Contribution of Nitric Oxide to Potassium Bromate-Induced Elevation of Methaemoglobin Concentration in Mouse Blood

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Bromate, an inorganic oxyhalide disinfection by-product, is known to cause kidney damage, haemolysis and methaemoglobinemia. In potassium bromate (KBrO₃)-treated mice (1.2 mmol/kg), elevation of methaemoglobin (MetHb) concentration in blood was observed simultaneously with an elevation of the NO concentration and attenuation of glutathione peroxidase (GPx) activity. Renal oxidative stress and kidney damage were also confirmed in the KBrO₃-treated mice. A pre-administered GPx-mimic ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) dose-dependently diminished the KBrO₃-induced changes in MetHb concentration and GPx activity. Renal oxidative stress and kidney damage caused by the KBrO₃ administration were also dose-dependently suppressed by ebselen. On the other hand, ebselen did not suppress the KBrO₃-induced elevation of the NO concentration. KBrO₃-induced methaemoglobinemia, renal oxidative stress and kidney damage, consequently, seemed to result from the attenuation of GPx activity. Besides, the enhancement of NO production was not likely to be a result but a cause for the KBrO₃-induced attenuation of GPx activity. In in vitro experiments, oxidation of human oxyhaemoglobin (HbO₂) to MetHb was observed in a reaction mixture containing HbO₂ and an NO donor, NOC-7 (1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene) or SIN-1 (3-(4-morpholinyl)sydnonimine), and this oxidation was inhibited by the NO scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide). However, no MetHb formation was observed in a reaction mixture containing HbO₂ and KBrO₃. These results suggest that KBrO₃-induced methaemoglobinemia results from the reduction of GPx activity in blood by the KBrO₃-induced increases in superoxide, NO and ONOO⁻.

Key words potassium bromate; methaemoglobin; ebselen; nitric oxide; glutathione peroxidase

MATERIALS AND METHODS

Chemicals Glutathione reductase from bakers yeast (EC 1.6.4.2), nitrate reductase from aspergillus species (EC 1.6.6.2), Hb, MetHb, reduced glutathione (GSH) and oxidized glutathione were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2,4-Dinitrophenylhydrazine (DNPH) obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). KBrO₃, potassium cyanide, sodium hydrosulfite and ebselen were purchased from Wako Pure Chemical Industries (Osaka, Japan). 1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7), 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide sodium salt (carboxy-PTIO) were obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals of the highest grade were obtained commercially.

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 h light

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and dark cycle.

**Treatment of Animals** Mice were administered orally 1.2 mmol/kg of KBrO₃ dissolved in 0.9% NaCl or 0.9% NaCl alone 15 min after intraperitoneal administration of ebselen. Ebselen dissolved in dimethyl sulfoxide (DMSO) or DMSO alone was injected to the abdominal cavity of the mice. All administrations were quickly and carefully carried out without anesthesia. The mice were sacrificed 6 h after the KBrO₃ administration. Kidneys were immediately excised after decapitation, and then were weighed, washed and homogenized with ice-cold PBS (pH 7.4). The homogenate was used for the measurement of protein carbonyl, thiobarbituric acid reactive substances (TBARS), GSH and enzyme activity. Whole blood removed by decapitation was used for the measurements of MetHb concentration, serum creatinine level, plasma NO level and plasma GPx activity. This experimental design was approved by the Animal Experimental 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 Concentration** Serum creatinine concentration was assayed spectrophotometrically with Wako Creatinine Test (Wako Pure Chemical Industries) using a Hitachi U-2000 Spectrophotometer (Hitachi Co., Tokyo, Japan).

**Measurement of Plasma NO Level** Nitrate was reduced to nitrite by nitrate reductase, and then the nitrite plus nitrate concentration was measured as the plasma NO level using Griess reagent.[5]

**Measurement of Plasma Methaemoglobin Concentration** Plasma MetHb concentration was determined spectrophotometrically according to the method of Matsuoka.[6] Absorption spectra of MetHb, HbO₂, COHb, and CNMetHb were measured and the percent ratio of MetHb in total Hb was calculated as described elsewhere.[6]

**Measurement of GPx Activity** Cumen hydroperoxide dissolved in water was used as the substrate to eliminate the effect of catalase contamination in the sample solution on the measurement of GPx activity in plasma and kidney.[7] Enzyme activity was calculated from the decrease in hydrogen peroxide concentration.

**Measurement of Catalase Activity** Catalase activity in the cytosol fraction of mouse kidney cell was measured as described previously.[14] Enzyme activity was calculated from the decrease in hydrogen peroxide concentration.

**Measurement of Protein Carbonyl Group** Protein carbonyl group in mouse kidney was assayed by the method of Reznick et al., using DNPH.[18] Finally, absorbance at 370 nm of guanidine hydrochloride solution was measured and carbonyl concentration was calculated as follows.

\[
\text{Carbonyl concentration (\mu M)} = \frac{\text{OD}_{370} \times 45.45}{5}
\]

**Measurement of TBARS Content** Kidney homogenate was heated with thiobarbituric acid, and then the red pigment that appeared was extracted with a mixture of n-butanol and pyridine (15 : 1 v/v). The absorbance at 535 nm of dehydrated organic solution was measured and the molar extinction coefficient of malondialdehyde (\(e = 1.56 \times 10^5 \text{mM}^{-1} \text{cm}^{-1}\)) was used to calculate TBARS concentration as described elsewhere.[19]

**Measurement of GSH Content** Renal GSH content was determined as described in the previous report using HPLC-UV detector system.[14]

**Measurement of Protein Concentration** Protein concentration was measured spectrophotometrically according to the method of Lowry et al., using bovine serum albumin as the standard protein.[20]

**Measurement of in Vitro MetHb Formation by NO or ONOO⁻** Commercially available human Hb was converted to oxy-haemoglobin (HbO₂) through deoxy-Hb by the method of Murphy et al.[21] To prepared HbO₂ solution (0.3 mg/ml PBS) was added 0.02 mM of NOC-7, SIN-1, KBrO₃, KBrO₃ plus GSH, sodium nitrate or sodium nitrite, and the MetHb formation was identified by the measurement of absorbance spectra at 400—500 nm.[22] The absorbance maximum of HbO₂ and MetHb were identified at 413.5 nm and 406.0 nm, respectively.[22]

**Statistics** Data were expressed as the mean±S.D. A one-way analysis of variance (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 AND DISCUSSION**

In the oxidative stress status Hb is oxidized to MetHb, that is, oxidation of ferrous ion in the haem rings of Hb. Thus, elevation of MetHb concentration is one of the markers of oxidative stress.[16,23] In the KBrO₃-treated mice a significant elevation of MetHb concentration was observed (Table 1), suggesting that oxidative stress status occurred in the erythrocytes of the mice. Therefore, KBrO₃-induced cyanosis[11] is likely to be caused as a consequence of the elevation of MetHb concentration in arterial blood. Moreover, KBrO₃ is

<table>
<thead>
<tr>
<th>Group</th>
<th>NO ((\mu M))</th>
<th>MetHb (%)</th>
<th>GPx activity (units/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>59.5±8.3a</td>
<td>0.95±0.03a</td>
<td>7.40±1.78a</td>
</tr>
<tr>
<td>KBrO₃ 1.2 mmol/kg</td>
<td>362.0±29.6b</td>
<td>6.95±0.05b</td>
<td>4.17±0.09b</td>
</tr>
<tr>
<td>KBrO₃ 1.2 mmol/kg + ebselen 0.15 mmol/kg</td>
<td>347.6±18.8b</td>
<td>5.42±0.07a</td>
<td>5.21±0.30b</td>
</tr>
<tr>
<td>KBrO₃ 1.2 mmol/kg + ebselen 0.3 mmol/kg</td>
<td>334.2±12.4b</td>
<td>2.44±0.01a</td>
<td>6.88±0.88b</td>
</tr>
<tr>
<td>Ebselen 0.3 mmol/kg</td>
<td>76.0±2.8a</td>
<td>0.99±0.08a</td>
<td>6.63±0.88a</td>
</tr>
</tbody>
</table>

Potassium bromate (KBrO₃) dissolved in 0.9% NaCl was administered orally to ddY male mice 15 min after ebselen administration. Ebselen dissolved in DMSO was administered intraperitoneally to mice. All mice were sacrificed 6 h after KBrO₃ administration. Whole blood was removed by decapitation and each measurement was performed as described in Materials and Methods. Values are expressed as mean±S.D. (\(n=4\)). *ab* Values not sharing a common letter are significantly different at \(p<0.05\) (ANOVA with Duncan’s multiple-range test).
known to cause haemolysis. Actually, lysis of red blood cells, of which membranes contain α-tocopherol as a chain-breaking antioxidant, is induced simultaneously with oxidative stress by vitamin E deficiency.

As shown in Table 1, the KBrO₃-induced elevation of MetHb concentration was prevented by the pre-administration of the GPx mimic ebselen. In the present paper, we indicated that reduction of GPx activity was responsible for the KBrO₃-induced renal oxidative stress in mice. In the present experiments, plasma GPx activity also decreased in the KBrO₃-treated mice (Table 1). The decrease in GPx activity would cause an elevation of the concentration of hydrogen peroxide, which interacts with transition metal ions to form hydroxyl radicals, a highly reactive oxygen-centered radical. It appears that ebselen suppressed the KBrO₃-induced oxidative stress in mouse blood by recovering GPx activity. Thus, it is likely that KBrO₃-induced methaemoglobinemia, cyanosis and haemolysis result from the oxidative stress status of blood mainly caused by the reduction of plasma GPx activity.

Nitric oxide production in the blood as indicated by the total of nitrite and nitrate concentrations was enhanced to approximately six-fold of the control by the KBrO₃ administration, simultaneously with the seven-fold elevation of MetHb concentration (Table 1). Formation of NO in the endothelium participates in the determination of blood pressure, which depends on the constriction and dilation of blood vessels. Thus, the serious depression of blood pressure known to be caused by KBrO₃ is likely to be associated with the rapid enhancement of NO production. Reduction of plasma GPx activity in KBrO₃-treated mice, shown in Table 1, seems to result from the enhancement of the production of NO, which is known to inactivate GPx in the form of ONOO⁻ when superoxide anion radical is present. Besides, pre-administered ebselen did not suppress the KBrO₃-induced elevation of NO (Table 1). While, the elevation of MetHb concentration and the reduction of plasma GPx activity were significantly abolished by ebselen (Table 1). These results suggest that ebselen scavenged ONOO⁻, which oxidizes HbO₂ and inactivates GPx.

Creatinine is excreted into urine at the glomeruli of the kidney and is not reabsorbed into the blood. Elevation of serum creatinine concentration indicates the attenuation of creatinine clearance in kidney. In our experiment (Fig. 1), elevation of serum creatinine concentration was observed in KBrO₃-treated mice, and this elevation was suppressed dose-dependently by the administration of ebselen, which is known to be distributed mainly to kidney, blood and bone. Potassium bromate-induced kidney damage, therefore, also appears to be caused by the reduction of cellular GPx activity under the oxidative stress status. In fact, cytoplasmic GPx activity in kidney was reduced remarkably by KBrO₃ administration (Table 2), as in the case of the blood. The reduction of cytoplasmic catalase activity was also observed in the KBrO₃-treated mice. Therefore, reduction of GPx and catalase activities appears to cause oxidative stress by elevating the cellular hydrogen peroxide concentration. In our experiment, recovery by pre-administered ebselen was not observed in KBrO₃-induced reduction of renal GPx activity contrary to plasma GPx (Table 2). Since four forms of GPx, classical GPx, phospholipid hydroperoxide GPx, gastrointestinal isoenzyme and plasma GPx, exist and differ in many properties, the effect of ebselen on renal GPx seems to be different from that on plasma GPx.

Generally, changes of tissue protein carbonyl concentration, TBARS content and GSH content are the indicators of oxidative stress, caused by the elevation of levels of reactive oxygen species (ROS). The TBARS value represents lipid peroxides level. Protein carbonyl is the product that is formed when amino acid residues in proteins are attacked by several reactive oxygen species. Reduced glutathione is a scavenging substance of ROS, and is known to play an important role in scavenging activity.

Table 2. Effect of Ebselen Administration on KBrO₃-Induced Changes of Protein Carbonyl Concentration, TBARS Concentration, GSH Concentration, GPx Activity and Catalase Activity in Mice Kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein carbonyl (nmol/g kidney)</th>
<th>TBARS (µmol/g kidney)</th>
<th>GSH (nmol/g kidney)</th>
<th>GPx (units/mg protein)</th>
<th>Catalase (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>52.5 ± 13.6ₐ</td>
<td>2.24 ± 0.1ₙ</td>
<td>81.3 ± 11.9ₐ</td>
<td>0.227 ± 0.02ₙ</td>
<td>471 ± 91ₙ</td>
</tr>
<tr>
<td>KBrO₃ 1.2 mmol/kg</td>
<td>188.2 ± 19.6ₐ</td>
<td>2.97 ± 0.1₈</td>
<td>52.7 ± 2.₁ₘ</td>
<td>0.064 ± 0.03₂ₙ</td>
<td>264 ± 40ₙ</td>
</tr>
<tr>
<td>KBrO₃ 1.2 mmol/kg + ebselen 0.15 mmol/kg</td>
<td>110.2 ± 32.₃ₘ</td>
<td>2.77 ± 0.1₉</td>
<td>56.9 ± 2.₅ₘ</td>
<td>0.045 ± 0.03ₘ</td>
<td>449 ± 12ₙ</td>
</tr>
<tr>
<td>KBrO₃ 1.2 mmol/kg + ebselen 0.3 mmol/kg</td>
<td>69.₃ ± 27.₃ₕ</td>
<td>2.69 ± 0.2₁ₙ</td>
<td>62.₄ ± 2.₃ₙ</td>
<td>0.04₅ ± 0.01₁ₙ</td>
<td>466 ± 4₁ₙ</td>
</tr>
<tr>
<td>Ebselen 0.3 mmol/kg</td>
<td>58.₅ ± 12.₇₉</td>
<td>2.37 ± 0.4₀ₕ</td>
<td>76.₇ ± 3.ₙ</td>
<td>0.22₁ ± 0.04ₙ</td>
<td>40ₙ ± 3ₙ</td>
</tr>
</tbody>
</table>

Potassium bromate (KBrO₃) dissolved in 0.9% NaCl was administered orally to ddY male mice 15 min after ebselen administration. Ebselen dissolved in DMSO was administered intraperitoneally to mice. All mice were sacrificed 6 h after KBrO₃ administration. Whole blood was removed by decapitation. Serum creatinine concentration was measured as described in Materials and Methods. Values are expressed as mean ± S.D. (n=4). Values not sharing a common letter are significantly different at p<0.05 (ANOVA with Duncan’s multiple-range test).
The KBrO₃-induced decrease in GPx activity appears to be produced decrease in GPx activity (Table 2). The major cause of lipid, protein and DNA bases. Thus, the elevation of ONOO⁻ production is known to bring about the elevation of transition metals 40,41) causes the oxidative modification of ONOO⁻. Methaemoglobin formation was confirmed by measurement of absorbance spectra at 400—500 nm. The absorbance maximum of HbO₂ and MetHb were identified at 413.5 nm and 406.0 nm, respectively. Reaction mixture was added 100 units of SOD or 20mM carboxy-PTIO, respectively. + : Complete shift of UV spectrum of HbO₂ to MetHb was observed. +: Shift of UV spectrum of HbO₂ to MetHb was insufficient (mixture of HbO₂ and MetHb). ND: Shift of UV spectrum of HbO₂ was not observed.

Oxy-haemoglobin (HbO₂) was prepared from marketed human Hb by the method of Murphy et al. 13) HbO₂ solution (0.3 mg/ml PBS) was added 0.02 mM of NOC-7, SIN-1, KBrO₃, KBrO₃ plus GSH, sodium nitrite or sodium nitrate and the methaemoglobin (MetHb) formation was confirmed in the presence of GSH and transition metal by ESR spectrometry, 14) our results of in vitro experiments suggest that the elevation of MetHb concentration in mouse blood induced by KBrO₃ did not result from the direct reaction of HbO₂ with KBrO₃, or superoxide in vessel but the KBrO₃-induced enhancement of NO generation (Chart 1).

In conclusion, our results suggest that KBrO₃-induced methaemoglobinemia results from the reduction of GPx activity in blood by the KBrO₃-induced increase in superoxide. 10) However, no MetHb production was observed in the reaction mixture containing HbO₂ and KBrO₃ in the presence or absence of GSH (Table 3). Chlorate-induced MetHb production has been considered to result from the direct reaction of HbO₂ with chloride. 15) Potassium bromate-induced elevation of MetHb in human blood also has been considered to occur in a similar manner as chloride, since KBrO₃ is also an inorganic oxy-halide and distributed mainly to kidney, blood and bone when it is orally taken. 25) Although superoxide generation from KBrO₃ was confirmed in the presence of GSH and transition metal by ESR spectrometry, 14) our results of in vitro experiments suggest that the elevation of MetHb concentration in mouse blood induced by KBrO₃ did not result from the direct reaction of HbO₂ with KBrO₃ or superoxide in vessel but the KBrO₃-induced enhancement of NO generation (Chart 1).

In conclusion, our results suggest that KBrO₃-induced methaemoglobinemia results from the reduction of GPx activity in blood by the KBrO₃-induced increase in superoxide, NO and ONOO⁻.

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