Dysfunction of Nrf2 decreases $KBrO_3$-induced oxidative DNA damage in Ogg1-null mice

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Abstract

Transcription factor Nrf2 regulates production of antioxidants and protects cells from oxidative/electrophilic stresses. Paradoxically, glutathione, one of the Nrf2-regulated antioxidants, has been assumed to promote genotoxicity of $KBrO_3$. To address this glutathione hypothesis, we examined roles Nrf2 plays in the cellular defense against $KBrO_3$-induced oxidative damage using *Nrf2*$^{-/-}$, *Ogg1*$^{-/-}$ and *Nrf2::Ogg1* double knockout mice. We found that upon $KBrO_3$ treatment *Nrf2::Ogg1* double knockout animals suffered from severe kidney damage, but unexpectedly the double knockout mice accumulated lower level of 8-hydroxyguanine than *Ogg1*$^{-/-}$ mice. Thus, $KBrO_3$-induced nephrotoxicity appears not to depend on the formation of 8-hydroxyguanine. Our data also indicate that both the $KBrO_3$-induced nephrotoxicity and formation of 8-hydroxyguanine are Nrf2-controlled processes, but the changes of the glutathione level are an Nrf2-independent. Based on these results we conclude that glutathione is a minor part of the mechanism promoting genotoxicity of $KBrO_3$ in *Ogg1* knockout mice.

Key words: Nrf2; Ogg1; 8-oxoguanine; oxidative stress; oxidative DNA damage; GSH; bromate

Potassium bromate ($KBrO_3$) is an unusual oxidizer, as it requires antioxidants to become harmful to DNA. $KBrO_3$ exerts a strong renal toxicity, inducing proliferative response and damage [1]. For instance, renal cell adenocarcinomas and adenomas were reported to develop in $KBrO_3$-treated rodents [2,3]. Interestingly, despite the primarily renal toxicity associated with $KBrO_3$, it was shown in rats that bromate deposition levels are highest in the liver [12]. Therefore, factors other than simple accumulation may be responsible for the nephrotoxicity of $KBrO_3$.

$KBrO_3$-treated animals accumulate the major oxidative DNA base modification, mutagenic 8-hydroxyguanine (or 7,8-dihydro-8-oxoguanine, 8-OH-G) [4-6]. Since antioxidants were found to play a protective role against $KBrO_3$-induced kidney damage [7], the carcinogenic properties of $KBrO_3$ seem to be related to oxidative stress and oxidative DNA damage. Interestingly, $KBrO_3$ does not react with DNA *in vitro*; it requires thiols to oxidize guanine, with a high genotoxicity-promoting potential demonstrated for glutathione ("glutathione hypothesis") [8-10]. The proposed mechanism assumes a reaction between SH-compounds and bromate anion to generate bromine oxides and radicals [11]. Chemicals depleting glutathione (GSH) were able to decrease $KBrO_3$-induced DNA damage in cultured cells [9-11], arguing for a link between GSH and $KBrO_3$-induced genotoxicity. However, no evidence supporting this link *in vivo* has been obtained.

The oxidation-promoting activity of thiols is highly unusual. GSH is an important antioxidant, and its levels in certain tissues are controlled by transcription factor Nrf2 [13], which plays a crucial role in cellular defense against oxidative and electrophilic stresses [14,15]. The Nrf2 pathway belongs to the central mechanism in coordinated response to electrophiles, and *Nrf2*$^{-/-}$ mice are highly susceptible to cyto- and/or genotoxicity induced by a variety of drugs [16,17]. However, roles Nrf2 plays in cellular response against oxidative DNA damage were scarcely studied. Levels of oxidized DNA bases (often expressed as levels of the major lesion 8-OH-G)

depend on two contrary processes; oxidation and lesion removal. It has been demonstrated that in wild-type mice treated with $KBrO_3$ 8-OH-G level was very low, whereas a high-level accumulation of the lesion was observed in *Ogg1*$^{-/-}$ animals [5,18]. Ogg1 (8-hydroxyguanine glycosylase/lyase) is a crucial mammalian enzyme responsible for removing 8-OH-G from DNA [19]. *Ogg1*$^{-/-}$ animals offer an advantage over wild-type models for the studies of dynamic damage-removal balance by excluding the repair component.

GSH may act as both pro- and antioxidant against $KBrO_3$ *in vivo*, so it is virtually impossible to predict how Nrf2-deficiency would shape the 8-OH-G level. Weakened antioxidative defense may increase oxidation of biomolecules, and possibly increase tissue damage. On the other hand, decrease in the glutathione level may reduce $KBrO_3$-induced oxidative DNA damage and decrease tissue damage.

In this study we examined how Nrf2-deficiency influences $KBrO_3$-induced oxidative DNA damage in the kidneys and livers (respectively the most sensitive target organ of $KBrO_3$ and the organ accumulating highest amount of bromate), using *Nrf2*$^{-/-}$, *Ogg1*$^{-/-}$ and *Nrf2::Ogg1* double knockout mice. Since the link between GSH level and capability of $KBrO_3$ to oxidize DNA *in vivo* has not been proven yet, we also attempted to clarify this issue. Finally, we examined how Nrf2-deficiency affects $KBrO_3$-mediated expression of the rate-limiting enzyme in the GSH biosynthesis, γ-glutamate-cysteine ligase (γ-GCS). The results provide a new insight into our understanding of pro- and antioxidative processes taking place in Nrf2-deficient models.

Materials and methods

Reagents. Unless stated otherwise, chemicals were purchased from WAKO Pure Chemicals.

Mice and treatment. Eight- to ten-week-old female *Nrf2*$^{-/-}$ mice (ICR/129SVJ [14]), *Ogg1*$^{-/-}$ mice (129sv/C57BL/6J [18]), homozygous *Nrf2::Ogg1* double knockout mice (obtained by mating F1 offspring of homozygous *Nrf2*$^{-/-}$ and *Ogg1*$^{-/-}$ mice) and wild-type (WT) offspring of *Nrf2*$^{+/-}$ and *Ogg1*$^{+/-}$ mice were used. Mice were housed under controlled temperature (23±2°C), humidity (40-60%), and lighting (14/10 h light/dark cycle), and fed with basic MF diet (Oriental Bio.) and water ad libitum. $KBrO_3$ was administered in drinking water (2 g/l); mice were sacrificed by cervical dislocation and dissected. Water-drinking animals were used as control.

Blood plasma kidney and liver damage markers. Blood was collected by the retro-orbital plexus method; Na_2EDTA was used as an anticoagulant. Activities of alanine aminotransferase (GPT), aspartate aminotransferase (GOT) and levels of total bilirubin (TBIL), creatinine (Cre) and blood urea nitrogen (BUN) were measured in plasma (obtained by centrifugation) with Dri-Chem 7000V Blood Plasma Analyzer and Fuji Dri-Chem Slides (Fuji-film).

Histopathology and TUNEL assay. Kidneys and livers were preserved in buffered formalin (for histopathology) or deep-frozen in Tissue-Tek OCT Compound (Sakura Finetechnical) for TUNEL. Paraffin specimens were prepared and 4-μm thin sections were stained with standard hematoxyline/eosine method and routinely evaluated by two expert histopathologists. Frozen sections 6 μm thin were used for TUNEL, using the standard horse radish peroxidase/DAB version and the In situ Apoptosis Detection Kit (TaKaRa); slides were counterstained with methyl green. In each slide, three randomly selected frames (each containing ca. 1000 cells) were captured under magnification 200×, using DP70 digital camera mounted to BX51 microscope and controlled by

DP Controller software (Olympus). Brown TUNEL-positive and green negative cells were counted manually.

8-Hydroxyguanine. Entire kidney and ca. 1/3 of liver from each mouse were used for 8-OH-G analysis in nuclear DNA as described previously [18].

Total GSH and GSH-depletion. Tissue samples were homogenized in 0.5 ml of 0.5% 5-sulfosalicylic acid, centrifuged and supernatants stored at -80°C before further processing. GSH was measured using the Total GSH Quantification Kit (Dojindo Molecular Technologies). To deplete glutathione in *Ogg1*$^{-/-}$ mice, animals were pretreated for 2, 4, 6 and 12 days with DL-buthionine-(S,R)-sulfoximine (BSO) at concentration of 20 mM in drinking water. After that time, BSO was replaced with $KBrO_3$ at 2 g/l in drinking water for subsequent 4 days. Mice were sacrificed and tissues collected for measurements of 8-OH-G.

γ-GCS heavy chain expression. $KBrO_3$ at 2 g/l was administered to the mice in drinking water for 5, 10 and 20 h, animals were sacrificed and RNA was isolated with Isogen (Nippon Gene). cDNAs were synthesized using standard procedure with Invitrogen Random Primer mix and SuperScript II polymerase (Invitrogen); real time PCR was performed using standard TaqMan protocol (Roche) and ABI PRISM 7700 Sequence Detector (Applied Biosystems), with two primers (5'-ATCTGCAAAGGCGGCAAC-3', 5'-ACTCCTCTGCAGCTGGCTC-3'), and FAM-probe (5'-ACGGGTGCAGCAAGGCCCA-3'), under the condition of 50°C/ 2 min, 95°C/ 10 min, 95°C/ 15 sec, 60°C/ 60 sec. Ribosomal RNA control was used as a reference (Ribosomal RNA Control Reagents, Roche).

Statistics. Comparisons were done by the Mann-Whitney test, Kruskall-Wallis ANOVA and the median test. Wherever in the results the word "significant" is used, it refers to statistically significant differences at $p>0.05$.

Results and discussion

Nrf2::Ogg1 double knockout mice show an increased susceptibility to $KBrO_3$-induced kidney damage

To examine toxic effects of *$KBrO_3$*, we first examined functions of kidneys and livers of the *Nrf2*$^{-/-}$, *Ogg1*$^{-/-}$ and *Nrf2::Ogg1*$^{-/-}$mice exposed to $KBrO_3$ for 4 days. Although differences between Cre and BUN levels taken separately were not statistically significant between the study groups, a highly increased BUN/Cre ratio was found in *Nrf2::Ogg1* double knockout mice (Fig. 1A), indicative for acute kidney failure. In contrast, BUN/Cre ratio was comparable in WT, *Nrf2*$^{-/-}$ and *Ogg1*$^{-/-}$ mice (Fig. 1A). The liver enzymes GPT and GOT, and total bilirubin, were found to be unchanged (data not shown). The elevated BUN/Cre ratio was accompanied by alterations in the kidney of *Nrf2::Ogg1* double knockout mice that consisted of slightly damaged distal nephritic tubules, dilated collecting ducts and local accumulation of cell debris, and such change was not found in other genotype mice (Fig. 1B). In the liver, several focal inflammatory cell infiltration regions were found, but such mild alterations were present in all animals regardless of the genotype.

Quantitative analysis of TUNEL-positive (i.e., mostly apoptotic) cells revealed significant differences in kidneys, but not in livers (Fig. 1C). In kidneys of all genotypes certain number of TUNEL-positive cells was found, with the lowest positive/negative cells ratio in WT, followed by *Ogg1*$^{-/-}$, *Nrf2*$^{-/-}$, and highest in *Nrf2::Ogg1* double knockout animals. All defective genotypes differed from wild-type animals at $p<0.01$, and double knockout mice differed from *Ogg1*$^{-/-}$ mice at $p=0.04$. No statistically significant difference was found in the case of *Nrf2*$^{-/-}$ vs. *Ogg1*$^{-/-}$ and *Nrf2*$^{-/-}$ vs. *Nrf2::Ogg1* double knockout mice. In livers, the number of TUNEL-positive cells in all groups was comparable to that found in kidneys of the double knockout mice, but it was uniform

in all genotypes.

These results indicate that kidneys of *Ogg1::Nrf2* double knockout mice are more susceptible to toxic action of bromate, but deficiency of Nrf2 and Ogg1 independently renders the mice more prone to $KBrO_3$-induced kidney damage. Since the wild-type and *Ogg1* knockout mice survive a long-term exposure to such a high concentration of $KBrO_3$ [5,6], the increase in TUNEL-positive cells in the kidneys in our short term experiment must be repairable and not critical for survival. We surmise that both the lack of oxidative DNA damage repair and deregulation of cellular defense against oxidative stress combined in *Ogg1::Nrf2* double knockout animals lead to highly elevated BUN/Cre levels in plasma and high apoptotic index in the kidneys. Collectively, these results clearly reflect increased $KBrO_3$ toxicity to kidneys of *Ogg1::Nrf2* double knockout mice.

Nrf2::Ogg1 double knockout mice show reduced levels of $KBrO_3$-induced 8-OH-G

We next measured 8-OH-G in the form of deoxynucleoside in kidneys and livers as a marker of oxidative stress to DNA. In the kidneys, although the baseline levels of 8-OH-G were very low in all cases, close to the detection limit of the HPLC-ECD technique, the result obtained for *Nrf2::Ogg1* double knockout mice was significantly elevated compared to all remaining genotypes (Fig. 2A). There was no significant difference in baseline level of 8-OH-G between WT, $Nrf2^{-/-}$ and $Ogg1^{-/-}$ mice. $KBrO_3$ significantly increased 8-OH-G in all genotype mice, with the lowest increase in the wild-type mice, followed by *Nrf2::Ogg1* double knockout and $Nrf2^{-/-}$, and it was highest in $Ogg1^{-/-}$ mice. Difference in $KBrO_3$-induced 8-OH-G between WT and $Nrf2^{-/-}$ mice was not statistically significant, whereas differences between the remaining genotypes were significant. Thus, upon treatment with $KBrO_3$, concomitant loss of Nrf2 decreased the accumulation of 8-OH-G facilitated by the loss of Ogg1, suggesting that certain factor under Nrf2 regulation exacerbates

the $KBrO_3$-induced 8-OH-G formation. At this stage, we speculated that one of the candidates of such factor was GSH.

In the case of livers, a similar pattern of 8-OH-G accumulation as that in kidneys was found (Fig. 2A). The baseline levels of 8-OH-G in livers were comparable to those obtained for kidneys in WT, *Nrf2*$^{-/-}$ and *Nrf2::Ogg1* double knockout mice, but nearly ten times higher in *Ogg1*$^{-/-}$ mice. Differences in the baseline level of 8-OH-G between the genotypes were again statistically significant, with the lowest level of 8-OH-G in WT, followed by *Nrf2*$^{-/-}$, *Ogg1*$^{-/-}$, and *Nrf2::Ogg1* double knockout mice. The difference in the baseline levels of 8-OH-G was not significant in the livers of *Nrf2*$^{-/-}$ vs. WT, but 8-OH-G in *Ogg1*$^{-/-}$ and *Nrf2::Ogg1* double knockout mice was significantly elevated. These results indicate that in homeostatic conditions both Nrf2 and Ogg1 act cooperatively to prevent accumulation of oxidative DNA damage in the liver.

While the $KBrO_3$-induced levels of 8-OH-G were nearly equal in livers and kidneys of WT mice, the 8-OH-G level was 1.9-fold lower in livers than in kidneys of *Nrf2*$^{-/-}$, 3.1-fold lower in *Ogg1*$^{-/-}$, and 2.1-fold lower in *Nrf2::Ogg1* double knockout mice. Treatment with $KBrO_3$ significantly increased the 8-OH-G level in three of the studied genotypes, with the highest increase found again in *Ogg1*$^{-/-}$, followed by wild-type, and *Nrf2*$^{-/-}$ mice. However, the increase was non-significant in the livers of double knockout mice (Fig. 2A). These results are in a good agreement with those observed for kidneys and they further support our contention that certain factor regulated by Nrf2 exacerbates the 8-OH-G formation.

Considering the enfeebled increase of 8-OH-G compared to *Ogg1*$^{-/-}$ mice, it is conceivable that the nephrotoxicity observed in *Nrf2::Ogg1* double knockout mice may not correspond directly to the genotoxicity of $KBrO_3$. Histologically the kidneys were proven to be more susceptible to $KBrO_3$ than the livers. However, the highest accumulation of 8-OH-G was observed in the kidneys

of *Ogg1*$^{-/-}$ mice, not in the kidneys of double knockout animals. Importantly, the additional knockout of *Nrf2* in *Ogg1*$^{-/-}$ background significantly reduced the susceptibility to guanine oxidation, indicating that the 8-OH-G level is Nrf2-dependent. The lack of difference between WT vs. *Nrf2*$^{-/-}$ mice can be attributed to the highly effective removal of 8-OH-G by Ogg1. Therefore, only in the Ogg1-defective background the protective effect of *Nrf2* knockout was possible to observe. In contrast, baseline levels of 8-OH-G both in kidneys and livers of *Nrf2::Ogg1* double knockout animals were elevated compared to other genotypes, indicating that highly impaired protection against oxidative stress caused by disabling two genes crucial for the process increased endogenous oxidative DNA damage (Fig. 2A).

BSO decreases $KBrO_3$-induced 8-OH-G in Ogg1$^{-/-}$ *mice*

Since several *in vitro* studies demonstrated a relationship between oxidizing properties of $KBrO_3$ and GSH [8,9,10], one plausible explanation for the observed reduction of 8-OH-G level in *Ogg1::Nrf2* double knockout mice seemed to be a downregulation of GSH synthesis due to the lack of Nrf2. However, since no protective effect of depleting GSH on oxidative DNA damage was found in a rat study [8], a prerequisite for further efforts to verify "the glutathione hypothesis" was to find a firm link between GSH and $KBrO_3$-induced oxidative lesions in an animal model. We addressed this issue utilizing the *Ogg1*$^{-/-}$ mice pretreated with a GSH synthesis inhibitor BSO, as the high efficiency of 8-OH-G removal may likely lay behind the lack of correlation in experiments performed in *Ogg1*$^{+/+}$ systems. Indeed, upon treatment with BSO the 8-OH-G level was clearly reduced in both the kidneys and livers (Fig. 2B). The effect was increasingly strong along with the pretreatment time, and reduction to 39.3% was observed in the kidneys of animals treated with BSO for 12 days. In the livers, a similar reduction was observed up to 6 days, when

the level of 8-OH-G decreased to 53.4%, but at 12 days 8-OH-G was increased. This may be due to high capacity of the liver to produce GSH in order to maintain the redox homeostasis. These results thus demonstrate that GSH is indeed involved in the 8-OH-G formation, at least in Ogg1-deficient animals.

Total GSH does not correspond to 8-OH-G in kidneys and livers of $KBrO_3$-treated mice

To elucidate the mechanism for the reduction of 8-OH-G level in *Ogg1::Nrf2* double knockout mice compared to *Ogg1*$^{-/-}$ genotype, we measured actual levels of GSH both in control and $KBrO_3$-treated mice (Fig. 3). We found that GSH levels were much higher in livers than in kidneys and there exist a significant difference in the GSH level in water-drinking and $KBrO_3$-treated mice. Importantly, alterations in GSH level caused by $KBrO_3$ were diversified. Both in kidneys and livers of WT and *Nrf2::Ogg1* double knockout mice $KBrO_3$ reduced total GSH, whereas no significant change was found in kidneys and livers of *Ogg1*$^{-/-}$ mice. The GSH depletion by $KBrO_3$ in WT mice is in a good agreement with results obtained in rats by Giri *et al.* [1]. In contrast, a small increase of GSH level was found in the livers and kidneys of *Nrf2*$^{-/-}$ mice treated with $KBrO_3$. Since Nrf2 regulates enzymes in the GSH biosynthesis pathway and indeed the GSH level was reduced in the water-drinking *Nrf2*$^{-/-}$ mice, this observation was against our expectation. Additionally, no correlation was found between GSH and 8-OH-G levels measured in the same mice. Therefore, GSH level alone is not sufficient to explain the susceptibility of cellular DNA toward $KBrO_3$-induced oxidation. This conclusion is further supported by the observation that livers contain more GSH but less 8-OH-G than kidneys.

$KBrO_3$-induced expression of γ-GCS is Nrf2-independent in kidneys and livers

$KBrO_3$ induced γ-GCS both in kidneys and livers of WT, *Nrf2*$^{-/-}$ and *Ogg1*$^{-/-}$ mice (Fig. 4). In *Nrf2::Ogg1* double knockout mice results were inconclusive due to a high variation, but still the highest measured values were much higher than those for wild-type untreated controls. Therefore, Nrf2 activity is not essential for the induction of γ-GCS by $KBrO_3$, and GSH appears to be an Nrf2-independent part in this experimental system. Apparently other mechanisms are involved in the induction of γ-GCS in this model, either permanent or compensatory. Factors other than Nrf2 may be responsible for upregulation of γ-GCS in kidneys and livers upon induction by $KBrO_3$. The compensatory variant would employ additional factors only when the Nrf2-centered pathway is defective or inefficient. Further study is needed to address this question.

Conclusion

Although GSH depletion experiments in *Ogg1*$^{-/-}$ mice suggest a direct link between GSH level and susceptibility to $KBrO_3$-induced DNA oxidation, the mechanisms responsible for promoting the oxidative potential of $KBrO_3$ appear to be more complex in Nrf2-deficient mice. Based on the present results, we conclude that i) both $KBrO_3$-induced nephrotoxicity and 8-OH-G level depend on Nrf2-controlled processes; ii) $KBrO_3$-induced nephrotoxicity does not depend on oxidative DNA damage, rather being related to other cytotoxic properties of bromate; iii) GSH is an Nrf2-independent and a minor part of the mechanism promoting genotoxicity of $KBrO_3$. We postulate existence of a yet unidentified Nrf2-dependent factor playing a major role in promoting $KBrO_3$-induced genotoxicity in Nrf2-defective mice.

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Figure legends

Fig. 1. Tissue damage markers in mice exposed to 2 g/l $KBrO_3$ for 4 days. (A) Blood nitrogen urea (BUN) / creatinine (Cre) ratio in blood plasma in the studied genotypes (means and SD values for 6 animals). (B) Tissue damage in HE-stained sections of kidneys (arrows indicate necrotic cell debris). (C) Apoptotic cells in kidneys and livers measured by HRP/DAB-TUNEL staining (means and SD values for 3 animals).

Fig. 2. Levels of 8-OH-G in nuclear DNA. (A) 8-OH-G in kidneys and livers of mice exposed to 2 g/l $KBrO_3$ for 4 days. (B) 8-OH-G in kidneys and livers of *Ogg1*$^{-/-}$ mice pretreated for 2-12 days with 20 mM of BSO, a GSH synthesis inhibitor, and subsequently with 2 g/l $KBrO_3$ for 4 days. Mean and SD values are shown for the indicated (n) number of animals.

Fig. 3. Changes in total GSH in kidneys (A) and livers (B) caused by treatment of the mice with 2 g/l $KBrO_3$ for 4 days. Mean and SD values are shown for the indicated (n) number of animals.

Fig. 4. Changes in expression of γ-GCS in kidneys and livers caused by treatment of the mice with 2 g/l $KBrO_3$ for 5, 10 or 20 hours. Mean and SD values for triplicates are shown.

Fig. 1

Fig. 2.

Fig. 3.

Fig. 4.