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Effects of Vitamin E Deficiency and Glutathione Depletion on Stress Protein Heme Oxygenase 1 mRNA Expression in Rat Liver and Kidney

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ABSTRACT. Heme oxygenase 1 (HO-1) is a stress protein and has been suggested to provide defense mechanisms against agents that may induce oxidative injury. Vitamin E (VE) is considered to function as an important cellular antioxidant. Rats were fed a VE-deficient (0E) or a VE-sufficient (10E) diet for 6 weeks and then were intraperitoneally administered buthionine sulfoximine (BSO), a glutathione (GSH)-depleting reagent. Whereas HO-1 mRNA levels were undetectable in untreated 0E and 10E rat livers, BSO administration induced HO-1 mRNA expression in both 0E and 10E rat livers. High levels of HO-1 mRNA expression were observed in particular in BSO-treated 0E rat livers. The time–course of changes in HO-1 mRNA expression in 0E rat liver after BSO administration showed that HO-1 mRNA expression was transiently induced at 2.5 hr after BSO treatment, the earliest time examined. In addition, to determine whether VE deficiency and GSH depletion affect the expression of HO-1 mRNA in other tissues, we also examined the time–course of HO-1 mRNA expression in BSO-treated 0E rat kidney. The expression pattern of HO-1 mRNA in the kidney was very similar to that in the liver, and the peak was also observed at about 2.5 hr after BSO administration. Interestingly, histologic assessment of liver and kidney showed that VE deficiency and GSH depletion induced injury in the kidney, but not in the liver. BIOCHEM PHARMACOL **54**;10:1081–1086, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. vitamin E; glutathione; heme oxygenase 1; stress protein; antioxidant.

HO¶ (EC 1.14.99.3), the rate-limiting enzyme in heme catabolism, oxidatively degradates heme into biliverdin, iron, and carbon monoxide [1, 2]. Two HO isozymes, products of two separate genes, are known [3, 4]. One is the inducible form (HO-1), which is distributed ubiquitously in mammalian tissues. The other is the constitutive form (HO-2), which is present in high concentrations in such tissues as the brain and testis and is believed to be noninducible. HO-1 is considered to be a stress protein and activated by oxidative stress in a wide variety of mammalian cells [5]; this induction may represent a protective response to oxidative stress [6]. In addition, HO-1 is induced by agents that are known to interact with or modify cellular GSH levels [5]. It is also known that BSO, an inhibitor of γ-glutamylcysteine synthetase which is the rate-limiting enzyme in GSH biosynthesis, induces HO-1 enzyme activity, HO-1 protein, and HO-1 mRNA in cultured liver cells [7–9] and in rat brain [10] and rat liver [11].

VE is considered to function as an important cellular antioxidant *in vivo* and it inhibits *in vitro* lipid peroxidation in a variety of membrane systems [12–14]. Several lines of evidence suggest that VE exerts its antioxidant activity by scavenging free radicals generated in lipid peroxidation [15]. It was reported that the content of α-tocopherol, which is a component of VE, in liver tissue decreases during hepatic ischemia and subsequent reperfusion, which suggests a consumption of this antioxidant by scavenging fatty peroxyl radical in biomembranes, including the mitochondrial membrane [16, 17]. In addition, it has been reported that α-tocopherol is effective in lessening acute pathologic changes observed in mouse and rat hearts [18]. In addition to the findings of VE, added GSH can inhibit microsomal lipid peroxidation and enhance the ability of VE to protect against lipid peroxidation. VE deficiency and GSH deficiency both affect the toxicity of oxidants in various tissues. Therefore, it is important to clarify the relationships among antioxidant stress protein HO-1, VE, and GSH.

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¶ *Abbreviations:* HO, heme oxygenase; GSH, glutathione; BSO, buthionine sulfoximine; VE, vitamin E; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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In this paper, we show that a combination of GSH and VE strongly regulated HO-1 mRNA expression in both the liver and kidney. These findings indicate that HO-1 may have protective functions in cells when defense mechanisms against oxidants are challenged in various tissues. However, histological analysis showed that both VE deficiency and GSH depletion induced renal injury, but not hepatic injury. At present, it still remains unknown why acute renal injury is induced only by this treatment.

MATERIALS AND METHODS

Materials

GSH and BSO were obtained from Sigma (St. Louis, MO, U.S.A.). [α-^{32}P]dCTP and Hybond-N nylon membrane were from Amersham (Arlington Heights, IL, U.S.A.). DTNB was from Wako Chemicals (Osaka, Japan). All other reagents were of analytical grade. Commercial safflower oil treated with activated charcoal powder was used as VE-deficient oil. A mixture of vitamins except for VE was prepared based on the composition of the vitamin mixture (AIN-76). The basal diet consisted of 20% casein, 50% sucrose, 5% cellulose powder, 3.5% mineral mixture (AIN-76), 1% vitamin mix, 0.2% sodium potassium bitartrate choline, 10.3% corn starch and 10% vitamin E-deficient safflower oil. Fatty acids of safflower oil consisted of C14:0, 1.5%; C16:0, 7.2%; C16:1, ND (not determined); C18:0, 2.6%; C18:1, 12.2%; C18:2, 76.5%; and C18:3, ND.

Animal Experiments

Four-week-old Wistar male rats (Clea, Japan) were fed a VE-deficient (0E) or a VE-sufficient (10E) diet for 6 weeks and then were administered, by intraperitoneal injection, BSO solution (0.2 mmol/mL) dissolved in 0.9% NaCl at 1 mmol/kg body weight. Control rats received the vehicle only. Fresh diets were given every day and water was given *ad lib*. Three animals per group were placed in a metabolic cage. The animals were kept in a temperature-controlled room with 12 hr alternating light and dark cycles. For a time–course experiment of BSO administration to 0E rats, the animals were anesthetized with ether, and killed by the collection of blood from the hearts at indicated times. The livers and kidneys were quickly removed, frozen in liquid nitrogen, and stored at $-80°$. For the histological analysis, each tissue was excised and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4–5 μm, and stained with hematoxylin and eosin for microscopic examination.

Biochemical Analyses

Total GSH (reduced and oxidized forms) concentration was measured with the GSH reductase–DTNB recirculating assay by the method of Tietze [19]. The tissue (0.2 g) was homogenized in 5 mL of 0.1 M phosphate buffer (pH 7.5) at 0° for 1 min 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. α-Tocopherol concentration was determined by an HPLC method [20]. Protein concentration was determined by the method of Lowry *et al.* [21].

Northern Blots

Total RNA was extracted according to Chomczynski and Sacchi [22]. RNA (20 μg/lane) was size-separated in 2.2 M formaldehyde-1% agarose gels and transferred onto nylon membranes as described [23]. Hybridization was carried out with a random primed cDNA probe labeled with [α-^{32}P]dCTP (3000 Ci/mmol) to a specific activity of 1–2 $\times$ 10^9 cpm/μg DNA. The rat HO-1 cDNA probe (approximately 0.9 kb pair fragment) was excised from rat HO-1 cDNA plasmid (pRHO1) with *Eco*RI and *Hin*dIII [24]. Membrane blots were prehybridized at 42° for 1 hr in 5$\times$ SSC (1$\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) solution containing 50% formamide, 5$\times$ Denhardt's solution (1$\times$ Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50 mM sodium phosphate (pH 7.0), denatured salmon sperm DNA (0.2 mg/mL) and then were hybridized at 42° overnight in the same solution containing radiolabeled probe. Washing was performed sequentially as follows: 2$\times$ SSC and 0.1% SDS solution twice at room temperature for 15 min, 1$\times$ SSC and 0.1% SDS solution twice at 65° for 30 min, and finally 0.3$\times$ SSC and 0.1% SDS solution twice at 65° for 30 min. After washing, the blots were air dried and exposed to X-ray films at $-80°$ with intensifying screens. Quantitative variabilities of isolation and transfer of RNAs were accounted for by reprobing the same filter with a cDNA probe for human GAPDH cDNA.

Statistical Analysis

Statistical significance was examined at the 0.05 level using Student's *t*-test.

RESULTS

We examined the effect of BSO on GSH and α-tocopherol contents in VE-deficient (0E) and VE-sufficient (10E) rat livers at 5 hr after BSO treatment. Table 1 shows that the α-tocopherol content in the livers of the 0E group was lower than that of the 10E group, but no differences in α-tocopherol contents were observed between the 0E and the BSO-treated 0E (0E-BSO) groups. Total GSH contents in the livers of the BSO-treated groups (0E-BSO and 10E-BSO) decreased as compared with those of the BSO-untreated groups (0E and 10E). Furthermore, total GSH

TABLE 1. Effect of BSO on contents of α-tocopherol and total GSH in vitamin E-deficient (0E) and vitamin E-sufficient (10E) rat livers

	α-Tocopherol (ng/mg protein)		Total GSH (μg/g wet tissue)	
BSO treatment	0E	10E	0E	10E
With	3.81 ± 0.97*	253.61 ± 51.71	128.4 ± 198.9*†	451.7 ± 185.4†
Without	4.79 ± 1.99*	278.15 ± 32.74	1490.8 ± 198.2*	1432.0 ± 96.2

Contents of α-tocopherol and total GSH were determined in livers of rats treated with or without BSO at 5 hr after treatment. Vitamin E is expressed in International Units. Values are means ± SD for five rats.

* Significant difference ($P < 0.05$) between the same treatment of 0E and 10E rat groups.

† Significant difference ($P < 0.05$) between the different treatments within the same vitamin E group.

content in the livers of the 0E-BSO group decreased to one-third of that of the 10E-BSO group (Table 1).

Because hepatic GSH levels decreased to near minimum level at 5 hr after the treatment as shown in Table 1, we examined the steady-state levels of antioxidant stress protein heme oxygenase-1 (HO-1) mRNA in rat livers (Fig. 1) at that time. Although HO-1 mRNA was not detectable in either the 0E group or the 10E group rat livers, BSO treatment induced HO-1 mRNA expression in these two groups (0E-BSO and 10E-BSO). In particular, higher levels of HO-1 mRNA (approximately 12-fold) were observed in the 0E-BSO group compared with the 10E-BSO group. These results show that a combination of VE deficiency and GSH depletion strongly activates the expression of the gene for HO-1 in the liver.

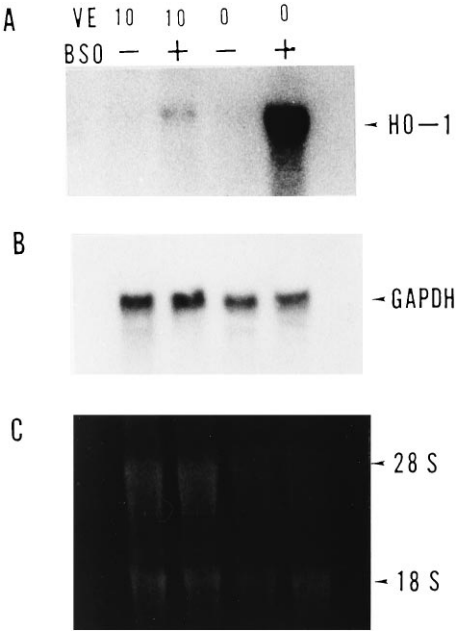

FIG. 1. Effect of BSO on HO-1 mRNA expression in vitamin E-deficient and vitamin E-sufficient rat livers. Total RNAs were extracted from rat livers at 5 hr after BSO treatment. RNAs (20 μg/lane) from vitamin E-deficient rats treated with (VE 0, BSO +) or without (VE 0, BSO −) BSO and vitamin E-sufficient rats treated with (VE 10, BSO +) or without (VE 10, BSO −) BSO were subjected to RNA blot analysis. (A) Northern blot analysis using rat HO-1 cDNA probe. (B) Northern blot analysis using human GAPDH cDNA probe. (C) Ethidium bromide staining pattern of RNA samples in agarose gel used in A and B.

To investigate further the effect of BSO on HO-1 mRNA expression in 0E rat liver, we examined a time–course of HO-1 mRNA expression after BSO treatment. As shown in Fig. 2, HO-1 mRNA expression was induced strongly at 2.5 hr, the earliest time examined, after BSO administration and then declined abruptly to an undetectable level. As shown in Fig. 2, the levels of HO-1 mRNA expression were extremely low at 5 hr after BSO treatment. Therefore, we needed a long exposure to obtain clear autoradiograms, as shown in Fig. 1.

To determine whether VE deficiency and GSH depletion induce the expression of HO-1 mRNA in other tissues, we examined a time–course of steady-state levels of HO-1 mRNA in VE-deficient rat kidneys (0E) after BSO administration (Fig. 3). The HO-1 mRNA expression was transiently induced at 2.5 hr after the treatment and dropped abruptly by 5 hr; then the levels became undetectable. This pattern was very similar to that of the liver. However, it is well known that BSO treatment induces HO-1 mRNA in

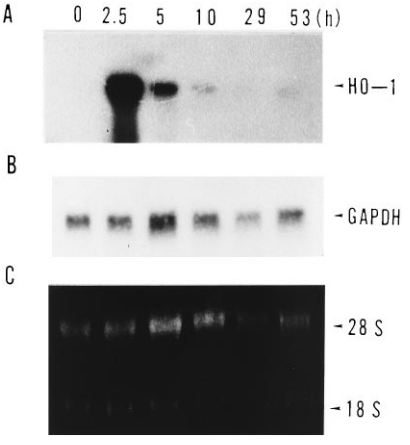

FIG. 2. Time–course of changes in HO-1 mRNA expression in livers of vitamin E-deficient rats treated with BSO. Total RNAs (20 μg/lane) from vitamin E-deficient rat liver (0 hr), 2.5, 5, 10, 29, and 53 hr after BSO administration were subjected to RNA blot analysis. (A) Northern blot analysis using rat HO-1 cDNA probe. (B) Northern blot analysis using human GAPDH cDNA probe. (C) Ethidium bromide staining pattern of RNA samples in agarose gel used in A and B.

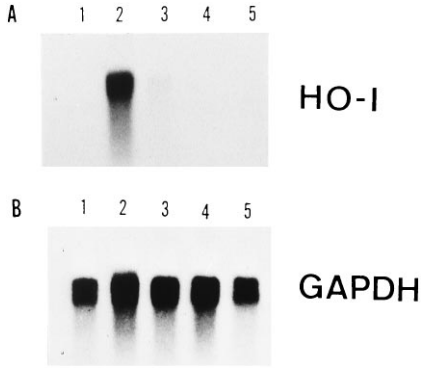

FIG. 3. Time–course of changes in HO-1 mRNA expression in kidneys of VE-deficient rats treated with BSO. Total RNAs (20 μg/lane) from VE-deficient rat kidney (0 hr), 2.5, 5, 10, and 29 hr after BSO administration were subjected to RNA blot analysis. (A) Northern blot analysis using rat HO-1 cDNA probe. (B) Northern blot analysis using human GAPDH cDNA probe. Lane 1, 0 hr; lane 2, 2.5 hr; lane 3, 5 hr; lane 4, 10 hr; and lane 5, 29 hr.

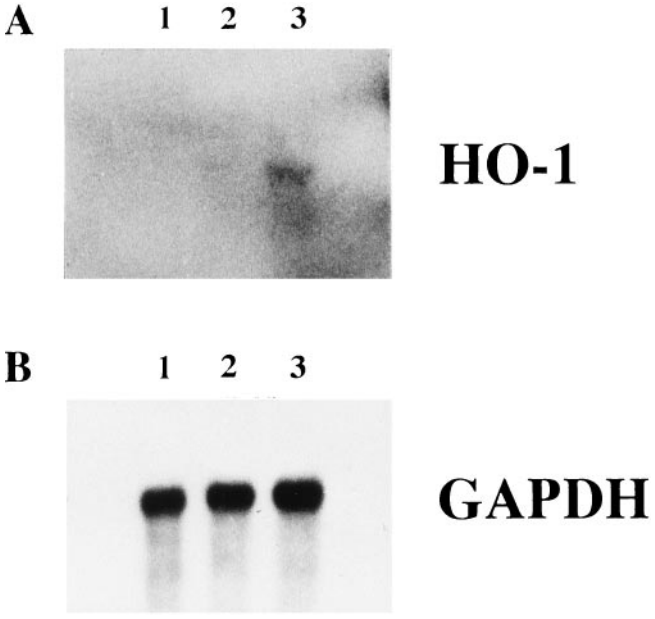

FIG. 4. Expression of HO-1 mRNA in rat kidneys after BSO treatment. Total RNAs (20 μg/lane) from rat kidneys at 0, 2.5, and 5 hr after BSO administration were subjected to RNA blot analysis. (A) Northern blot analysis using rat HO-1 cDNA probe. (B) Northern blot analysis using human GAPDH cDNA probe. Lane 1, 0 hr; lane 2, 2.5 hr; and lane 3, 5 hr.

various tissues. Therefore, to examine whether or not this induction of HO-1 in the kidney is due only to the BSO treatment, we determined the levels of HO-1 mRNA expression after BSO treatment (Fig. 4). At 2.5 hr after the treatment, the band of HO-1 mRNA was not detectable and a minor amount of HO-1 mRNA was only observed at 5 hr after a long exposure of the X-ray film. Therefore, the induction of HO-1 mRNA observed in VE-deficient and BSO-treated rat kidneys was caused by these treatments. In addition, we determined the changes in the contents of GSH and α-tocopherol in 0E rat kidneys after BSO administration (Table 2). The content of α-tocopherol in vitamin E-deficient rat kidneys decreased to one-twenty-fifth of the control value, whereas the content of kidney GSH was not affected by the VE-deficient diet after 6 weeks of feeding. However, BSO administration abruptly decreased the GSH content to approximately one-fifth at 5 hr after BSO treatment, and then the GSH content gradually decreased with time. At 24 hr after BSO administration, GSH content in kidney reached approximately one-tenth of the control value.

Figure 5 shows the light microscopic photographs of the kidneys of VE-deficient rats after BSO administration. In contrast to the VE-deficient rat kidney (Fig. 5C), the injury was observed exclusively in proximal tubules as early as 2.5 hr after BSO administration (Fig. 5D). Furthermore, extensive necrosis, desquamation, and hyalinization were observed in proximal tubule cells at 5 and 24 hr after BSO treatment (Fig. 5E and F, respectively). Of course, when BSO was administered to VE-sufficient rat kidneys, renal injury was not observed at 5 hr after the treatment (Fig. 5B). On the contrary, hepatic injury was not observed in either VE-deficient or BSO-treated rats (data not shown). These results show that a combination of VE deficiency and GSH depletion is needed to induce acute renal injury.

DISCUSSION

BSO is a specific inhibitor of GSH synthesis and is useful to investigate the cellular function of GSH. Several reports have indicated that BSO administration induces the expression of HO-1 mRNA in human fibroblasts [9], newborn rat brain [10], and mouse liver [11] via GSH depletion. In addition to GSH, vitamin E is also considered to be an important cellular antioxidant [12–15]. In this experiment, our results show that the expression of HO-1 mRNA was induced by GSH depletion, but was not induced by VE deficiency alone. Unexpectedly, both GSH depletion and VE deficiency strongly induced the expression of HO-1 mRNA in the liver and kidney. Therefore, both VE and GSH may play an important role as cellular antioxidants in the liver and kidney. It is suggested that HO-1 may have protective functions *in vivo* when defense mechanisms against oxidants are challenged. An understanding of the way in which VE and GSH protect against oxidative stress may shed light on the regulation of cellular defense mechanisms. GSH depletion in various cells has been observed to increase susceptibility to oxidative and chemical stress [25, 26]. Similarly, lipid peroxidation has been implicated as a major process in cellular damage [27, 28]. Reduced GSH is considered to be a potent inhibitor of lipid peroxidation *in vivo*. In addition, McCoy *et al.* [29] reported that the renal injury in reperfusion after ischemia was caused by the hydrogen peroxide produced and this injury was closely related to the GSH content in the kidney. These reports indicate that the maintenance of the GSH content is important for the prevention of oxidative dam-

TABLE 2. Effect of BSO on contents of α-tocopherol and total GSH in vitamin E-deficient and vitamin E-sufficient rat kidneys

Time after BSO administration (hr)	VE	BSO	Total GSH (μg/g wet tissue)	α-Tocopherol (ng/mg protein)
Control (normal rats)	+	−	582.4 ± 91.1	125.88 ± 30.67
0	−	−	628.2 ± 58.2	5.44 ± 0.55
5	−	+	124.4 ± 13.6	5.08 ± 0.50
10	−	+	83.8 ± 34.3	5.49 ± 0.63
24	−	+	60.6 ± 11.7	6.01 ± 0.50

Values are means ± SD for five rats.

age to the kidney. Furthermore, VE has also been shown to inhibit lipid peroxidation of cell membranes *in vitro* [30]. Our previous results showed that depletion of GSH by BSO administration does not, by itself, cause acute tubular necrosis, whereas depletion of GSH combined with VE deficiency causes severe necrosis in the proximal tubules of the kidney [31]. It is tempting, therefore, to suggest that interaction between the water-soluble GSH and the lipid-soluble VE may play an important role in protection against oxidative injury in the kidney.

Various oxidative stress-inducing agents, such as metals, UV light, heme, and hemoglobin, have been implicated in the pathogenesis of the inflammatory process. Heme moieties released from heme proteins and hemoglobin released from damaged erythrocytes at tissue sites of hemorrhage or injury have been shown to promote the formation of oxygen radicals, generating reactive species toxic to endothelial cells [32, 33]. In addition, oxidant-mediated tissue damage may contribute significantly to the pathogenesis of diseases such as atherosclerosis and to reperfusion injury after myocardial ischemia and strokes.

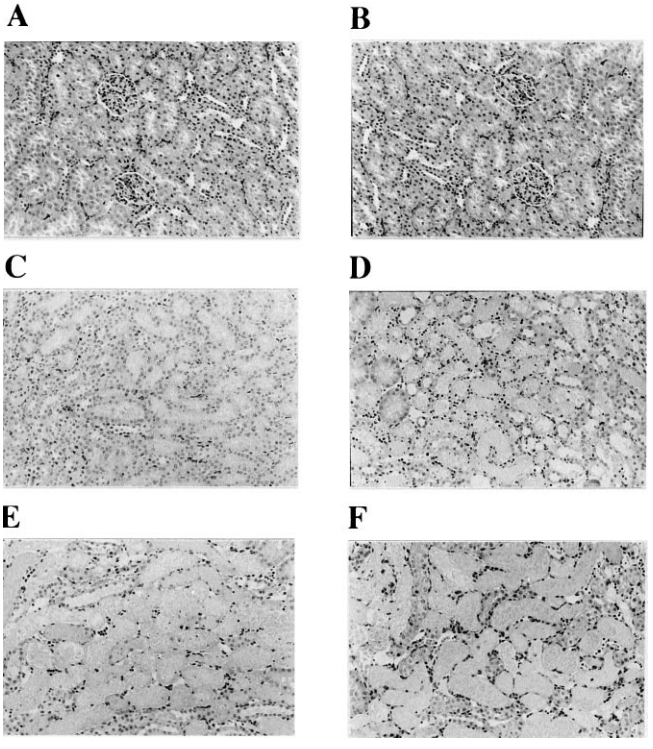

FIG. 5. Histological changes in kidneys of VE-deficient rats treated with BSO. Kidney sections were fixed in formalin and stained with hematoxylin and eosin. (A) VE-sufficient rat kidney. (B) VE-sufficient rat kidney treated with BSO (5 hr). (C) VE-deficient rat kidney. (D) VE-deficient rat kidney treated with BSO (2.5 hr). (E) VE-deficient rat kidney treated with BSO (5 hr). (F) VE-deficient rat kidney treated with BSO (24 hr). Original magnification, 50×.

HO-1 is a stress protein [34–36] and has been suggested to participate in defense mechanisms against treatments that may induce oxidative injury [37–40]. Induction of HO-1 also has been suggested to be an adaptive response to oxidative stress agents [32]. For example, overexpression of HO-1 may offer a means of cellular protection against heme/hemoglobin oxidative injury by enhancing the degradation of these pro-oxidants to bile pigments, which themselves have antioxidant properties [37, 39].

In the state of VE deficiency, GSH depletion may cause a high risk of renal damage. To clarify the mechanism of the renal injury under conditions of VE deficiency and GSH depletion, we should investigate further the relationship between oxidative stress and its cellular responses.

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