Dose-related changes of oxidative stress and cell proliferation in kidneys of male and female F344 rats exposed to potassium bromate

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(Received December 15, 2003/Revised March 3, 2004/Accepted March 5, 2004)

It is still of importance to investigate renal carcinogenesis by potassium bromate ($KBrO_3$), a by-product of water disinfection by ozonation, for assessment of the risk to man. Five female F344 rats in each group were given $KBrO_3$ at a dose of 300 mg/kg by single i.g. intubation or at a dose of 80 mg/kg by single i.p. injection, and were killed 48 h after the administration for measurements of thiobarbituric acid-reactive substances (TBARS) and 8-oxodeoxyguanosine (8-oxodG) levels in the kidney. Both levels in the treated animals were significantly elevated as compared with the control values. In a second experiment, 5 male and female F344 rats in each group were administered $KBrO_3$ at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. $KBrO_3$ in the drinking water did not elevate TBARS in either sex at any of the doses examined, but 8-oxodG formation in both sexes at 250 ppm and above was significantly higher than in the controls. Additionally, the bromodeoxyuridine-labeling index for proximal convoluted tubules was significantly increased at 30 ppm and above in the males, and at 250 ppm and above in the females. α2u-Globulin accumulation in the kidneys of male rats was increased with statistical significance at 125 ppm and above. These findings suggest that DNA oxidation induced by $KBrO_3$ may occur independently of lipid peroxidation and more than 250 ppm $KBrO_3$ in the drinking water can exert a carcinogenic effect by way of oxidative stress. (Cancer Sci 2004; 95: 393–398)

Potassium bromate ($KBrO_3$) was at one time widely used as a maturing agent for flour and as a dough conditioner.[1)] It was, however, demonstrated to induce renal cell tumors in male and female F344 rats after oral administration for 2 years in the drinking water[2)] and the use of $KBrO_3$ as a food additive is now limited or prohibited, so that exposure of humans via food is very low.[3)] Nevertheless, there is still concern regarding this chemical in the environment. In order to avoid the formation of trihalomethanes, major by-products in the process of drinking water chlorination[4)] that are carcinogenic in rodents,[5)] ozone disinfection has been proposed as an alternative method.[6)] However, it has been shown that ozonation of surface water can generate bromate as one of various by-products in treated drinking water,[7)] implying a potential hazard.

$KBrO_3$ has been classified as a genotoxic carcinogen based on positive mutagenicity in the Ames,[8)] chromosome aberration[9)] and micronucleus tests.[10)] It has the potential to induce 8-oxodeoxyguanosine (8-oxodG) formation both *in vitro* and *in vivo*,[11–14)] and since ribo- and deoxyribonucleosides of 8-oxodG induce sister chromatid exchange in human lymphocytes[15)] and 8-oxodG pairs with adenine as well as cytosine, generating GC-to-TA transversion upon replication by DNA polymerases,[16)] it has been postulated that this oxidized base is responsible for the mutagenicity and carcinogenicity.[17, 18)] The formation of oxidized base also indicates that the intra-nuclear redox status is altered in an oxidative direction, and this may lead to the induction of aberrant transcriptional events. However, except for our previous paper,[19)] we know of no data showing a direct correlation between actual carcinogenic doses and 8-oxodG formation in kidney DNA. Likewise, although it has been proposed that reactive free radicals resulting from the oxidizing property of $KBrO_3$ also attack membrane lipids to induce cellular lipid peroxidation (LPO) in male rats,[20, 21)] it remains uncertain whether LPO indeed occurs concomitantly with DNA oxidation during carcinogenesis. In view of the possible role of various reactive aldehydes as end products of LPO in tumorigenesis,[22, 23)] it is necessary to assess their participation in $KBrO_3$ carcinogenesis.

A two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine (EHEN) as an initiator has supplied clear evidence that $KBrO_3$ has promoting activity for renal carcinogenesis in male and female rats.[24, 25)] We have also shown that numbers of bromodeoxyuridine (BrdU)-incorporating cells in kidney tubules are elevated in male and female rats exposed to $KBrO_3$ at a dose of 500 ppm in the drinking water.[19, 26)] While we have hypothesized the involvement of oxidative stress induced by $KBrO_3$ in the promoting activity, our previous data also suggest that the promoting action observed in male rats may be dependent on cell proliferation due to accumulation of α2u-globulin, a male rat specific urinary protein.[26)] Elimination of this possibility as a factor contributing to $KBrO_3$ promoting activity is a prerequisite for accurate assessment of the carcinogenic risk in humans.

In the present study, in order to confirm a positive correlation between oxidized DNA base formation and occurrence of LPO, we measured the levels of 8-oxodG and thiobarbituric acid-reactive substances (TBARS) in kidneys of F344 female rats given $KBrO_3$ by single administration at high doses. Secondly, we examined the dose-response effects with reference to 8-oxodG levels, TBARS, BrdU-labeling and α2u-globulin accumulation in kidney, as well as serum creatinine (CRN) level, of male and female rats, employing the same doses and route as used in the previous carcinogenicity tests and promoting activity assays. The aim was to clarify the possibility that LPO and oxidative DNA damage participate in $KBrO_3$ initiation and to cast light on the effects of oxidative stress in the promotion phase.

Materials and Methods

Chemicals. $KBrO_3$ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Alkaline phosphatase and BrdU were obtained from Sigma Chemical Co. (St. Louis, MO) and nuclease P1 was from Yamasa Shoyu Co. (Chiba).

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Animals, diet and housing conditions. The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female F344 rats (specific pathogen-free) were purchased from Charles River Japan (Kanagawa) and housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility maintained under conditions of controlled temperature (23±2°C), humidity (55±5%), air change (12 times per h) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Charles River Japan) and tap water, and were used after a 1-week acclimation period.

Animal treatments.

Experiment I: Five female rats in each group were given $KBrO_3$ at a single dose of 300 mg/kg by i.g. administration or 80 mg/kg by i.p. injection. Control animals received saline at the same volume as the i.p. administration group. All animals were killed 48 h after the administration under ethyl ether anesthesia, and the right and half of the left kidneys were immediately removed and frozen with liquid nitrogen and stored at −80°C until measurement of 8-oxodG in nuclear DNA and TBARS levels. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and H&E staining. The doses and experimental period followed reported conditions under which the 8-oxodG and TBARS levels in kidney were significantly increased.[27]

Experiment II: Five male and female rats in each group were administered $KBrO_3$ solution at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. For analysis of CRN, the animals were anesthetized with ethyl ether and blood was collected from the aorta. Determination of CRN was carried out at SRL, Inc. (Tokyo). At necropsy, the right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The left kidneys were frozen and stored as in Experiment I until measurement of 8-oxodG in nuclear DNA, and TBARS levels and α2u-globulin contents in the homogenates.

Measurement of nuclear 8-oxodG. The 8-oxodG levels in kidney DNA were determined according to the method of Nakae *et al.*[28] Nuclear DNA was extracted with a DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-oxodG (8-oxodG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA).

Measurement of TBARS. Malondialdehyde (MDA, nmol/g) was assessed as an index of LPO by the method of Uchiyama and Mihara.[29] A 0.1 g portion of kidney was homogenized with 0.9 ml of 1.15% KCl solution and the TBARS content was measured.

α2u-Globulin content. α2u-Globulin accumulation in kidneys was measured using a commercially available ELISA kit (Quatikinine M, R&D Systems, Inc., MN). Absorbance at 450 nm was determined using a microplate reader (Thermo Labsystems, Vantaa, Finland), with the reference wavelength set at 590 nm.

Immunohistochemical procedures. For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton Dickinson) (1:100), biotin-labeled horse anti-mouse IgG (1:400) and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4 *N* HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg *et al.*[30] using L-glutamyl-4-methoxy-β-naphthylamide (Polysciences, Ltd., Warrington, PA) as a substrate in order to assist in distinguishing the three kinds of tubules, as previously described.[26] The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

Cell proliferation quantification. Cells of the three kinds of tubules in the kidney were identified on the basis of γ-GT activity and morphology as previously described.[31] At least 3000 tubular cells in each kidney were counted. The labeling index (LI) was calculated as the percentage of cells positive for BrdU incorporation.

Statistics. The significance of differences in the results from Experiment I was evaluated with Student's *t* test. For Experiment II ANOVA was used, followed by Dunnett's multiple comparison test.

Results

Experiment I. The data for 8-oxodG and TBARS levels in kidneys of female rats given $KBrO_3$ by single administration at doses of 300 mg/kg (i.g.) or 80 mg/kg (i.p.) are summarized in Fig. 1. Values for both parameters were significantly ($P<0.01$) elevated as compared with the controls, in line with previous data. Histopathological examination revealed severe nephrotoxicity characterized by hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules, and extensive necrosis of collecting ducts (Fig. 6, A and B).

Experiment II. As shown in Fig. 2, $KBrO_3$ in the drinking water did not cause elevation of TBARS in kidneys of either sex at any of the doses examined. However, 8-oxodG levels in male

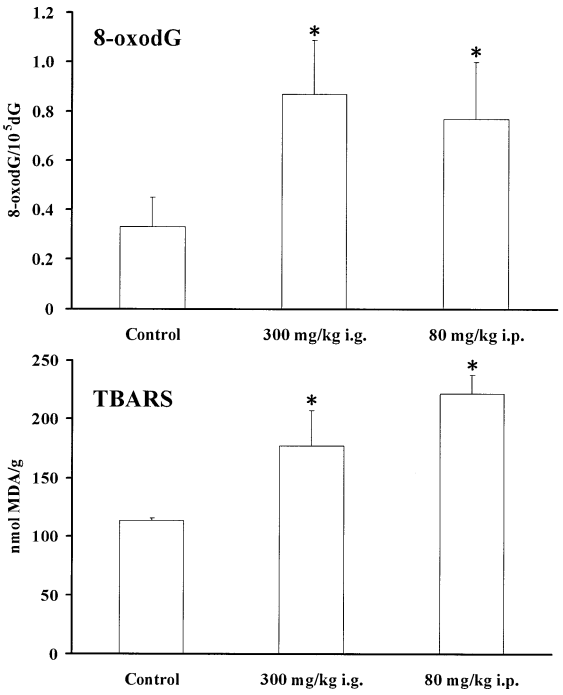

Fig. 1. 8-OxodG and TBARS levels in kidneys of female rats 48 h after single i.g. or i.p. administration of $KBrO_3$ at the dose of 300 or 80 mg/kg, respectively. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group treated with saline alone.

rats exposed to $KBrO_3$ in the drinking water were elevated at concentrations of 250 ppm and above in a clearly dose-dependent manner (250 ppm, $0.57\pm0.19/10^5$ dG, $P<0.01$; 500 ppm, $0.71\pm0.21/10^5$ dG, $P<0.01$) as compared to the control value ($0.31\pm0.06/10^5$ dG). Likewise, 8-oxodG levels in female rats were $0.51\pm0.10/10^5$ dG at 250 ppm and $0.70\pm0.16/10^5$ dG at 500 ppm, which were statistically significantly higher ($P<0.01$) than the control value ($0.25\pm0.05/10^5$ dG). Histopathologically, although degeneration of proximal tubules was dose-dependently observed in the males at 60 ppm and above, there were no overt nephrotoxicity in the females at any of the doses examined. Fig. 3 illustrates changes in BrdU-LI for each tubule type in male and female rats treated with $KBrO_3$ in the drinking water at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm for 4 weeks. BrdU-LIs of proximal convoluted tubular cells (PCT) in the males were elevated in a dose-dependent manner, with significant increases at 30 ppm (1.69±0.32%, $P<0.01$), 60 ppm (2.67±0.45%, $P<0.01$), 125 ppm (4.23±0.80%, $P<0.01$), 250 ppm (6.11±2.23%, $P<0.01$) and 500 ppm (9.10±1.40%, $P<0.01$), as compared to the control value (0.87±0.32%). In the females, although there was no change up to 125 ppm, dose-dependent increase was subsequently observed to 1.29±0.39% at 250 ppm and 2.22±0.37% at 500 ppm, both of which were statistically siginificant ($P<0.01$) as compared to the control value (0.59±0.14%) (Fig. 6C). On the other hand, no change in BrdU-LIs for other tubules was found at any dose in either sex. Fig. 4 summarizes data for α2u-globulin accumulation in kidneys of male and female rats given $KBrO_3$ in the drinking water. In the males, increase was evident at 30 ppm and above in a dose-dependent fashion, the elevation being statistically significant at 125 ppm (1.37±0.18 mg/ml, $P<0.01$), 250 ppm (1.96±0.24 mg/ml, $P<0.01$) and 500 ppm (3.50±0.26 mg/ml, $P<0.01$) as compared to the control value (0.80±0.16 mg/ml). In contrast, α2u-globulin contents in the females were much lower than those in the males and were not changed by $KBrO_3$ exposure. Fig. 5 shows the changes of serum CRN levels in rats of both sexes given $KBrO_3$ in the drinking water. In contrast to the male data, revealing a slight, but statistically significant elevation at 250 ppm and above, there was no change among the female groups.

Discussion

It is generally accepted that oxygen radicals can attack DNA to produce damaged bases, including 8-oxodG, and/or initiate the oxidative decomposition of cellular membranes by LPO,[23]

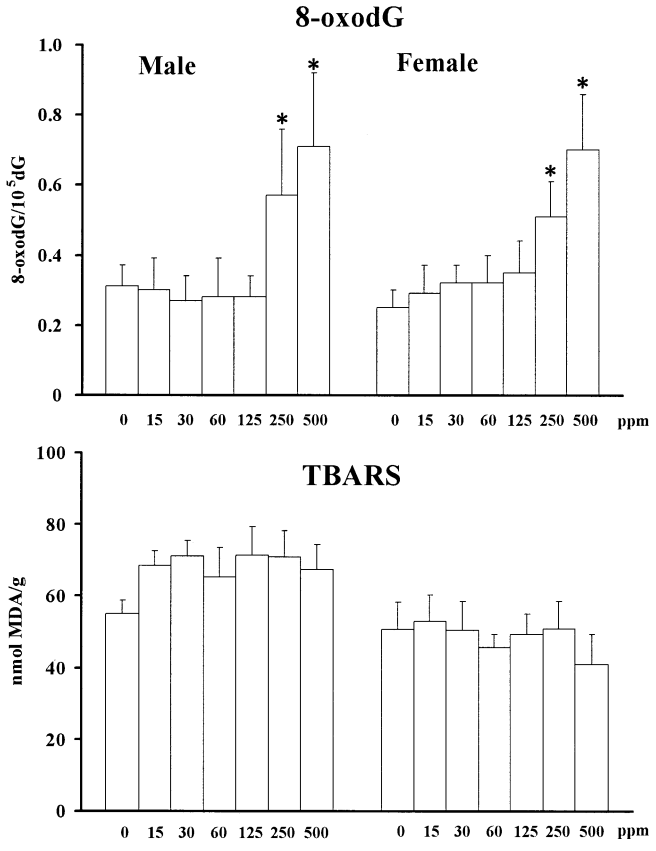

Fig. 2. 8-OxodG and TBARS levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

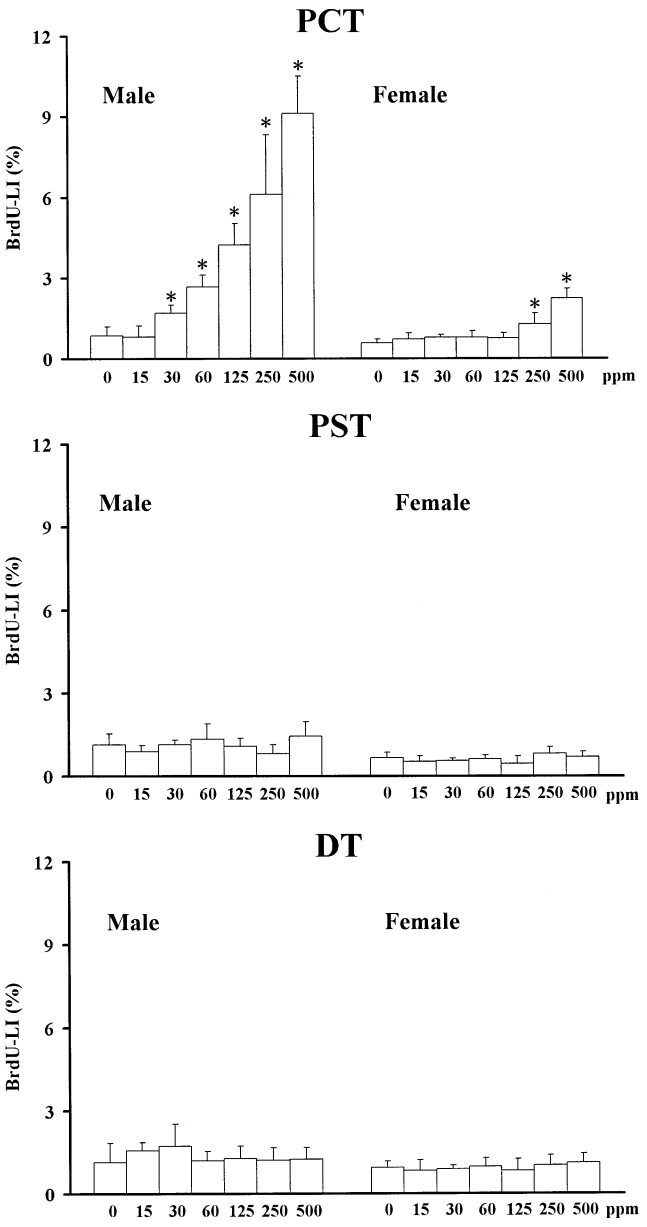

Fig. 3. BrdU-LIs for the proximal convoluted, straight and distal tubules (PCT, PST, DT) of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

which not only act as intermediates for free radical chain reactions, but also generate various reactive aldehydes, such as malondialdehyde and *trans*-4-hydroxy-2-nonenal, which directly form exocyclic DNA adducts.[22, 32] A single exposure of male rats to $KBrO_3$ at high doses causes an increase of TBARS along with 8-oxodG formation,[27] which was also confirmed in the present study using female rats. However, exposure to carcinogenic doses in the drinking water failed to increase TBARS, in spite of the elevation of 8-oxodG levels. Another group has also reported that a single dose of $KBrO_3$ at a low dose did not elevate etheno-DNA adducts formation or TBARS levels in the kidneys of male rats.[21] In the light of the finding of no initiating activity of $KBrO_3$ with a single i.g. administration at 300 mg/kg,[33] our present data indicate that LPO might not be involved in the renal carcinogenesis due to this compound. Instead, histological findings in the present study suggest an involvement of LPO in the nephrotoxicity induced by $KBrO_3$. It has recently been reported that reduction of $KBrO_3$ by sulfhydryl compounds such as glutathione and cysteine yields bromine oxides and bromine radicals, which can effectively oxidize guanine.[34] A large amount of cysteine is supplied as a result of metabolism of glutathione by γ-glutamyltransferase on the proximal tubule brush borders,[35] where $KBrO_3$ reduction might give rise to bromine oxides. Since they are stable in comparison with radicals, they might move into the nuclei, where further reduction could generate bromine radicals in close proximity to nuclear DNA, leading to formation of 8-oxodG without any necessity for intervention of cellular LPO.

Kurokawa *et al*. earlier reported significantly elevated incidences of renal cell tumors in male and female rats given $KBrO_3$ at 250 and 500 ppm in the drinking water for 110 weeks.[2] A further dose-response study using only male rats showed 125 ppm to also be a carcinogenic dose.[36] However, a recent study by another group demonstrated that while $KBrO_3$

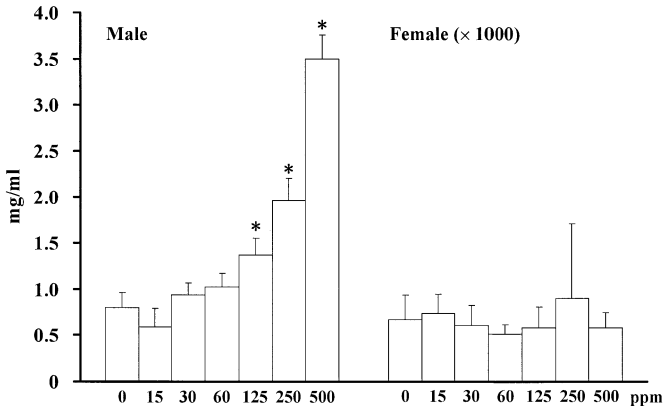

Fig. 4. α2u-Globulin levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Note the values for females are μg/ml. Significantly different (* $P<0.01$) from the control group (0 ppm).

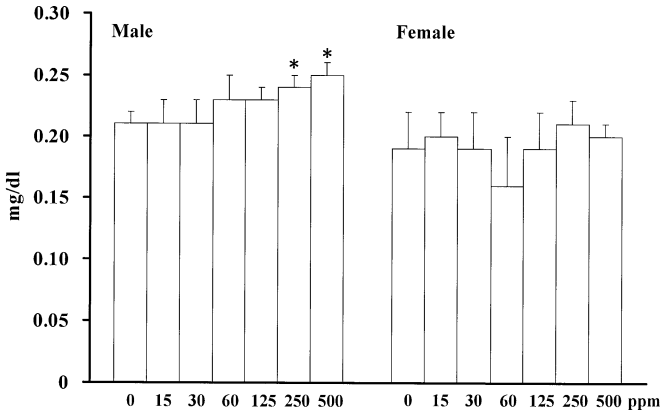

Fig. 5. Serum CRN levels in male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (*, ** $P<0.05$, 0.01) from the control group (0 ppm).

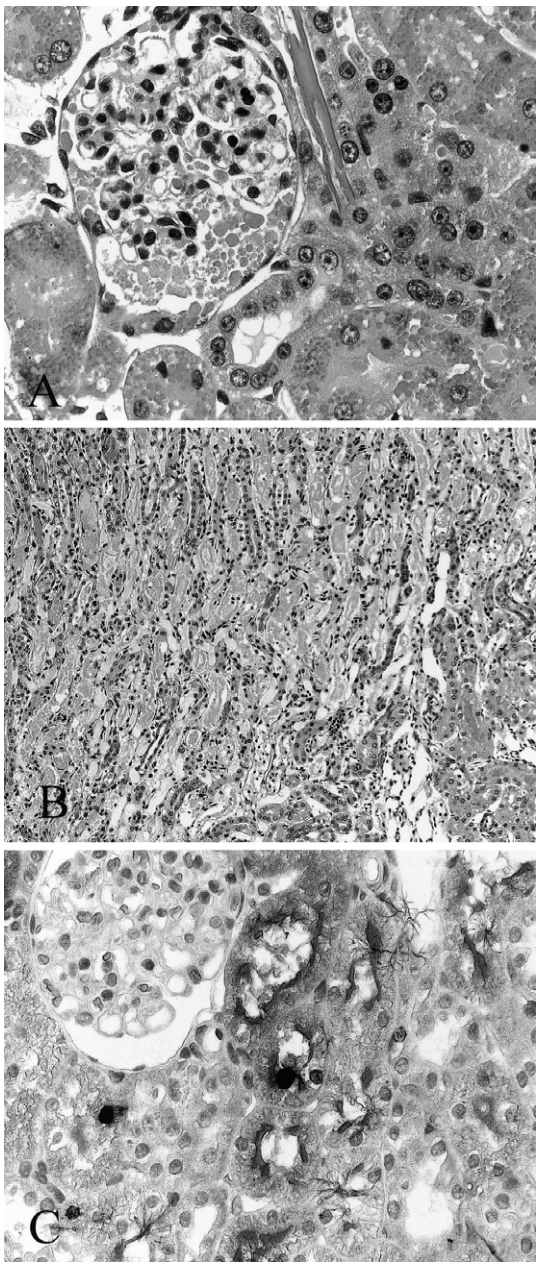

Fig. 6. (A) Renal cortex of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules. H&E staining at ×720 original magnification. (B) Renal medulla of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note extensive necrosis of collecting ducts. H&E staining at ×180 original magnification. (C) Renal cortex of a female rat treated with $KBrO_3$ at 500 ppm in drinking water for 4 weeks. BrdU-positive cells were seen in PCT (positive enzymatic reaction for γ-GT), but not in DT (negative enzymatic reaction for γ-GT). γ-GT-BrdU immunohistochemical staining at ×720 original magnification.

at 400 ppm in the drinking water was able to induce tumors in male rats with significant incidences, this was not the case with 200 ppm.[37] For the present, it seems equivocal whether 125 ppm has a carcinogenic potential. Accordingly, the present demonstration of increased 8-oxodG formation in kidney DNA of male and female rats given $KBrO_3$ at 250 and 500 ppm, but not at 125 ppm and below, seem to be in accordance with the carcinogenic data. In addition, the fact that $KBrO_3$-induced renal cell tumors originate from the proximal tubules[37] allows us to hypothesize that oxidative stress participates in the carcinogenesis. In a previous carcinogenicity study, the mean induction time for tumors in males was much shorter than in females,[2] but there was no sex difference with regard to doses inducing 8-oxodG formation in the present study. Therefore, variation in the tumor latency period might be explained by differential susceptibility to $KBrO_3$-induced cell proliferation, rather than oxidative stress.

In the two-stage rat renal carcinogenesis model using EHEN as an initiator, promoting activity of $KBrO_3$ was apparent in both sexes of rats.[24, 25] In particular, in males, a dose of 30 ppm in the drinking water was sufficient for development of dysplastic foci from initiated cells. We also showed, in the present study, that $KBrO_3$ at the same dose was able to cause degeneration and increase of BrdU-LI in the PCT in the males. α2u-Globulin accumulation in the kidney of male rats exposed to $KBrO_3$ was also observed in a dose-dependent manner at 30 ppm and above, even though the increases at 30 and 60 ppm were not statistically significant. It has been established that this is associated with eventual cell death and subsequent cell proliferation.[38] Despite negative mutagenicity,[39, 40] exposure to this kind of chemical can lead to renal cell tumors in male rats, which implies that α2u-globulin-mediated cell proliferation might be sufficient for tumor development.[41] Thus, it is highly probable that $KBrO_3$-induced cell proliferation in PCT and subsequent tumor-promoting activity observed in males might involve α2u-globulin accumulation. However, the finding that $KBrO_3$ exposure of female rats also increases BrdU-LI in PCT at doses of 250 and 500 ppm in spite of the absence of α2u-globulin indicates an involvement of some other mechanism. Considering that $KBrO_3$ might be reduced to form more reactive species at PCT,[34] the good correlation between the doses inducing 8-oxodG formation and elevation of BrdU-LI enables us to hypothesize that the cell proliferation observed in female rats might result from oxidative stress.[19, 25] Since the histopathological findings and serum biochemical parameters indicate no obvious nephrotoxicity in female rats treated with $KBrO_3$ in the drinking water at any dose tested, oxidative stress might act via mitogenic stimulation.[42–44]

Judging from the female data, it appears that the cell proliferation observed in male rats at 125 ppm and below might be attributed to α2u-globulin accumulation and not to oxidative stress. In other words, the increase at 250 ppm and above in the males might reflect the combined effects of the two. For risk assessment of $KBrO_3$ in the human situation, it is essential to focus on oxidative stress and to ignore α2u-globulin-mediated effects.[45] The overall data allow us to hypothesize that more than 250 ppm of $KBrO_3$ in the drinking water is able to exert both initiating and promoting activities in the kidney of rats of both sexes by means of the generated oxidative stress. Long-term studies now appear warranted for confirmation.

This study was supported by Grants-in-Aid from the Ministry of the Environment.

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