BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 93–98 (1997)
ARTICLE NO. RC977573

Regulation and Immunohistochemical Analysis of Stress Protein Heme Oxygenase-1 in Rat Kidney with Myoglobinuric Acute Renal Failure

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Received September 21, 1997

Intramuscular injection of hypertonic glycerol solution to rats results in acute renal injury. In this model, the proximal tubules are characteristically damaged. After glycerol injection renal glutathione (GSH) levels drastically decreased. On the other hand, stress protein heme oxygenase-1 (HO-1) was induced. When N-acetyl cysteine was administered to rats before 1 h glycerol injection, renal function was obviously improved. In this condition, the renal GSH content were sustained in the normal levels and HO-1 protein levels were decreased compared with those of glycerol-treated rats. Induction of HO-1 was accompanied by reduced renal GSH content. In addition, to investigate whether the location of HO-1 protein induced by glycerol injection is restricted to injured region or not in the kidney, we determined the localization of HO-1 protein using immunohistochemical staining. HO-1 protein was identified in the epithelia of the distal tubules, Henle's loop and collecting ducts, but not in the injured proximal tubules.

Glycerol-induced acute renal failure (glycerol-ARF) in rats is a model of acute trauma in which intramuscular injection of hypertonic glycerol solution causes rapid myoglobinuria, oliguria, and a rapid reduction in glomerular filtration rate. Organ damage may arise when heme proteins, such as myoglobin in muscle or hemoglobin in erythrocytes, escape from the intracellular space. Such hememediated organ injury underlies the rhabdomyolysis, a disease instigated by muscle necrosis and dominated by renal failure. Recently, a role of reactive oxygen metabolites in glycerol-ARF has been suggested based on the protective effect of hydroxyl radical scavengers (1) and deferoxamine (1,2), which chelates iron that participates in hydroxyl radical formation.

Heme oxygenase (HO) (EC.1.14.99.3) is the enzyme involved in the rate-limiting degradation of heme, a most effective prooxidant (3,4), to biliverdin and carbon monoxide (CO), the former being subsequently converted to bilirubin by biliverdin reductase (5). Two HO isozymes, products of two separate genes are known (6). One is a inducible form (HO-1), the other is a constitutive form (HO-2). The promotor for rat HO-1 contains a heat-shock consensus element (7), NF-κB(8), and AP-1(9) binding sites, thus triggering HO-1 induction by inflammatory stimuli, including cytokines, oxidative stress and heat shock. In addition, HO-1 is induced by agents which are known to interact with or modify cellular glutathione (GSH) (10). GSH exists in high concentrations in almost all mammalian cells and is the most prevalent intracellular thiol (11). GSH has many diverse functions, one of which is the protection of tissues against oxidative damage. The importance of GSH in protecting cells against oxidative injury has been delineated in numerous *in vivo* studies and depletion of GSH results in markedly enhanced toxicity (12,13). Due to the high expression of HO-1 in response to oxidative stress-related agents, it has been suggested that HO-1 acts as an intracellular protective system against oxidative stress (14).

In this report, we have examined the regulation of HO-1 expression by GSH in the kidney with glycerol-ARF. To our knowledge we have first shown that the

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0006-291X/97 $25.00

localization of HO-1 is restricted to the distal tubules, Henle's loop and collecting ducts in the kidney with glycerol-ARF. Our findings suggest that the induction of HO-1 in these sites may be closely correlated with the protection against glycerol-ARF occurred in the proximal tubules.

MATERIALS AND METHODS

Glycerol-ARF. Male Wistar rats, weighing 100–150 g, were deprived of water for 24 h with free access to rat chow before glycerol injection. Under ether anesthesia, rats were injected intramuscularly with 10 ml/kg of 50% glycerol solution distributed equally in both hind limbs. Control group received saline only. In some protocols, N-acetyl cysteine (NAC) (St. Louis, MO, USA) was administered orally by gavage at a dose of 1, 2, and 4 g/kg body weight before 1 h glycerol injection. NAC was dissolved in water in aid of 0.1N NaOH. Control rats were received the same volumes of vehicle solution. At the indicated times, rats were anesthetized with ether and sacrificed by the collection of blood from the heart. All animal experiments were performed in strict accordance with the institution's criteria for the care and use of laboratory animals as approved by an institutional governing committee.

Biochemical analyses. Total renal GSH contents were determined by the method of Tietze (15). Protein concentration was determined by the method of Bradford (16).

Western blot analysis. Tissue extracts from kidneys were prepared as follows. Kidneys were homogenized in 10 volumes of 20 mM Hepes (pH 7.5), 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM orthovanadate, 1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and centrifuged at 8,000 $\times$ g for 10 min at 4°C, and the supernatants were used as tissue extracts. The extracts (200 μg protein/sample) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (17) and electrophoretically blotted onto nitrocellulose filters. Western blot analysis was performed using antibody to rat HO-1 (StressGen, Victoria, Canada) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendation. The immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride.

Histological examination. The organs including the kidney which were sampled from the control and experimental group rats were fixed with 10% formalin, dehydrated and embedded in paraffin for histopathological examination. Paraffin sections were stained with hematoxylin and eosin staining.

Immunohistochemical analysis. For immunohistochemistry of HO-1, the sections were deparaffinized, dehydrated, and immersed in hydrogen peroxide containing methanol to inactivate intrinsic peroxidase. The sections were then boiled in 10 mM citrate buffer (pH 6.0) with a microwave for 5 min to retrieve the antigen (18), and then incubated with antibody to rat HO-1 at 37°C for 1 h, following treatment with 10% normal goat serum-containing PBS. The labeled antigen was visualized with streptavidin-biotin complex method (HistoFine kit, Nichirei, Tokyo, Japan) and diaminobenzidine reaction. The sections were counterstained with methylgreen. The histiocytes in the spleen was employed as the positive control.

Statistics. Significance of experimental results between animals was evaluated using the unpaired Student's *t*-test. For comparisons involving more than two groups, ANOVA was applied. The differences were considered statistically significant when the P value was less than 0.05.

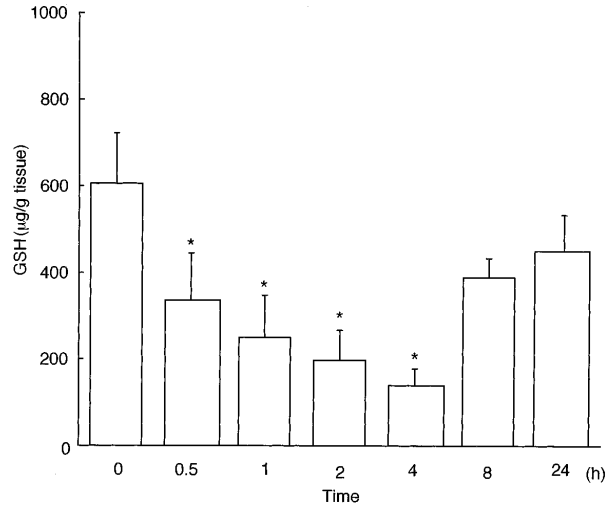

FIG. 1. Effect of glycerol injection on total renal GSH levels. Rats were sacrificed at 0.5, 1, 2, 4, 8 and 24 h after 50 % glycerol injection (10 ml/kg body weight) intramuscularly. Values were expressed as mean ± SEM (n=6)(*$p<0.05$). As control rats were injected with 0.9 % saline (n=6) at time 0.

RESULTS

Cellular GSH Levels

After glycerol injection, renal GSH levels promptly decreased and reached a minimum at least by 4 h (control rats 605 ± 116.2 vs. glycerol-injected rats 139.6 ± 37.5 μg/g tissue, $p<0.05$), and then returned to about two-thirds of control level after 24 h (control rats 608±109.8 vs. glycerol-injected rats 449.3±84.9 μg/g tissue, $p>0.05$) (Figure 1). As markers for renal function both serum creatinine and blood urea nitrogen levels also increased in glycerol-injected rats compared with control rats (data not shown).

Induction of HO-1 in Glycerol-Injected Rat Kidneys

To examine whether the intramuscular glycerol injection induces renal HO-1 or not, we first determined the time course of the levels of the protein using immunoblot analysis (Figure 2). Using a specific antibody to rat HO-1, we detected a band of 32 kDa protein (corresponding to rat HO-1 protein). HO-1 protein was not detected in rat kidneys from control, 0, 1 and 2 h after glycerol injection. Whereas, after 4 h of the treatment HO-1 protein was faintly detected, then gradually increased, and the levels reached a maximum at about 8 to 12 h after glycerol injection. At 48 h after the treatment, HO-1 protein levels decreased about one fourth or fifth compared with the maximum level (data not shown).

Renal GSH and HO-1 Protein Levels in NAC-Treated Rats with Glycerol-Induced ARF

To examine the effect of NAC on renal GSH levels in glycerol-injected rats, rats were pretreated with NAC

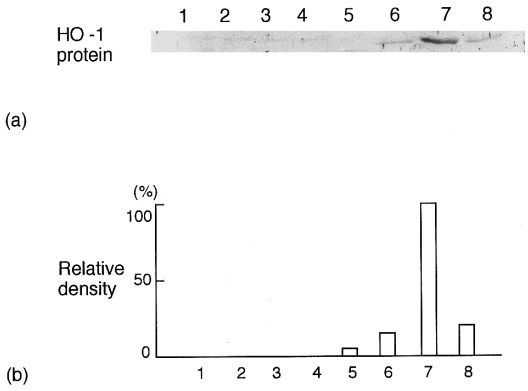

FIG. 2. Time course of HO-1 protein levels. (a) Tissue extracts (200 μg protein/lane) from rat kidneys at the indicated times after glycerol injection were separated by SDS-PAGE and then analyzed by immunoblot analysis using specific antibody to rat HO-1. Lane 1, normal; lane 2, 0 h; lane 3, 2 h; lane 4, 4 h; lane 5, 8 h; lane 6, 12 h; lane 7, 16 h; lane 8, 24 h. HO-1 denotes HO-1. (b) Relative amounts of HO-1 protein. Densitometric scanning was performed using the data as shown in (a). A relative amount was expressed as compared with a maximum level.

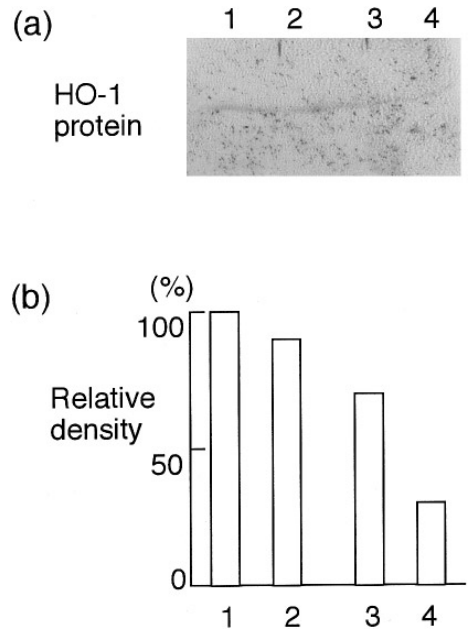

FIG. 4. Effect of NAC on the levels of HO-1 protein in the kidney of glycerol-treated rats. (a) The immunoblot analysis of tissue extracts (200 μg protein/lane) from kidneys of glycerol-injected rats (50% glycerol 10 ml/kg body weight, intramuscularly) and of NAC-pretreated and glycerol-treated rats as described in Materials and Methods. Control rats were received vehicle solution orally 1 h before glycerol injection. Lane 1, control; lane 2, NAC 1 g/kg; lane 3, NAC 2 g/kg; lane 4, NAC 4 g/kg. (b) Relative amounts of HO-1 protein levels. Densitometric scanning was performed using the data as shown in (a). A relative amount was expressed as compared with maximum level.

before 1 h glycerol injection. Figure 3 shows that NAC prevents the decrease of renal GSH contents at 4 h compared with those of glycerol-injected rats (NAC-treated rats 360.9±23.2 vs. glycerol-injected rats 150.0±33.4 μg/g tissue, $p < 0.05$). Serum creatinine levels at 24 h after glycerol injection in NAC-treated rats were improved compared with glycerol-injected rats (NAC-treated rats 3.4±0.08 vs. glycerol-injected rats 4.5±0.14 mg/dl, $p<0.05$).

In addition, we have examined the effect of NAC on the levels of HO-1 protein in the kidney after glycerol injection. When NAC (1, 2, and 4 g/kg body weight) was administered to the rats before 1 h glycerol injection, HO-1 protein levels at 24 h were reduced in NAC-treated rats compared with glycerol-treated rats. Figure 4 shows that the reduction of HO-1 protein is depend on the dose of NAC.

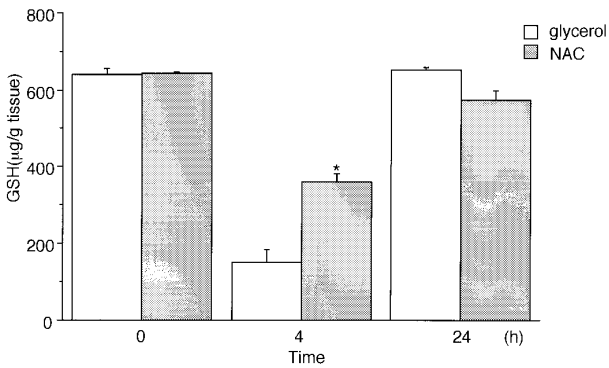

FIG. 3. Effect of NAC on renal GSH levels in glycerol-ARF. Rats were sacrificed at 0, 4, 24 h after 50 % glycerol injection (10 ml/kg body weight) intramuscularly. Some of them were treated with NAC (4 g/kg body weight, orally) at 1 h before glycerol injection. Values were expressed as mean ± SEM (n=9)(*$p<0.05$).

Histopathological Analysis

The kidneys obtained from glycerol-injected rats showed marked swelling and were dark red in color. Histologically, 1 h after glycerol injection, the proximal tubules especially in the corticomedullary junction began to be dilated with heme casts. At 2 h after the injection, the casts became obvious in the collecting ducts. Then retention of heme casts was obvious in the entire nephron at 2–4 h after the injection. At 8 h after the injection, the proximal tubules began to dilated (Figure 5a). Heme casts were uptaken to the proximal tubular epithelia to show intracytoplasmic hyaline globular bodies. After 12 h, the number of casts decreased and dilatation of the proximal tubules seemed to become milder. Immunohistochemical staining showed that immunoreactivity for HO-1 in the macrophages and histiocytes scattered in the various organs (data not shown). The normal kidney failed to show obvious positivity (Figure 5b-1). At 8 h after the injection, the distal tubules showed obvious intracytoplasmic positivity for heme oxygenase-1, but not in the

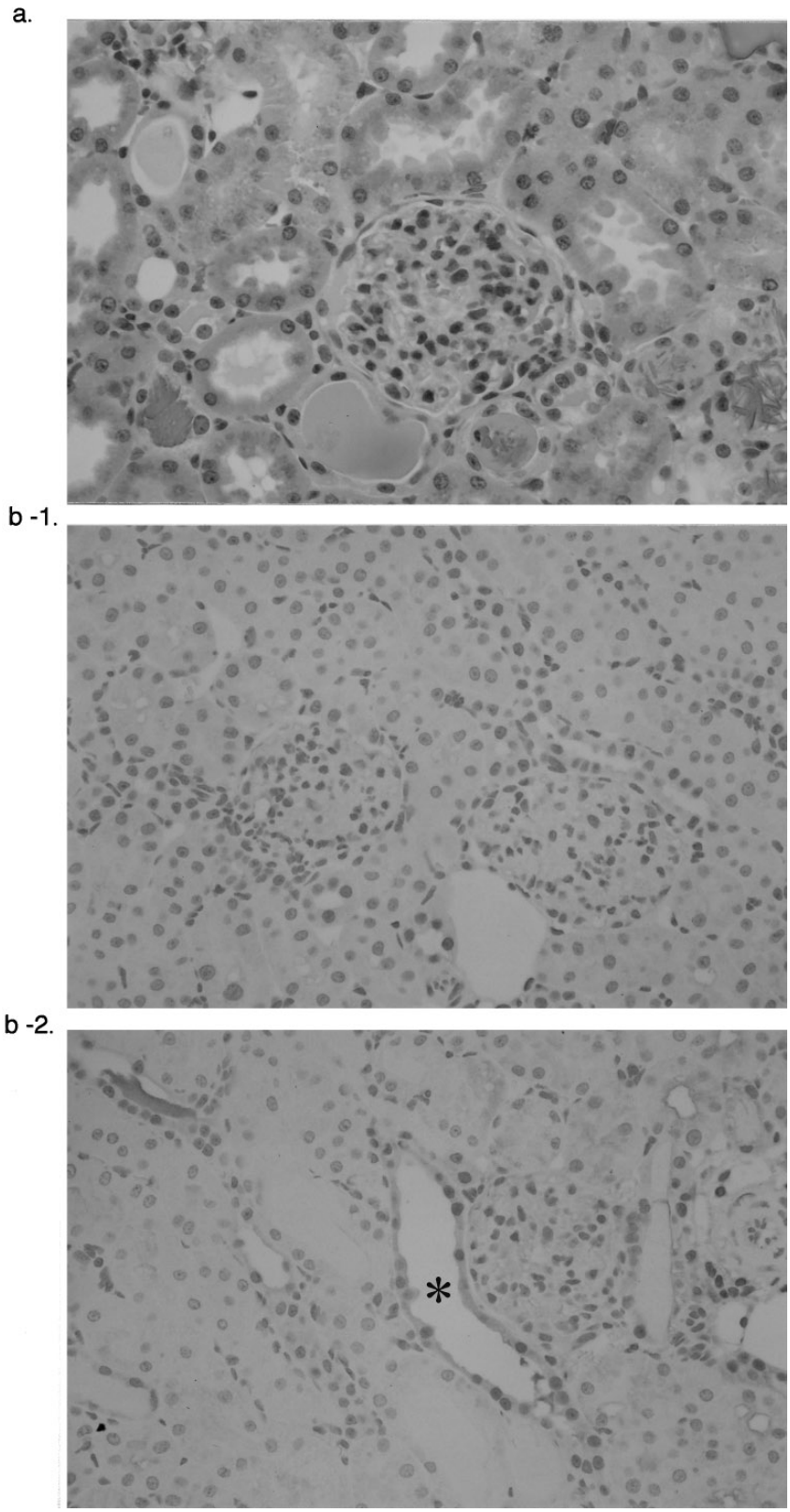

FIG. 5. (a) Light micrograph of the rat kidney. At 8 h after glycerol injection, the proximal tubules were markedly dilated and contained numerous heme casts. Note the relatively intact distal tubules and lower nephron (Hematoxylin-Eosin staining, original magnification; ×100). (b) Immunohistomicrograph for HO-1. (b-1) In the control rat kidney, there was no obvious immunoreactivity for HO-1 (original magnification; ×100). (b-2) The rat kidney of 8 h after glycerol injection showed immunoreactivity in the distal tubules (original magnification; ×100). Asterisk (*) denotes the distal tubules.

proximal tubules (Figure 5b-2). In addition, the positive staining was also observed in Henle's loop and collecting ducts in glycerol-treated rat kidneys.

DISCUSSION

The intramuscular injection of hypertonic glycerol causes regional myolysis and hemolysis, leading to acute tubular injury through the contraction of renal vessels (19). Glycerol-ARF in rats is a well established model for acute trauma and recognized as a model of rhabdomyolysis. At present, many explanations have been reported about the pathogenic mechanism of this model. Oxidative stress is believed to be related to glycerol-ARF and the generation of hydroxyl radicals induced by released myoglobin in the kidney may be associated with this injury (1). In our study, many proximal tubules became necrotic and distal nephron segments were stuffed by casts containing heme pigments. Heme proteins directly constrict renal vessels by scavenging NO (14), and iron from heme proteins promotes free radical formation (9). Depletion of cellular GSH also increase the cytotoxic effects of H_2O_2, implicating the GSH-oxidized glutathione (GSSG) cycle in protecting cells against H_2O_2-induced toxicity (10). Our results showed that renal GSH contents abruptly decreased with time till 4 h after glycerol injection, then gradually returned to the control level. These findings strongly suggest that oxidative stress is one of the causes of glycerol-ARF and GSH contents may be correlated with defence system against the injury of glycerol-ARF.

HO-1 activity was induced by its substrate heme or heme analogues, and by a variety of chemical agents and physical types of stress, for example H_2O_2 (8), and the depletion of GSH (10). NAC might act as a direct scavenging agent (20). It sustained GSH synthesis and reduced lipopolysaccharide-induced HO-1 mRNA accumulation (20). Our results indicated that the GSH levels affected renal function and HO-1 protein induction in glycerol-ARF. Previously, Nath et al. showed that HO-1 mRNA was induced in the kidney by intramuscular glycerol injection and had a protective ability for glycerol-ARF (21). Recently, Vogt et al. reported that nephrotoxic serum, which caused glomerular inflammation, was administered intravenously to rats induced HO-1 protein expression in the renal tubules where were not injured region (22). Therefore, to examine whether HO-1 expression induced by intramuscular injection of hypertonic glycerol is localized in injured region (proximal tubules) or not, we have determined the location of HO-1 protein using immunohistochemical analysis. In our histological analysis, the proximal tubules are the most damaged region in the kidney with glycerol-ARF. Our results show that the localization of HO-1 protein is obviously restricted to the distal tubules, Henle's loop and collecting ducts. Morphologically, this experimental model for ARF seemed to recover since 8 h after injection because of decrease of heme casts and of degrees of tubular dilatation.

Proximal tubule cells possess epidermal growth factor (EGF) receptors and EGF is a mitogen to proximal tubular cells *in vitro* (23). In addition, Humes et al. reported that exogenously administered EGF enhanced renal tubular cell regeneration and repair and accelerated the recovery of renal function in postischemic ARF (24). In fact, EGF is synthesized in Henle's loop and distal tubules (25). It is supposed that EGF synthesized in these regions seems to play an important role to promote the recovery of the injured proximal tubules. Similarly, in addition to protective function, HO-1 may be considered to be an important enzyme for recovering from ARF via intoxication and catabolism of heme. Therefore, we need further investigation to clarify the function of HO-1 in the distal nephron in the pathological condition of glycerol-ARF.

ACKNOWLEDGMENT

We thank Dr. Shibahara (Tohoku Univ. Japan) for kind gift of rat HO-1 cDNA plasmid.

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