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The Protective Role of Glutathione, Cysteine and Vitamin C against Oxidative DNA Damage Induced in Rat Kidney by Potassium Bromate

Kimie Sai,[1] Takashi Umemura, Atsuya Takagi, Ryuichi Hasegawa and Yuji Kurokawa

Division of Toxicology, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158

The roles of glutathione (GSH), cysteine, vitamin C, liposome-encapsulated superoxide dismutase (L-SOD) and vitamin E in preventing oxidative DNA damage and cytotoxicity in the rat kidney after administration of potassium bromate ($KBrO_3$) to male F344 rats were investigated by measuring 8-hydroxydeoxyguanosine (8-OH-dG), an oxidative DNA product, lipid peroxidation (LPO) levels and relative kidney weight (RKW). Combined pre- and posttreatment of animals with 2 × 800 mg/kg GSH i.p. inhibited the increase of 8-OH-dG, LPO levels and RKW caused by 80 mg/kg $KBrO_3$ i.p. administration. In contrast, pretreatment with 0.3 ml/kg diethylmaleate (DEM) i.p., a depletor of tissue GSH, was associated with elevation of 8-OH-dG, LPO levels and RKW after a 20 mg/kg $KBrO_3$ i.p. treatment, which itself caused no change. Administration of $KBrO_3$ itself reduced renal non-protein thiol levels, but this was inhibited by the two doses of exogenous GSH. Combined treatment with DEM and $KBrO_3$ lowered the non-protein thiol level in the kidney more than did DEM treatment alone. Protective effects against the oxidative damage caused by $KBrO_3$ were also observed for pre- and posttreatment with 400 mg/kg cysteine i.p., another sulfhydryl compound, and daily i.g. application of 200 mg/kg vitamin C for 5 days. However, no influence was evident after pre- and posttreatment with 18,000 U/kg L-SOD i.p. or daily i.g. 100 mg/kg of vitamin E for 5 days. The results suggest that intracellular GSH plays an essential protective role against renal oxidative DNA damage and nephrotoxicity caused by $KBrO_3$.

Key words: Cysteine — Glutathione — Potassium bromate — Lipid peroxidation — 8-Hydroxydeoxyguanosine

$KBrO_3$, a food additive, has been demonstrated to induce rat renal cell tumors[1-3] and also to exert promoting activity in renal tumorigenesis.[4] In addition, after administration of $KBrO_3$ to rats, enhancement of renal LPO[2] levels was observed.[5] Because $KBrO_3$ has oxidizing properties, the involvement of active oxygen species in its initiation/promotion of neoplasia as well as the increment of LPO has been suggested. $KBrO_3$ was the first chemical carcinogen for which significant increase of 8-OH-dG, an oxidative DNA product formed by active oxygen species,[6] was recognized in the kidney (target organ) DNA, but not in the liver (non-target organ) DNA after administration to rats.[7] Subsequently several carcinogens were demonstrated to produce 8-OH-dG in their respective target organ DNAs,[8-14] and a close relationship between carcinogenesis and 8-OH-dG formation has been indicated.[15]

We previously reported that the levels of LPO and 8-OH-dG after $KBrO_3$ treatment to rats were increased in a dose-dependent manner, suggesting the involvement of LPO in the process of 8-OH-dG formation.[14] In addition, exogenous GSH was found to inhibit the mortality and nephrotoxicity induced by $KBrO_3$ treatment, while pretreatment with DEM, a depletor of tissue GSH, enhanced the above toxic effects,[3,5] indicating that GSH can play a protective role against $KBrO_3$-induced nephrotoxicity. The general protective functions of GSH against oxidative stress have been widely investigated,[16-21] and therefore the details of how GSH acts on $KBrO_3$-induced oxidative DNA damage are of interest.

In the present experiment, the effects of exogenous and endogenous GSH, and some other biological scavengers on renal oxidative DNA damage caused by $KBrO_3$ were therefore examined. Protective roles for GSH, cysteine and vitamin C against both DNA damage and nephrotoxicity were revealed.

MATERIALS AND METHODS

Animals Five-week-old male F344 rats (specific-pathogen-free, Charles River Japan, Inc., Kanagawa) were given F-2 pellet basal diet (Funabashi Farm Co., Chiba) and tap water freely, and were used after 1 week of acclimation.

Chemicals and use $KBrO_3$ (food additive grade) was purchased from Matsunaga Chemical Industry, Hiroshima. GSH, vitamin C and vitamin E were obtained

[1] To whom all correspondence should be addressed.

[2] The abbreviations used are: LPO, lipid peroxidation; DEM, diethyl maleate; GSH, glutathione; L-SOD, liposome-encapsulated superoxide dismutase; 8-OH-dG, 8-hydroxydeoxyguanosine; RKW, relative kidney weight.

from Wako Pure Chemical Industries, Ltd., Osaka and DEM was from Tokyo Kasei Industry Co. Ltd., Tokyo. Cysteine and alkaline phosphatase were obtained from Sigma Chemical Co., St. Louis, MO, and nuclease P1 was from Yamasa Shoyu Co. Ltd., Chiba. L-SOD was kindly supplied by Toyo Jozo Co. Ltd., Shizuoka. All other chemicals used were of specific analytical or HPLC grade. $KBrO_3$, GSH and cysteine were dissolved in saline. Vitamin C, Vitamin E, DEM and L-SOD were dissolved in distilled water, corn oil, dimethylsulfoxide (DMSO) and phosphate buffer (pH 7.0), respectively.

Treatment of animals with $KBrO_3$, and GSH or DEM Administration of $KBrO_3$ for the combination study with GSH or DEM was carried out to groups of 5 rats as follows. Rats were given a single i.p. administration of $KBrO_3$ at doses of 80 mg/kg and 20 mg/kg for the GSH and DEM treatments, respectively. For the GSH-treatment study, rats were injected i.p. with GSH at a dose of 800 mg/kg both 30 min before and 30 min after i.p. treatment with $KBrO_3$. With the DEM treatment, DEM was injected i.p. at a dose of 0.3 ml/kg 60 min before $KBrO_3$ administration. Control animals were given saline or DMSO i.p. simultaneously with the $KBrO_3$, GSH or DEM treatments. Animals were killed 48 h after the $KBrO_3$ treatment, both kidneys being immediately excised, weighed and used for the measurement of 8-OH-dG and LPO levels. Rats were also killed at 1, 2 and 3 h after the $KBrO_3$ treatment, and the kidneys were excised and immediately used for measurement of renal non-protein thiol content.

Treatment of animals with $KBrO_3$, and cysteine, L-SOD, vitamin C or vitamin E For the cysteine or L-SOD treatments, rats were injected i.p. with 400 mg/kg cysteine or 18,000 U/kg L-SOD both 30 min before and 30 min after i.p. treatment of $KBrO_3$ at a dose of 60 mg/kg. With the vitamin C or vitamin E treatments, rats were daily given 200 mg/kg of vitamin C or 100 mg/kg vitamin E i.g. for 5 days and $KBrO_3$ i.p. on the 4th day. Control animals were treated with saline or each of the antioxidants alone. Rats were killed 48 h after treatment with $KBrO_3$, for analysis of 8-OH-dG and LPO levels.

Measurement of 8-OH-dG Kidney DNA was immediately isolated by the method previously described,[14] and digested to deoxynucleotides with nuclease P1 and alkaline phosphatase. 8-OH-dG levels (8-OH-dG/10^5 deoxyguanosine) were analyzed by HPLC with an electrochemical detection system (SPD-6A, Shimadzu Co., Ltd., Japan; Coulochem Model-5100A, ESA, USA).

Measurement of LPO levels Malondialdehyde levels (MDA, nmol/g wet tissue) were assessed as an index of LPO by the method of Uchiyama *et al.*[22] A kidney sample (0.1 g) was homogenized with 0.9 ml of 1.15% KCl solution and the content of thiobarbituric acid-reactive substances was measured.

Measurement of non-protein thiol levels A kidney sample (0.1 g) was homogenized with 1 ml of 6% trichloroacetic acid and non-protein thiol was estimated by the method of Ellman.[23]

Statistical analysis All data were expressed as mean ± SD for five rats/group. The data were analyzed for inter-group significance by using Student's *t* test.

RESULTS

Effects of exogenous GSH The effects of GSH treatment on 8-OH-dG and LPO levels in the kidney, and RKW were examined 48 h after $KBrO_3$ administration, when these parameters reach maximum levels.[14] The 8-OH-dG level was significantly increased to three-fold the control level by the treatment with 80 mg/kg of $KBrO_3$. Additional GSH treatment significantly diminished this increment to near the control level (Fig. 1 (A)). Elevation of LPO level to three times that of the controls by administration of $KBrO_3$ was also completely inhibited by exogenous GSH (Fig. 1 (B)). With regard to RKW, the 40% increase observed with $KBrO_3$ treatment was not evident when combined with exogenous GSH (Fig. 1 (C)). GSH alone did not exert any significant effect on renal 8-OH-dG and LPO levels or RKW (Fig. 1 (A)–(C)).

Effects of GSH depletion To examine the relationship between intracellular GSH level and oxidative DNA damage caused by $KBrO_3$ in the kidney, animals were pretreated with DEM to effect depletion of tissue GSH. Because pretreatment with DEM enhanced the toxicity of $KBrO_3$, dose levels of 0.3 ml/kg for DEM and 20 mg/kg for $KBrO_3$ were finally selected as appropriate for the combination study. DEM + $KBrO_3$ induced an approximately 4-fold significant increase of 8-OH-dG level in the kidney DNA (Fig. 2 (A)). The renal LPO level was elevated approximately 6 times, and also a 50% increase in RKW was observed (Fig. 2 (B) and (C)). Neither DEM nor $KBrO_3$ alone caused any significant change in levels of 8-OH-dG, LPO and RKW as compared to control values (Fig. 2 (A)–(C)).

Changes in renal non-protein thiol content after treatment with $KBrO_3$, GSH or DEM alone or in combination Renal non-protein thiol content 1, 2 and 3 h after $KBrO_3$ treatment was determined to elucidate the role of intracellular GSH level in protection against the oxidative stress. Changes of non-protein thiol content in the kidneys after treatment with $KBrO_3$, GSH or DEM alone, and GSH or DEM in combination with $KBrO_3$ are shown in Table I.

The administration of 80 mg/kg $KBrO_3$ alone significantly reduced the renal non-protein thiol level until 3 h after the treatment. Renal non-protein thiol levels remained very high 1 h after administration of exogenous GSH (800 mg/kg × 2) both with and without $KBrO_3$

treatment. Pretreatment with DEM significantly lowered the level to 63% at 1 h, significantly low levels persisting over 3 h. The combined treatment with DEM and 20 mg/kg of $KBrO_3$ reduced the non-protein thiol content still further, with a reduction in the rate of return to normal, as evidenced by a significantly lower value at 3 h than that with DEM pretreatment alone. $KBrO_3$ alone (20 mg/kg) did not cause any significant change in renal non-protein thiol content (99% of the control value) 1 h after the treatment (data not shown).

Effects of cysteine, L-SOD, vitamin C and vitamin E To examine the effects of other radical scavengers on oxidative damage caused by $KBrO_3$, cysteine, L-SOD, vitamin C and vitamin E were administered to rats treated with 60 mg/kg of $KBrO_3$. Treatment with each scavenger alone exerted no influence on 8-OH-dG levels in the

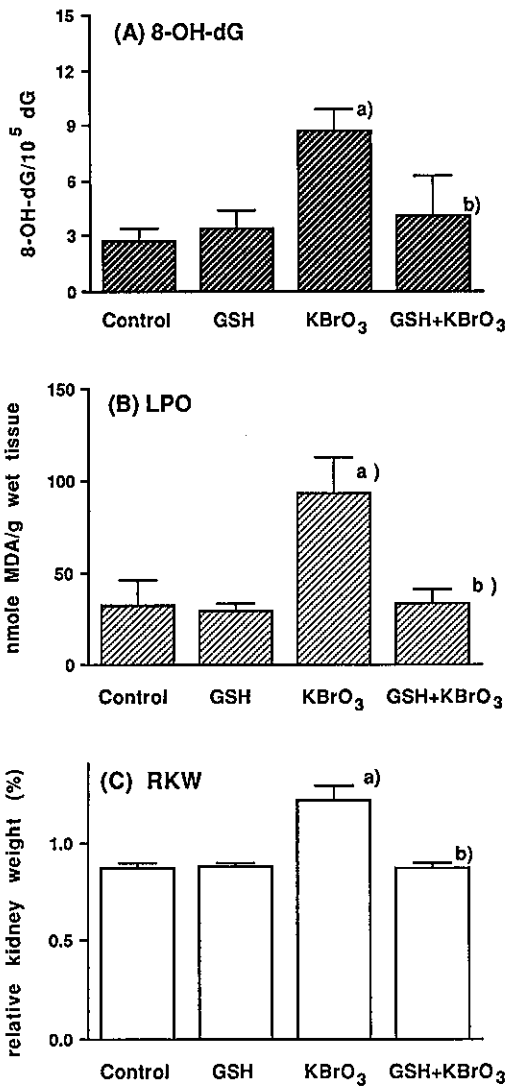

Fig. 1. Effects of GSH treatment on (A) 8-OH-dG, (B) LPO levels in the kidney and (C) RKW after a single i.p. administration of $KBrO_3$ (80 mg/kg). GSH (800 mg/kg) was administered i.p. both 30 min before and 30 min after $KBrO_3$ treatment. Control animals were treated with vehicle alone, and all animals were killed 48 h after $KBrO_3$ injection. a), b) Significantly different from control group and $KBrO_3$-treated group values at $P<0.01$.

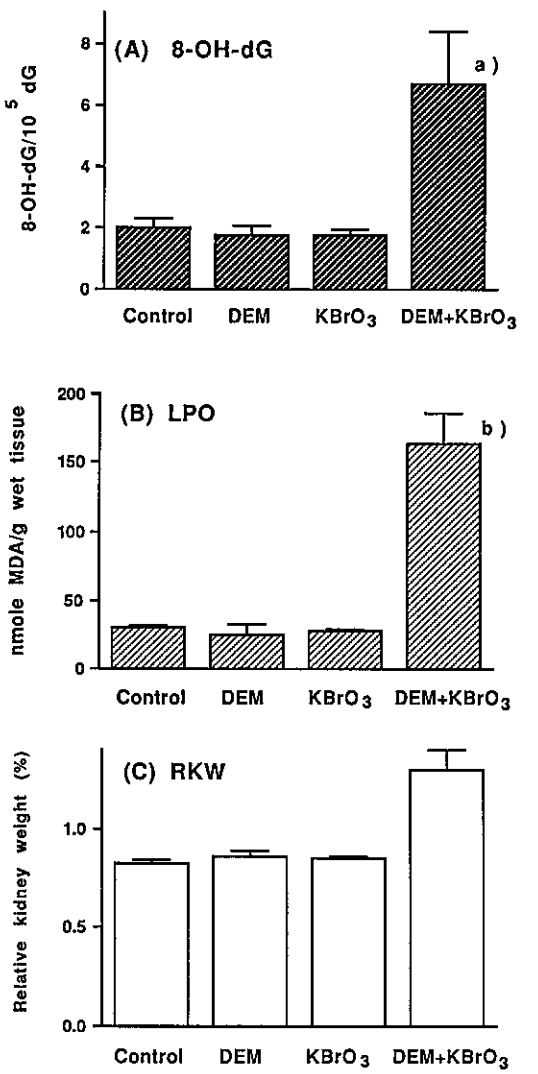

Fig. 2. Effect of DEM treatment on (A) 8-OH-dG, (B) LPO levels in the kidney and (C) RKW after a single i.p. administration of $KBrO_2$ (20 mg/kg). DEM (0.3 ml/kg) was administered i.p. 60 min before $KBrO_3$ treatment. Control animals were treated with vehicle clone, and all animals were killed 48 h after $KBrO_3$ injection. a) Significantly different from the control group value at $P<0.01$, and from the $KBrO_3$-treated group value at $P<0.05$. b) Significantly different from control group and $KBrO_3$-treated group values at $P<0.01$.

Table I. Changes in Renal Non-protein Thiol Levels after $KBrO_3$, GSH or DEM Alone or after Combined Treatment with GSH or DEM and $KBrO_3$

Treatment	1	2	3 (h)
Saline→$KBrO_3$(*)→saline	$3.00\pm0.13^{a)}$	$3.03\pm0.36^{a)}$	$3.11\pm0.14^{a)}$
GSH→saline→GSH	$10.45\pm1.25^{b)}$	$5.69\pm0.88^{b)}$	$2.91\pm0.37^{b)}$
GSH→$KBrO_3$(*)→GSH	$9.85\pm0.52^{b)}$	$5.67\pm0.80^{b)}$	3.26 ± 0.33
DEM→saline	$2.17\pm0.24^{b)}$	$2.51\pm0.61^{b)}$	$3.03\pm0.32^{a)}$
DEM→$KBrO_3$(**)	$1.84\pm0.89^{b)}$	$2.22\pm0.53^{b)}$	$2.40\pm0.33^{b,c)}$

Treatment procedure as described in "Materials and Methods." Dosage: $KBrO_3$(*), 80 mg/kg; $KBrO_3$(**), 20 mg/kg; GSH, 800 mg/kg; DEM, 0.3 ml/kg.
a) Significantly different from the non-treated value (3.44 ± 0.13) at $P<0.05$.
b) Significantly different from the non-treated value at $P<0.01$.
c) Significantly different from the DEM-alone treatment 3 h value at $P<0.05$.

kidney. In contrast to treatment with $KBrO_3$ alone, when 8-OH-dG level was significantly enhanced approximately 2.5 times, a combination of cysteine and $KBrO_3$ resulted in near to control levels. Similarly with vitamin C treatment, the increase in 8-OH-dG level was also diminished. With L-SOD or vitamin E in combination with $KBrO_3$, however, no significant differences from $KBrO_3$ treatment alone were evident (Fig. 3 (A)). Exogenous cysteine completely prevented the significant elevation of LPO by $KBrO_3$ treatment alone, slight inhibition also being observed with vitamin C but not L-SOD or vitamin E treatment (Fig. 3(B)). $KBrO_3$ treatment alone induced an increase of RKW, and this increase was significantly inhibited by the combined treatment with cysteine. Vitamin C treatment also reduced the increased level, although the effect was not significant. L-SOD or vitamin E treatment with $KBrO_3$ did not exert any influence on RKW as compared to $KBrO_3$ treatment alone (Fig. 3 (C)).

DISCUSSION

Protective roles for antioxidants in general against free radicals have been demonstrated in a number of *in vitro* and *in vivo* experiments.[24-28] Among the species acting as scavengers, GSH's importance has been widely stressed,[3, 5, 16-21] depletion of tissue GSH causing hypersusceptibility to some toxic chemicals[16-19] and radiation.[20] Renal function, as indicated by glomerular filtration rate, etc., is also easily affected by depletion of GSH.[21]

In a previous study, it was found that mortality caused by $KBrO_3$ could be effectively blocked by application of exogenous GSH or cysteine, while being strongly enhanced by pretreatment with DEM.[5] These results have indicated a protective role for GSH against $KBrO_3$-induced cytotoxicity in the kidney, but no light was cast on the mechanisms involved.

In the present work, protection by GSH against $KBrO_3$-induced oxidative DNA damage as well as nephrotoxicity, as represented by RKW increase, was again demonstrated. From the time-course study of renal non-protein thiol level (Table I), intracellular non-protein thiol, mostly GSH, content within the first few hours after $KBrO_3$ treatment seems to be of particular importance for determining whether oxidative stress will occur.

As for the action of exogenous GSH, at least two possible mechanisms require consideration. Firstly, it might cause decomposition of $KBrO_3$ in the blood stream prior to reaching the kidneys, because it was earlier shown that GSH strongly catabolizes $KBrO_3$ *in vitro*.[29] However, it was also reported that exogenous GSH is rapidly removed from the blood by the kidney and other organs that contain transpeptidase.[30, 31] After glomerular filtration. GSH is degraded by γ-glutamyl transferase and dipeptidase located on the outside of the renal brush-border membranes in the proximal convoluted tubules, the amino acids being quickly incorporated into the renal tubular cells and the GSH resynthesized.[30] Secondly, exogenous GSH might act on $KBrO_3$ or $KBrO_3$-induced LPO inside renal tubular cells. The latter speculation seems to be highly probable because our preliminary experiment revealed blood non-protein thiol levels 30 min after the exogenous GSH administration, when $KBrO_3$ was to be administered, to be already restored to near control levels, at a time when non-protein thiol levels in the kidney remained markedly increased. Although Inoue *et al.* reported that exogenous GSH (0.1 mmol/kg) could not increase the intracellular GSH level in the kidney,[31] the present result is different, probably because an extremely large amount of GSH (2.6 mmol/kg) was administered. The increase in this study might have resulted from enhancement of GSH synthesis by a large amount of GSH supplement. Furthermore, since active transport of intact GSH through the basal lateral region was demonstrated,[32] there is a possibility that

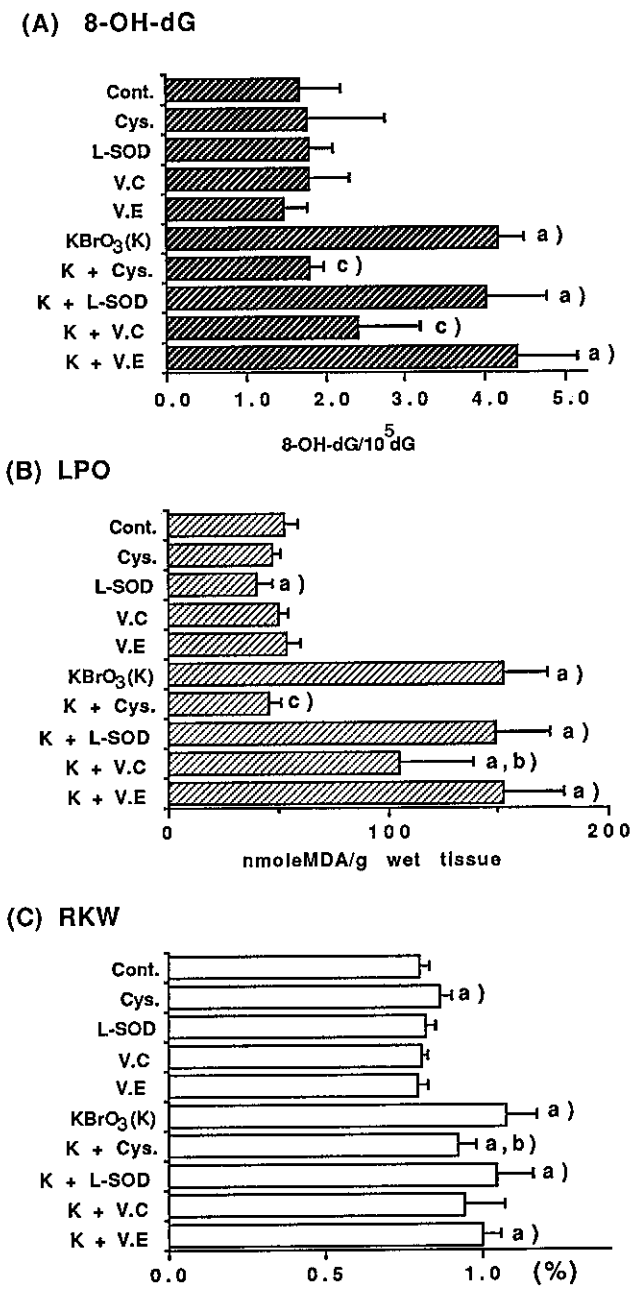

Fig. 3. Effects of cysteine (Cys.), L-SOD, vitamin C (V. C) and vitamin E (V. E) on (A) 8-OH-dG, (B) LPO levels in the kidney and (C) RKW after a single i.p. administration of $KBrO_3$ (K) (60 mg/kg). Cysteine (400 mg/kg) or L-SOD (18,000 U/kg) was administered i.p. both 30 min before and 30 min after $KBrO_3$ treatment. Vitamin C (200 mg/kg) or vitamin E (100 mg/kg) was administered daily i.g. for 5 days and $KBrO_3$ (60 mg/kg) was given i.p. on the 4th day. Control animals were treated with vehicle or each scavenger alone, and all animals were killed 48 h after $KBrO_3$ injection. a) Significantly different from the control group value at $P<0.01$. b), c) Significantly different from the $KBrO_3$-treated group value at $P<0.05$ and $P<0.01$, respectively.

exogenous GSH might also be directly incorporated into renal tubular cells, especially when a large amount of GSH is administered. Thus, the increase of non-protein thiol compounds in the kidney may work as a protector against $KBrO_3$-caused oxidative damage.

Previously we demonstrated that $KBrO_3$ caused dose-dependent increases in LPO and 8-OH-dG levels and that the elevation of LPO levels occurred first, followed by the increase of 8-OH-dG level, suggesting that oxidative DNA damage might be mediated by intracellular LPO.[14] Exogenous GSH treatment in the present study clearly inhibited this elevation of LPO. Probably a sufficiency of intracellular GSH, supplied by exogenous GSH, might react enzymatically or non-enzymatically with $KBrO_3$ and/or the $KBrO_3$-induced LPO first and thus protect DNA from eventual oxidative damage.

Two further experiments in this study provided additional support for the essential role of GSH in protecting against $KBrO_3$-induced DNA oxidative damage in the kidney. DEM pretreatment itself caused a temporary depletion of non-protein thiol content in the kidney without any resultant oxidative damage in renal DNA (Fig. 2). In combination with $KBrO_3$ the result was severe depletion of renal non-protein thiol content, suggesting consumption by $KBrO_3$ and subsequent 8-OH-dG production. This finding suggests that intracellular GSH can protect against $KBrO_3$-induced oxidative damage. Oxidative DNA damage induced by 80 mg/kg $KBrO_3$ was severer than that by 20 mg/kg $KBrO_3$ plus DEM pretreatment despite the fact that the latter caused severe non-protein thiol depletion. The severity of oxidative damage may be related to the level of residual $KBrO_3$ not decomposed by intracellular GSH rather than remaining non-protein thiol level itself.

Cysteine is known as a rate-limiting precursor of GSH biosynthesis, and administration of cysteine to rats provides protection against the necrotic effects of carbon tetrachloride[24] or other hepatotoxins[25] on the liver. Cysteine inhibition of endrin-induced hepatic toxicity was reported to be mediated by influence on LPO increase and GSH depletion in the liver, cysteine itself being considered to interact with endrin or its metabolites.[26] In the present study, exogenous cysteine treatment also protected against the oxidative DNA damage and nephrotoxicity of $KBrO_3$. In a preliminary study, non-protein thiol levels in the blood and kidney 30 min after administration of exogenous cysteine were not significantly increased, in line with earlier findings.[13, 26] This phenomenon might result from the facts that cysteine is easily oxidized and exists mostly as cystine extracellularly,[33] which can not be detected as non-protein thiol, and cystine is incorporated into several organs, including the kidney, and incorporated cystine in the kidney is used as a substrate for the γ-glutamyl cycle

that is involved in synthesis and degradation of GSH.[34] When intracellular GSH in the kidney was consumed by $KBrO_3$, GSH might be synthesized from cystine in the blood after incorporation into the kidney, resulting in the protective effect of cysteine treatment.

In order to assess the involvement of O_2^- in $KBrO_3$-induced toxicity, the effects of L-SOD were investigated. Protection by L-SOD against acetaminophen-induced liver necrosis *in vivo* suggested direct participation of O_2^-.[27] In this study, however, $KBrO_3$-induced oxidative damage was not influenced by the treatment with L-SOD. This finding might suggest that O_2^- production may not be related to the $KBrO_3$-induced changes. This result is reminiscent of a finding in an *in vitro* study using ESR measurement that active oxygen species generation by the reaction of $KBrO_3$ with rat kidney cells was not inhibited by the addition of SOD (unpublished data).

Protective potential of vitamin C against oxidative stress was also reported from *in vivo* experiments,[26] and similarly, in the present work, vitamin C treatment resulted in significant reduction of $KBrO_3$-induced oxidative DNA damage and nephrotoxicity, albeit less pronounced than with GSH and cysteine.

Vitamin E is easily incorporated into the liver and oxidative damage induced by a hepatotoxic agent was effectively inhibited by vitamin E treatment.[26] However, vitamin E exerted no such influence in the kidney in this study, presumably because administered vitamin E was trapped mainly in the liver and its uptake into the kidney was too small to operate on the $KBrO_3$-acting site. Further study is needed to evaluate precisely the effect of exogenous vitamin E in the kidney, in terms of its administration period and the amount to be used.

As a possible contribution of $KBrO_3$-induced oxidative damage to renal carcinogenesis, the following pathway can be considered. Peroxidized fatty acids and/or reactive derivatives, produced in nuclear membrane directly by $KBrO_3$ or through cytoplasmic oxidation, might react with nuclear DNA, resulting in 8-OH-dG production as observed in a recent *in vitro* experiment.[35] The mutagenic activity of 8-OH-dG in DNA[36–38] finally may lead to renal carcinogenesis.

In conclusion, the present examination demonstrated strong protection by GSH and cysteine against $KBrO_3$-induced 8-OH-dG formation. Research is in progress on the effects of these radical scavengers *in vitro* on the 8-OH-dG production induced by $KBrO_3$.

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