Contribution of Glutathione Peroxidase and Nitric Oxide to Potassium Bromate-Induced Oxidative Stress and Kidney Damage in Mice

Satoshi Watanabe,*,[a] Yoshihiro Yoshimura,[b] and Tetsuya Fukui[a]

[a]Department of Health Chemistry, [b]Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University 4–41, Ebara 2-chome, Shinagawa-ku, Tokyo 142–8501, Japan

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In order to confirm the participation of peroxynitrite in potassium bromate ($KBrO_3$)-induced oxidative stress and kidney damage in mice, we investigated effects of administration of nitric oxide (NO) synthase inhibitor on them. Cytoplasmic glutathione peroxidase (GPx) activity remarkably decreased within 3 hr after $KBrO_3$ administration, and then oxidative stress started to occur. However, kidney damage occurred 24 hr after $KBrO_3$ administration. Pre-administered N^G-monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor, suppressed $KBrO_3$-induced oxidative stress and kidney damage. However, no effect of L-NMMA was observed on the $KBrO_3$-induced reduction of cytoplasmic GPx activity. These results suggest that reduction of cytoplasmic GPx activity resulted from the $KBrO_3$ administration initiates oxidative stress and that NO also participates in the promotion of $KBrO_3$-induced oxidative stress and kidney damage.

Key words —— potassium bromate, oxidative stress, glutathione peroxidase, superoxide, kidney damage

INTRODUCTION

Potassium bromate ($KBrO_3$) is added to flour as a maturing agent, to dough and fish paste as a conditioner, and also added to beer or cheese.[1)] $KBrO_3$ has also been used as a constituent in cold-wave hair solutions.[1,2)] In addition, bromate is generated as a disinfection by-product from bromide in the process of ozonation and chlorination of raw water.[2,3)] Ingestion of $KBrO_3$ has been reported to induce renal oxidative stress in human and other mammals,[1–4)] which is known to cause renal failure, methemoglobinemia and kidney cancer. This carcinogenesis has been suggested to be associated with oxidative DNA damage, which was induced by BrO_3^- in the presence of intracellular sulfhydryl compound such as reduced glutathione (GSH) or cysteine.[1,3,4)] We previously reported that *in vitro* lipid peroxidation by $KBrO_3$ progressed in the presence of GSH but not the oxidized form.[5)] These results suggested that reactive species responsible for the induction of renal oxidative stress were generated from $KBrO_3$. However, the reactive species in the $KBrO_3$-induced oxidative stress are not clear yet. In the present study, we have demonstrated the *in vitro* generation of superoxide anion radical (O_2^-) from $KBrO_3$ using electron spin resonance (ESR) spectrometry, and have shown the participation of peroxynitrite ($ONOO^-$) possibly produced from O_2^- and physiological nitric oxide (NO) in the $KBrO_3$-induced renal oxidative stress and kidney damage by administering NO synthase inhibitor to mice.

*To whom correspondence should be addressed: Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University 4–41, Ebara 2-chome, Shinagawa-ku, Tokyo 142–8501, Japan. Tel.: +81-3-5498-5773; Fax: +81-3-5498-5771; E-mail: satoshi@hoshi.ac.jp

MATERIALS AND METHODS

Chemicals —— Glutathione reductase from bakers yeast (EC 1.6.4.2), xanthine oxidase from milk (EC 1.1.3.22), GSH and oxidized glutathione, xanthine, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2,4-Dinitrophenylhydrazine was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Dojindo Laboratories (Kumamoto, Japan). $KBrO_3$ and N^G-monomethyl-L-arginine acetate (L-NMMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals of the highest grade were obtained commercially.

ESR Spectrometry —— ESR spectra were recorded on a JEOL JES-RE1X spectrometer using a flat quartz cell designed for aqueous solution. Conditions of ESR spectrometry were as follows : magnetic field, 336.3 ± 5 mT; power, 8.0 mT; modulation frequency, 100 kHz; frequency, 9.425 GHz; modulation amplitude, 0.063 mT; gain, 500; time scan, 1 min; time constant, 0.03 s.

Animals —— Five-week-old male ddY mice purchased from Tokyo Experimental Animal Supply Co. (Tokyo, Japan) were given MF pellet basal diet (Oriental Yeast Co., Tokyo, Japan) and tap water freely, and were used after 1 week of acclimation. Mice were housed in an air-conditioned room with temperature of 23 ± 1°C, a humidity of 50 ± 3%, and a 12 hr light and dark cycle.

Treatment of Animals —— In the time-course experiments on the oxidative stress status caused by $KBrO_3$, mice received a single i.p. administration of $KBrO_3$ dissolved in 0.9% NaCl at a dose of 200 mg/kg and were sacrificed at 0, 3, 6, 9 and 24 hr after treatment. The experiments for effects of L-NMMA on the $KBrO_3$-induced oxidative stress and kidney damage were carried out by the injection of 20 mg/kg or 40 mg/kg of L-NMMA dissolved in DMSO or DMSO alone to the abdominal cavity of mice at 30 min before $KBrO_3$ administration. The mice were sacrificed 6 hr after the $KBrO_3$ administration. In both experiments, kidneys were immediately excised, weighed and used for the measurement of protein carbonyl, thiobarbituric acid-reactive substances (TBARS), GSH and antioxidative enzyme activities. Whole blood was removed and used for the measurement of serum creatinine level. This experimental design was approved by the Animal Experiment Committee of Hoshi University and the mice were cared for in accordance with the Guidelines Concerning the Care and Use of Laboratory Animals.

Measurement of Serum Creatinine Level —— Serum creatinine level was measured with Creatinine Test-Wako (Wako Pure Chemical Industries, Japan).

Measurement of Protein Carbonyl Group —— Protein carbonyl group in kidney was assayed by the method of Reznick *et al.*[6] Absorbance at 370 nm of guanidine hydrochloride solution was measured using Hitachi U-2000 Spectrophotometer (Hitachi Co., Tokyo, Japan). Carbonyl concentration was calculated as follows, C (μM) = $OD_{370} \times 45.45$.[6]

Measurement of TBARS Content —— TBARS content in kidney was measured by the method of Ohkawa *et al.*[7] Absorbance at 535 nm of dehydrated organic layer was measured and the molar extinction coefficient of malondialdehyde ($\varepsilon = 1.56 \times 10^2$ mM^{-1} cm^{-1})[7] was used to calculate TBARS concentration.

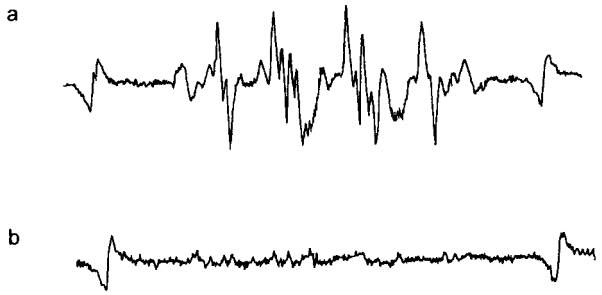

Fig. 1. ESR Signal of DMPO-OOH Adduct Produced from $KBrO_3$ in the Presence of GSH and Transition Metal

The reaction mixture contained 1 mM $KBrO_3$, 1 mM GSH, 0.1 mM $FeSO_4$ and 100 mM DMPO (a), 1 mM oxidized glutathione instead of 1 mM GSH was added to the reaction mixture (b). ESR conditions were described in "MATERIALS AND METHODS."

Measurement of GSH Content —— GSH content in kidney was determined as described in the previous report[8] using HPLC-UV detector system.

Measurement of Antioxidative Enzyme Activities —— Copper/zinc-containing superoxide dismutase (Cu/Zn-SOD), catalase and glutathione peroxidase (GPx) activities were measured spectroscopically as described previously.[8]

Measurement of Protein Concentration —— Protein concentration was determined by the method of Lowry *et al.*,[9] using bovine serum albumin as the standard protein.

Statistics —— Data were expressed as the mean ± S.D. One-way ANOVA was used to determine any significant differences ($p < 0.05$) between means. When significant differences were found, Duncan's multiple range test was used to determine the exact nature of the difference.

RESULTS

In Vitro O_2^- Generation from $KBrO_3$

Reactive species generated from $KBrO_3$ in the presence of sulfhydryl group were identified by ESR spectrometry. ESR signal of O_2^- adduct to DMPO (DMPO-OOH) was detected in the presence of $KBrO_3$, GSH and transition metal (Fig. 1a). While, no DMPO-OOH signal was observed when oxidized glutathione instead of GSH was added to the reaction mixture (Fig. 1b).

Table 1. Changes of Parameters for Oxidative Stress and Kidney Damage after $KBrO_3$ Administration to Mice

Time (h)	BW (g)	KW (g)	RKW	TBARS (μmol/g tissue)	Carbonyl (nmol/g tissue)	GSH (nmol/g tissue)	GPx (unit/mg protein)	Catalase (unit/mg protein)	Cu/Zn-SOD (unit/mg protein)
0	31.6±1.4$^{a)}$	0.51±0.02$^{a)}$	1.63±0.09$^{a)}$	2.56±0.68$^{a)}$	99.9±27.3$^{a)}$	85.0±7.2$^{a)}$	0.39±0.07$^{a)}$	153±23$^{a)}$	15.0±1.9
3	32.3±0.5$^{a)}$	0.56±0.04$^{a,b)}$	1.73±0.15$^{a)}$	4.09±0.92$^{b)}$	163.8±39.3$^{a,b)}$	74.6±6.3$^{b)}$	0.14±0.04$^{b)}$	116±11$^{b)}$	12.3±2.4
6	32.3±2.0$^{a)}$	0.56±0.04$^{a,b)}$	1.74±0.23$^{a)}$	7.26±0.40$^{c)}$	245.4±38.7$^{b)}$	60.4±8.2$^{c)}$	0.12±0.02$^{b)}$	92±15$^{c)}$	16.3±1.8
9	29.0±1.0$^{b)}$	0.55±0.08$^{a)}$	1.90±0.21$^{a)}$	3.29±1.27$^{a,b)}$	362.4±83.4$^{c)}$	54.3±4.6$^{c)}$	0.13±0.02$^{b)}$	84± 9$^{c)}$	15.1±3.0
24	28.2±0.6$^{b)}$	0.63±0.04$^{b)}$	2.23±0.19$^{b)}$	2.99±0.41$^{a,b)}$	420.0±74.7$^{c)}$	52.3±4.3$^{c)}$	0.11±0.03$^{b)}$	70±10$^{c)}$	14.4±2.9

$KBrO_3$ (200 mg/kg) dissolved in 0.9% NaCl was administered intraperitoneally to male ddY mice. Each parameter was measured as described in "MATERIALS AND METHODS." BW, KW and RKW indicate body weight, kidney weight and relative kidney weight, respectively. RKW was calculated as follows, KW×100/BW. Values are mean ± S.D. ($n = 4$). a, b, c) Values not sharing a common letter are significantly different at $p < 0.05$ (ANOVA with Duncan's multiple-range test).

Table 2. Effect of L-NMMA Administration on the $KBrO_3$-Induced Oxidative Stress and Kidney Damage in Mice

Group	BW (g)	KW (g)	RKW	Creatinine (mg/l serum)	TBARS (μmol/g tissue)	Carbonyl (nmol/g tissue)	GSH (nmol/g tissue)	GPx (unit/mg protein)	Catalase (unit/mg protein)	Cu/Zn-SOD (unit/mg protein)
Vehicle	30.8±1.5	0.45±0.02	1.46±0.12$^{a)}$	3.06±0.69$^{a)}$	1.78±0.18$^{a)}$	98±15$^{a)}$	83.7±6.3$^{a)}$	0.48±0.04$^{a)}$	154±10$^{a)}$	17.8±2.4
$KBrO_3$ 200 mg/kg	29.6±0.6	0.50±0.04	1.87±0.15$^{b)}$	10.22±2.36$^{b)}$	2.38±0.08$^{b)}$	228±21$^{b)}$	57.7±1.6$^{b)}$	0.07±0.01$^{b,c)}$	70±17$^{b,d)}$	18.9±2.7
$KBrO_3$ 200 mg/kg + L-NMMA 20 mg/kg	29.2±2.1	0.46±0.02	1.58±0.08$^{a,c)}$	6.47±1.71$^{c)}$	2.36±0.06$^{b)}$	242±31$^{b)}$	68.0±2.6$^{c)}$	0.12±0.02$^{b)}$	52±15$^{d)}$	14.5±4.4
$KBrO_3$ 200 mg/kg + L-NMMA 40 mg/kg	29.2±0.8	0.50±0.03	1.69±0.06$^{c)}$	4.43±2.58$^{a,c)}$	2.06±0.06$^{c)}$	125±26$^{a,c)}$	80.5±8.8$^{a)}$	0.05±0.02$^{c)}$	88±15$^{b,c)}$	16.2±3.4
L-NMMA 40 mg/kg	30.3±1.8	0.47±0.04	1.56±0.06$^{a,c)}$	2.72±1.11$^{a)}$	2.12±0.12$^{c)}$	141±10$^{c)}$	82.3±6.6$^{a)}$	0.46±0.07$^{a)}$	105±19$^{c)}$	17.3±3.6

$KBrO_3$ dissolved in 0.9% NaCl was administered intraperitoneally to ddY male mice at 30 min after L-NMMA administration. L-NMMA dissolved in DMSO was administered intraperitoneally to mice as low dose (20 mg/kg) and high dose (40 mg/kg) at 30 min before $KBrO_3$ administration. All mice were sacrificed 6 h after $KBrO_3$ administration. Each parameter was measured as described in "MATERIALS AND METHODS." BW, KW and RKW are the same in Table 1. Values are mean ± S.D. ($n = 4$). a, b, c, d) Values not sharing a common letter are significantly different at $p < 0.05$ (ANOVA with Duncan's multiple-range test).

Time-Course of Oxidative Stress Status after $KBrO_3$ Administration

Effect of administration of $KBrO_3$ to mice on antioxidative enzyme activities which are known to be influenced by O_2^- and/or other reactive species and on kidney damage was investigated. Cytoplasmic catalase and GPx activities were remarkably decreased by the $KBrO_3$ administration. In particular, GPx activity reached near the lowest level within 3 hr after the treatment (Table 1). Protein carbonyl group, which is known to be an important parameter to discriminate the oxidative stress status, gradually elevated, and the highest level was observed at 24 hr after the treatment under the experimental condition (Table 1). However, renal TBARS started to increase within 3 hr and reached the maximum level at 6 hr after the treatment, and then gradually decreased (Table 1). Renal GSH slowly decreased, and the lowest level was observed 24 hr after the treatment (Table 1). Kidney weight and relative kidney weight, which are parameters of kidney damage, significantly increased at 24 hr after the treatment (Table 1).

Effect of L-NMMA on the $KBrO_3$-Induced Oxidative Stress and Kidney Damage

Next, in order to confirm the involvement of NO and $ONOO^-$ in the $KBrO_3$-induced oxidative stress, L-NMMA, a NOS inhibitor, was pre-administered to mice. As shown in Table 2, L-NMMA treatment alone caused the increases in TBARS and protein carbonyl group, and the decrease in catalase activity. In other parameters measured in the experiment, no significant effect of L-NMMA alone was observed (Table 2). In $KBrO_3$-treated mice, the decreased cytoplasmic catalase activity was recovered by 40 mg/kg L-NMMA to nearly the same level as that obtained by L-NMMA alone. However, no effect of L-NMMA on the $KBrO_3$-induced decrease of cytoplasmic GPx activity was observed under the experimental condition (Table 2). Increases in protein carbonyl group and TBARS, parameters of oxidative stress, were prevented in L-NMMA-treated mice (Table 2). L-NMMA also prevented the $KBrO_3$-induced decrease in GSH and increases in relative kidney weight and serum creatinine level (Table 2).

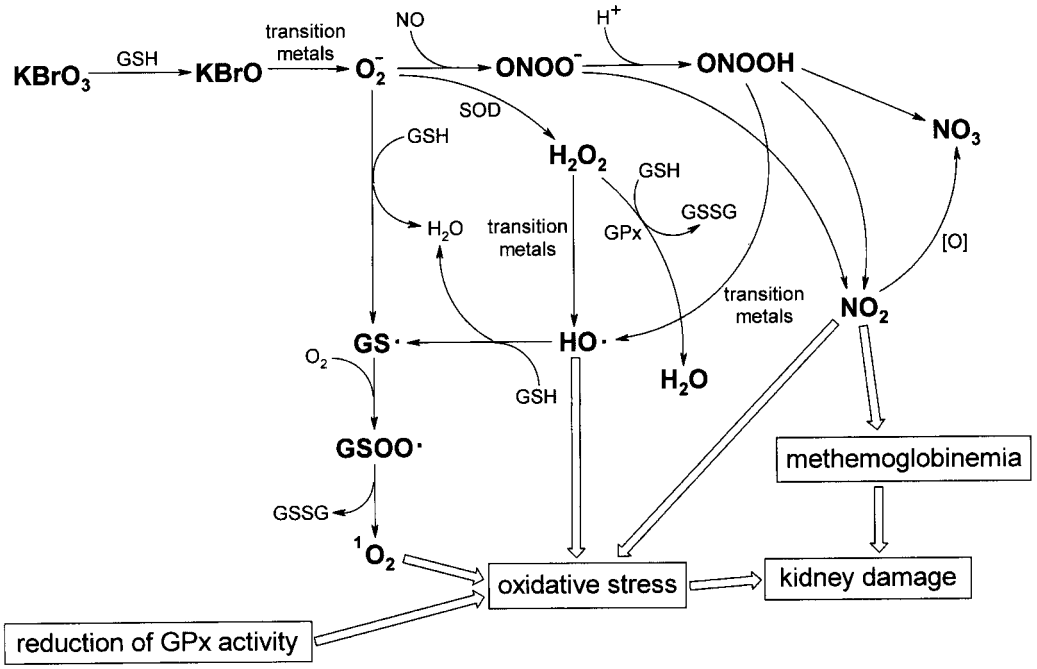

Fig. 2. Possible Pathways for the $KBrO_3$-Induced Generation of Reactive Species which Cause Oxidative Stress, Methemoglobinemia and Kidney Damage in Mouse

DISCUSSION

In the oxidative stress status, increase in TBARS and decrease in GSH in various tissues have been generally observed.[10] TBARS and protein carbonyl group are useful markers for the detection of cellular damage caused by reactive oxygen species (ROS) if they are taken together with other markers such as cellular concentrations of various antioxidants.[11,12] Our results of the time-course of TBARS suggest that renal oxidative stress is induced within 6 hr after $KBrO_3$ administration. In addition, the level of oxidatively-modified protein in kidney also increased remarkably. Elevation of the level of oxidatively-modified protein has been observed in several oxidative stress-related diseases, such as Alzheimer's disease[13] and Parkinson's disease.[14]

Cu/Zn-SOD, catalase and GPx are known as antioxidative enzymes in various tissues which physiologically suppress the oxidative stress by catalyzing the removal of ROS. In our experiment, cytoplasmic catalase and GPx activities were distinctly reduced within 3 hr after $KBrO_3$ administration. Therefore, the $KBrO_3$-induced oxidative stress status was likely to have resulted from the reductions of cytoplasmic catalase and GPx activities.[15] That is, the reductions of cytoplasmic catalase and GPx activities caused the elevation of cellular hydrogen peroxide, which is mainly removed by these enzymes.

Relative tissue weight is a marker which generally elevates when tissue is damaged.[16] Since relative kidney weight elevated after the increases in renal TBARS and protein carbonyl group, $KBrO_3$-induced kidney damage appears to occur following oxidative stress. Intraperitoneally-injected L-NMMA, an inhibitor of NOS, is known to cause decrease in NO generation in liver and kidney by nonselectively inhibiting the constitutive and inducible NOS activities.[17] Since $KBrO_3$-induced increases in relative kidney weight and serum creatinine levels were significantly suppressed by L-NMMA, NO seemed to be involved in the $KBrO_3$-induced kidney damage.

Pre-administration of L-NMMA significantly prevented the $KBrO_3$-induced elevations of renal TBARS and protein carbonyl group. Furthermore, $KBrO_3$-induced reduction of renal GSH content was also significantly prevented by L-NMMA. These results suggest that L-NMMA exhibits the suppressive effect on the oxidative stress status caused by $KBrO_3$. Since L-NMMA is not a free radical scavenger,[18] suppressive effect of L-NMMA on the oxidative stress is likely to have resulted from the reduction of NO generation. NO is known to rapidly react with O_2^- to form $ONOO^-$, which is a powerful oxidant and has cytotoxicity.[19] Although being a result of *in vitro* experiment, O_2^- generation from $KBrO_3$ in the

presence of GSH but not oxidized glutathione indicates that one of the causes of $KBrO_3$-induced oxidative stress is the elevation of the level of cellular $ONOO^-$ produced from O_2^- and NO.

Reduction of not cytoplasmic GPx activity but cytoplasmic catalase activity in $KBrO_3$-treated mice was significantly prevented by L-NMMA administration. However, catalase is known to possess much lower hydrogen peroxide-removing ability than GPx in the cytoplasmic fraction of the cell.[20] Therefore, it is possible that incomplete suppression by L-NMMA of $KBrO_3$-induced oxidative stress was mainly resulted from its inability to recover the cytoplasmic GPx activity. It has been reported that element selenium in the active site of GPx nonenzymatically reacts with $ONOO^-$ to bring about the inactivation of the enzyme[15,20] However, since L-NMMA had no influence on the reduction of cytoplasmic GPx activity caused by $KBrO_3$ administration, it is likely that O_2^- generated from $KBrO_3$ rather than NO is directly involved in the $KBrO_3$-induced reduction of GPx activity.

$KBrO_3$ is also known to cause methemoglobinemia,[1,3] and mechanism for its induction by $KBrO_3$ is not clear yet. According to our results, one of the mechanisms could be explained on the basis of the elevation of $ONOO^-$ level in blood.[21] Thus, tentative mechanisms of $KBrO_3$-induced oxidative stress, kidney damage and methemoglobinemia are shown in Fig. 2. The pathway of the generation of singlet oxygen from $KBrO_3$, previously proposed by us,[5] is also shown in Fig. 2. In the recent paper, Rahman *et al*. proposed that NO acts as an antioxidant in $KBrO_3$-induced renal oxidative stress.[18] However, present results surely suggest that NO and $ONOO^-$ act as accelerator on $KBrO_3$-induced oxidative stress and kidney damage.

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