. This pharmacological preconditioning seems to be clinically useful because a single injection of DOX ameliorates subsequent hepatic injury. Therefore, it is tempting to speculate that DOX preconditioning may generally exhibit the advantage of promoting tolerance to subsequent severe oxidative injury.

HO is the rate-limiting enzyme in the degradation of heme to biliverdin, iron, and carbon monoxide [16,17]. Biliverdin is then converted to bilirubin by biliverdin reductase. Bilirubin and biliverdin both act as antioxidants *in vivo*. HO exists as two isozymes [18,19]. HO-2 is expressed constitutively, and HO-1 is induced by a variety of stimuli including ROS, irradiation, heavy metals, chemotherapeutic agents, microbial products such as endotoxin, hypoxia, hyperoxia, hormones, various cytokines, and heme itself [16, 17,20–22]. In addition, HO-1 is induced by agents that are known to interact with or modify cellular antioxidant GSH levels [23]. HO-1 is recognized as one of the heat shock proteins and is also referred to as HSP32 [23]. Induction of HO-1 may specifically decrease cellular heme (pro-oxidant) and elevate bilirubin (antioxidant) levels [24,25]. Induction of HO-1 has been suggested to be an adaptive response to oxidative stress.

To investigate the application of pharmacological preconditioning with DOX against various types of oxidative stress injury, we examined the effect of DOX pretreatment on subsequent DOX-induced hepatic injury. The present study shows that pharmacological preconditioning with DOX prevents subsequent DOX-induced hepatic insult. Our findings indicate that DOX preconditioning stimulates an oxidative stress-inducible defense system against subsequent hepatic injury. Consistent with our hypothesis, preinduction of HO-1 in the liver may play an important role in this model.

2. Materials and methods

2.1. Drugs

DOX was obtained from Kyowa Hakko. Antibodies to rat HO-1 and HO-2 were from StressGen. Zinc-protoporphyrin IX was from the Sigma Chemical Co. Hybond-C extra nitrocellulose filters were from Amersham. Alkaline phosphatase-conjugated goat anti-rabbit IgG was from Vector Laboratories. 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride were from Promega. All other drugs and reagents were of analytical grade.

2.2. Animal experiments

Male Wistar rats weighing 250–300 g were used and housed in cages in an air-conditioned room (23 ± 1^{o}) with controlled 12-hr light/dark cycles. All animal experiments were performed in strict accordance with the criteria of our institution for the care and use of laboratory animals. To examine the levels of HO-1 protein in the livers after DOX administration, DOX (5 mg/kg body weight) was injected i.v. via the tail vein into ether-anesthetized rats, and their livers were resected at the indicated times. Hepatic injury was induced in rats, anesthetized with sodium pentobarbital (50 mg/kg body weight), by the injection of DOX (10 mg/kg body weight) via the portal vein. DOX was dissolved in sterilized water to 20 mg/mL. Twenty-four hours after the injection, rats were anesthetized with ether and then killed by the collection of blood from the hearts. When zinc-protoporphyrin IX, a specific inhibitor of HO-1 [26], was administered to rats, it (20 μmol/kg body weight) was delivered i.p. 3 hr before the injection of DOX (10 mg/kg body weight). In this experiment, animals were divided into the following groups: Group 1, control (vehicle alone); Group 2, zinc-protoporphyrin IX treatment alone; Group 3, DOX treatment alone; and Group 4, zinc-protoporphyrin IX and DOX treatment. For the DOX preconditioning, a low dose of DOX (5 mg/kg body weight) was administered i.v. via the tail vein 3 days before the injection of a high dose of DOX (10 mg/kg body weight) via the portal vein. Rats used in these experiments were anesthetized and killed as described above. Sampled blood was then centrifuged to obtain serum for biochemical analysis. Liver tissues were sampled at indicated times and stored at -80^{o} before use.

2.3. Assessment of hepatic injury

For the assessment of hepatic function, serum AST and ALT levels were measured in a clinical chemistry laboratory and used herein as markers for hepatic injury.

2.4. Biochemical analysis

Total GSH (reduced and oxidized forms) concentration was measured by the method of Tietze [27] with the GSH reductase-DTNB recirculating assay. The tissue (0.2 g) was homogenized in 5 mL of 0.1 M phosphate buffer (pH 7.5) at 0^{o} and mixed with 2.5 mL of 10% trichloroacetic acid solution. The mixture was centrifuged at 1500 *g* for 15 min. One milliliter of supernatant was extracted three times with 2 mL of cold ether. Each assay contained 0.2 mM NADPH,

0.6 mM DTNB, 0.6 U of GSH reductase and 100 μL of sample in a final volume of 1 mL. The reaction was started by the addition of GSH reductase. The rate of formation of reduced DTNB was followed at 415 nm.

2.5. Western blot analysis

For detection of HO-1 and HO-2 immunoreactive proteins, livers were homogenized in 10 vol. of homogenization buffer containing 20 mM Tris–HCl buffer (pH 7.4), 2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 10 μg/mL of aprotinin, and 1% Tween 20. The homogenates were centrifuged at 8000 *g* for 10 min, and the resulting supernatants (50 μg protein/lane) were separated by 12.5% SDS–PAGE [28]. The sample preparation step was performed at 0–4°. After electrophoresis, the fractionated proteins were electrophoretically blotted onto nitrocellulose filters. The membranes were first treated with 3% (w/v) nonfat dry milk in TBST solution (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, and 0.05% Tween 20) for 1 hr and then incubated with antibody against rat HO-1 or HO-2 (1:1000 dilution) in 3% nonfat dry milk in TBST for 1 hr at room temperature. The filters were washed three times with TBST solution for 10 min each to remove any unbound antibodies and then were incubated for 1 hr at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG in 3% nonfat dry milk in TBST. After washing three times with TBST solution for 10 min each and once with 100 mM Tris–HCl (pH 9.5) containing 100 mM NaCl and 10 mM $MgCl_2$, the immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride as recommended by the supplier.

2.6. Immunohistochemical analysis

The livers of the control and experimental rats were fixed in neutral buffered 10% formalin immediately after sampling. The fixed organs were embedded in paraffin and subjected to immunohistochemistry for HO-1, according to a previous report [29]. Briefly, deparaffinized sections were immersed in 0.3% hydrogen peroxide-containing methanol for inactivation of the intrinsic peroxidase activity. The sections were incubated with 10% normal goat serum at 37° for 15 min, and then treated with antibody against HO-1 (1:500 dilution) at 4° overnight. The labeled antigens were detected using the HistoFine kit (Nichirei) and diaminobenzidine reaction. The sections were counterstained with methyl green.

2.7. Statistical analysis

Data are expressed as means ± SEM for each group. Statistical analysis was performed by ANOVA with a multiple comparison test (Bonferroni's multiple *t*-test). A *P* value of less than 0.05 was considered to be significant.

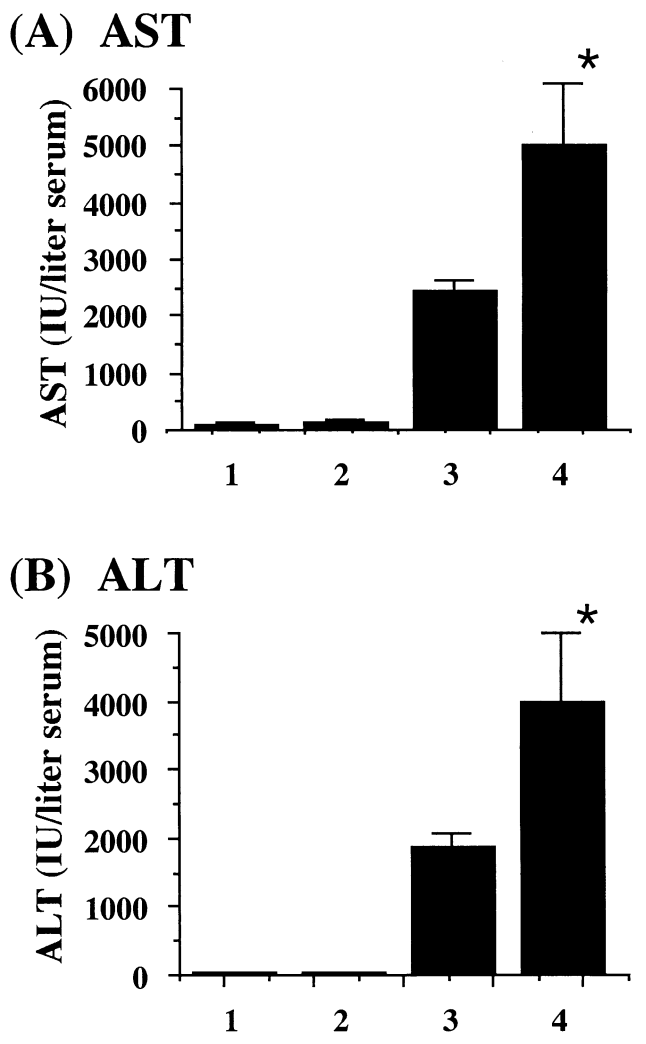

Fig. 1. Effect of zinc-protoporphyrin IX on DOX-induced hepatic injury. DOX (10 mg/kg body weight) was injected into rats via the portal vein. Zinc-protoporphyrin IX (20 μmol/kg body weight) was injected s.c. 3 hr before the injection of DOX. AST (A) and ALT (B) levels were determined 24 hr after the DOX injection. Treatments: 1, normal rats; 2, zinc-protoporphyrin IX; 3, DOX; and 4, zinc-protoporphyrin IX plus DOX. Data represent the means ± SEM of 5–6 animals per group. Key: (*) $P < 0.05$, DOX vs zinc-protoporphyrin IX plus DOX.

3. Results

3.1. Effect of zinc-protoporphyrin IX on DOX-induced hepatic injury

To investigate the relationship between the role of HO-1 and DOX-induced hepatic injury, we examined the effect of zinc-protoporphyrin IX, a specific inhibitor of HO-1, on liver injury in rats (Fig. 1). When a single dose of DOX (10 mg/kg body weight) was injected directly into the rat liver via the portal vein, serum AST and ALT levels, which are markers for hepatic injury, increased 24 hr after the injection (AST, 2431 ± 190 IU/L serum; ALT, 1878 ± 208 IU/L

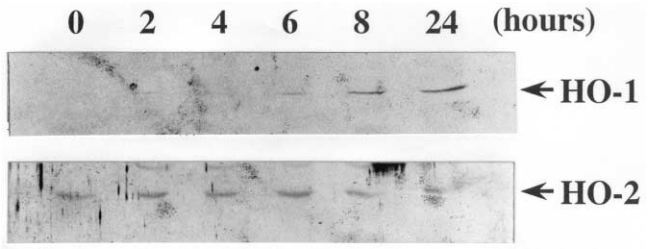

Fig. 2. Time courses of the levels of HO-1 and HO-2 proteins in rat livers after DOX administration. DOX (5 mg/kg body weight) was injected i.v. into rats via the tail vein. Protein samples (50 μg/lane) isolated from rat livers at the indicated times were analyzed by western blots.

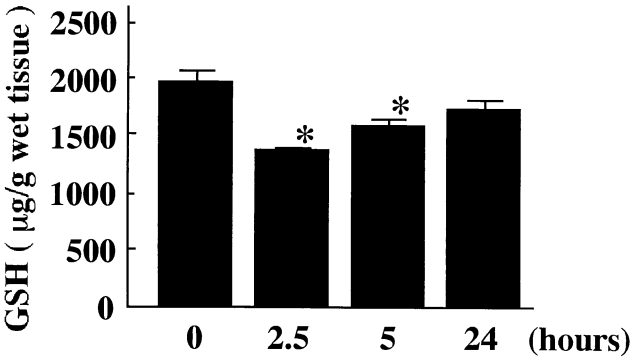

Fig. 3. Changes in the hepatic GSH levels in rat livers after DOX administration. DOX (5 mg/kg body weight) was injected i.v. into rats via the tail vein. At the indicated times, hepatic GSH levels (μg/g wet tissue) were determined in rats as described in "Materials and methods." Data represent the means ± SEM of 5–6 animals per group. Key: (*) $P < 0.05$, normal vs DOX-treated rat livers.

serum) as compared with the control rats (AST, 108 ± 11.2 IU/L serum; ALT, 42 ± 10.8 IU/L serum). When zinc-protoporphyrin IX was administered s.c. to rats 3 hr before DOX injection, the levels of AST and ALT were elevated further (AST, 5023 ± 1058 IU/L serum; ALT, 4000 ± 996 IU/L serum) as compared with the DOX-treated rats. On the other hand, zinc-protoporphyrin IX treatment alone had no effect on the levels of AST and ALT (AST, 156 ± 28 IU/L serum; ALT, 53 ± 1.87 IU/L serum). These results suggest that HO-1 may be protective against DOX-induced hepatic injury.

3.2. Induction of HO-1 protein by DOX administration

It is tempting to speculate that prior induction of HO-1 by pharmacological preconditioning with DOX may protect against the subsequent severe DOX-induced hepatic injury. To test this, a low dose of DOX was administered i.v. to rats via the tail vein in an attempt to reduce hepatic injury. Using western blot analysis, we examined the time course of the induction of the HO-1 protein in rat liver after injection of a low dose of DOX (5 mg/kg body weight). Tissue extracts prepared from the livers at the indicated times were subjected to SDS–PAGE and then were immunoblotted with antibody specific for rat HO-1 or HO-2, respectively (Fig. 2). The HO-1 protein was first detected at 4 hr after the injection of DOX, and the levels gradually increased to 24 hr. In contrast, the levels of HO-2 protein were not affected by the administration of DOX during the experimental period.

To investigate whether the action of DOX is mediated by the generation of ROS, we examined the levels of GSH in rat livers after injection of a low dose of DOX (5 mg/kg body weight) (Fig. 3). At 2.5 hr after the injection, GSH levels were decreased significantly (1363.6 ± 21.7 μg/g wet tissue) compared with those of normal rat livers (1942 ± 92.0 μg/g wet tissue). Similarly, the levels of GSH at 5 hr (1559.7 ± 57.9 μg/g tissue) were reduced significantly compared with the normal rat livers. However, at 24 hr the GSH levels (1715.9 ± 85.0 μg/g tissue) had returned to nearly normal. These results suggested that the induction of HO-1 protein by DOX administration may be caused by oxidative stress.

Next, we examined the expression of HO-1 protein for 3 days following the administration of DOX (5 mg/kg body weight) (Fig. 4). Although the level of HO-1 protein was extremely low 3 days after the injection of DOX, expression was still higher than that in normal rats. On the other hand, the levels of HO-2 were not changed during the experimental period (data not shown).

In addition, we examined the localization in the liver of HO-1 protein induced by administration of a low dose of DOX (5 mg/kg body weight) via the tail vein (Fig. 5). Although there was no remarkable change of the liver observed with routine histological examination, immunohistochemically the livers of the experimental groups showed that histiocytes infiltrating the periportal areas presented intense positivity for HO-1 (Fig. 5, B and C). The control liver did not show such findings (Fig. 5A).

3.3. Effect of DOX preconditioning on subsequent DOX-induced hepatic injury

To examine the effect of pharmacological preconditioning with DOX on the hepatic injury induced by a high dose of this anthracyclin, rats were preconditioned with a low dose of DOX (5 mg/kg body weight; administered i.v. via the tail vein) 3 days before injection of a high dose (10

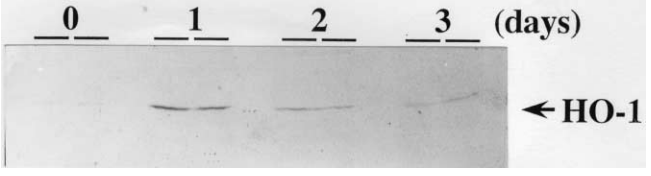

Fig. 4. Time course of the levels of HO-1 protein in rat livers after DOX administration. DOX (5 mg/kg body weight) was injected i.v. into rats via the tail vein. Two samples were analyzed in each group. Each lane (50 μg/lane) represents the tissue extract prepared from the liver of an individual rat. This western blot is representative of three separate experiments.

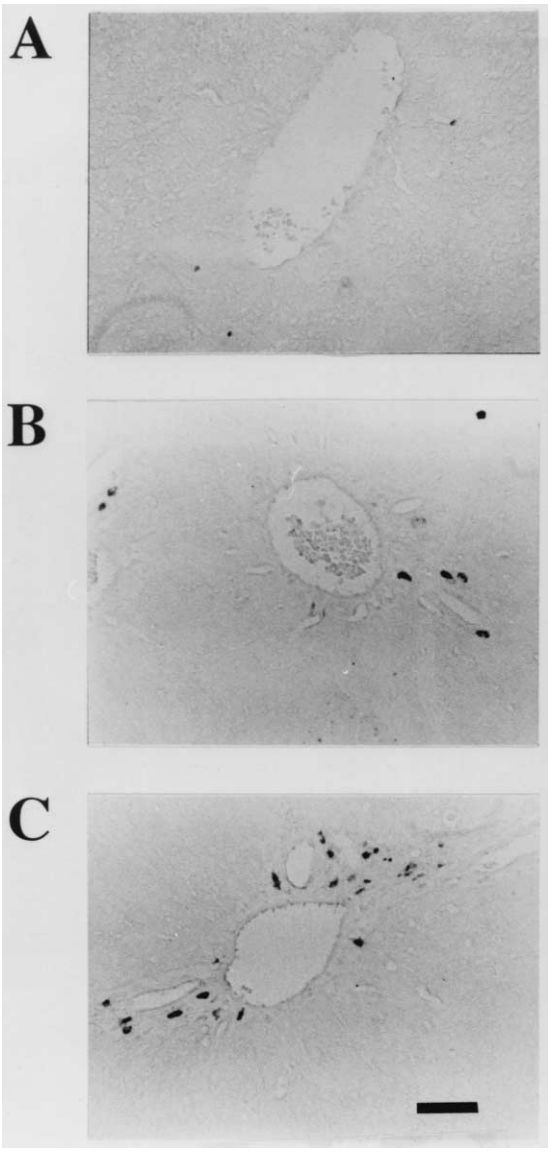

Fig. 5. Immunohistochemical micrographs of HO-1. DOX (5 mg/kg body weight) was injected i.v. into rats via the tail vein. (A) (control group): in the control liver, there were few cells positive for HO-1. (B and C) (experimental groups): (B) 6 hr after injection, and (C) 24 hr after injection. In the experimental livers, there were several histiocytes presenting positivity in the periportal areas. Their number and intensity were more remarkable in (C) than in (B). The bar represents 50 μm.

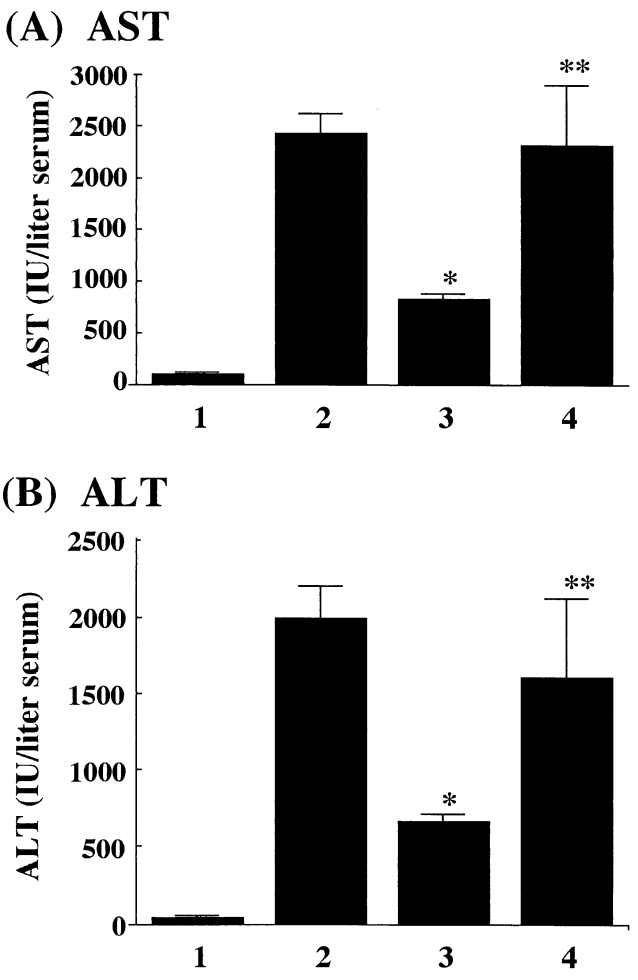

Fig. 6. Effect of preconditioning with DOX on subsequent DOX-induced hepatic injury. Rats were preconditioned with DOX (5 mg/kg body weight, i.v. injection via tail vein) 3 days before injection of a high dose of this anthracyclin (10 mg/kg body weight, i.v. injection via the portal vein). Zinc-protoporphyrin IX (20 μg/kg body weight) was injected s.c. into rats 3 hr before preconditioning with DOX. AST (A) and ALT (B) levels were determined 24 hr after the second DOX injection. Column 1, normal rats; column 2, hepatic injury without preconditioning; column 3, hepatic injury with DOX preconditioning; column 4, zinc-protoporphyrin IX treatment before the DOX preconditioning. Data represent the means ± SEM of 5–6 animals per group. Key: (*) $P < 0.05$, hepatic injury without preconditioning vs hepatic injury with preconditioning; and (**) $P < 0.05$, hepatic injury with preconditioning vs zinc-protoporphyrin IX-pretreated hepatic injury with preconditioning.

mg/kg body weight) was delivered via the portal vein (Fig. 6). Preconditioning with DOX significantly suppressed serum levels of AST and ALT in rats with DOX-induced hepatic injury (AST, 820 ± 63 IU/L serum; ALT, 667 ± 47.3 IU/L serum) compared with the DOX-treated rats without the preconditioning (AST, 2431 ± 190 IU/L serum; ALT, 1978 ± 208 IU/L serum). When zinc-protoporphyrin IX was administered s.c. to rats prior to DOX preconditioning, the effectiveness of DOX preconditioning disappeared (zinc-protoporphyrin IX pretreatment: AST, 2318 ± 585 IU/L serum; ALT, 1603 ± 497 IU/L serum). These results clearly suggest that DOX preconditioning decreases DOX-induced hepatic injury by mediating HO-1 induction. On the other hand, when rats were preconditioned 1 day before exposure to a high dose of DOX, hepatic injury was not alleviated (AST, 2661 ± 451 IU/L serum; ALT, 2648 ± 730 IU/L serum) (data not shown).

In addition, we examined the effect of preconditioning with DOX on the level of hepatic HO-1 protein with DOX-induced hepatic injury (Fig. 7). DOX preconditioning 3 days before a subsequent DOX injection reduced the levels of HO-1 protein induced by a high dose of DOX. This result

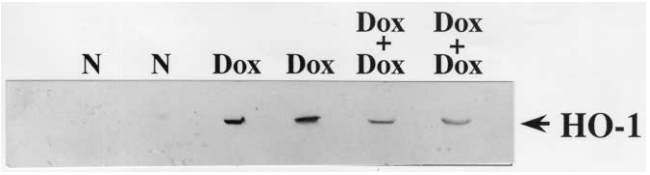

Fig. 7. Effect of preconditioning with DOX on the level of HO-1 protein in rat livers with DOX-induced hepatic injury. Rats were preconditioned with DOX (5 mg/kg body weight, i.v. injection via the tail vein) 3 days before injection of a high dose of this anthracyclin (10 mg/kg body weight, injection via the portal vein). The tissue extracts (50 μg/lane) were isolated from the liver of individual rats at 24 hr after the injection of a high dose of DOX. The levels of HO-1 protein were determined by western blot analysis. Key: (N) normal; (DOX) high dose of DOX injection without preconditioning; and (DOX-DOX) high dose of DOX injection with preconditioning.

suggested that DOX preconditioning lessened the subsequent severe DOX-induced hepatic injury.

4. Discussion

Cells primed by mild oxidative stress induce stress proteins and thereby develop tolerance to the next severe oxidative stress. This phenomenon is known as preconditioning. Cells can be preconditioned by exposure to mild ischemia, heat shock, and low doses of pharmacological agents. Murry *et al.* [1] first reported that preparatory brief ischemia before subsequent long ischemia could protect the heart effectively from ischemia–reperfusion injury. In the liver, ischemic or heat shock preconditioning exerts a protective effect against the next hepatic ischemia–reperfusion injury [2–7,25,30–32]. In addition, heat shock preconditioning protects livers from free radical-mediated injury induced by the administration of carbon tetrachloride [33]. Although little is known about the molecular basis of these preconditioning mechanisms, oxidative stress seems to be one of the causes. Peralta *et al.* [3] recently reported that adenosine exerts a protective role in rat liver ischemic preconditioning by activation of adenosine A_2 receptors and by eliciting an increase in NO generation. In addition, HSP72 protein induced by heat-shock or ischemic preconditioning may also play a critical role in subsequent hepatic injury [6–8,32]. Although both ischemic and hyperthermic preconditioning are effective tools, these maneuvers seem to be clinically complicated methods. We have reported recently that DOX induces the expression of HO-1 and this induced HO-1 plays a protective role against hepatic ischemia–reperfusion injury [15]. HO-1 is known as one of the stress proteins, and its production has been suggested to participate in the defense mechanisms against oxidative stress such as endotoxins, heat shock, ischemia–reperfusion, and heavy metals. Therefore, it seems that the preinduction of HO-1 by various mild oxidative stresses such as preconditioning may play an important role in subsequent oxidative injuries. In addition, Kume *et al.* [34] also reported that DOX administration induces the expression of HSP72 and HSP73 in the liver and that DOX has the potential to provide the liver with tolerance against ischemia–reperfusion injury.

The cardiomyopathy produced by DOX, a widely used anticancer drug, is believed to be related to the production of ROS and the consumption of GSH during redox cycling of the drug. This side-effect of DOX is attributed to the pro-oxidant action of DOX, and its potential usefulness is restricted because of its cardiotoxic side-effect. It was found that when DOX was administered to animals, intracellular GSH content was reduced [35,36]. Our present experiment confirmed these results. In addition, when a high dose of DOX (10 mg/kg body weight) was administered i.v. to rats, lipid peroxidation products in urine increased gradually and peaked 12 hr after injection [37]. In addition, DOX-induced lipid peroxidation in mitochondria and microsomes from rat livers also increased 24 hr after the treatment [36]. HO-1 is well known to be induced by agents that interact with or modify cellular GSH levels [23]. The administration of DOX seemed to induce the HO-1 through a decrease of GSH content and/or free radical generation. In our previous report, DOX (10 mg/kg body weight) administration via the tail vein induced HO-1 in the liver [15]. Together with the previous report, our present experiments clearly show that DOX (5 mg/kg body weight) induces HO-1 protein in the liver. Therefore, administration of a small amount of DOX seems to be an effective agent for pharmacological preconditioning. Pharmacological preconditioning with DOX is a unique and simple method. If it could be used in human beings, this approach would be more easily implemented clinically than either ischemic or heat shock preconditioning. It will be important to extend these studies to determine the optimal dosage and time of preconditioning with DOX in order to reduce the side-effects associated with DOX therapy, e.g. its cardiotoxicity. Moreover, a number of acute phase proteins, antioxidative enzymes, and other HSPs may also be influenced or induced by preconditioning.

In conclusion, liver HO-1 is induced by preconditioning with DOX, and this preconditioning contributes to the amelioration of subsequent DOX-induced hepatic injury. These results seem to allow the speculation that the HO-1 induced by DOX preconditioning plays some role in the acquisition of tolerance for oxidative stress, irrespective of the type of secondary insult, with the associated beneficial effects on protection mechanisms. Therefore, particularly in the field of hepatic surgery, induction of tolerance by pharmacological preconditioning is a promising novel strategy for preventing the critical problems associated with oxidative stress.

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