Toxicology and Applied Pharmacology 272 (2013) 391–398

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Association of brominated proteins and changes in protein expression in the rat kidney with subcarcinogenic to carcinogenic doses of bromate ☆

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ARTICLE INFO

Article history:
Received 25 April 2013
Revised 13 June 2013
Accepted 17 June 2013
Available online 26 June 2013

Keywords:
Kidney
Nephrotoxicity
Bromate
Water disinfection byproduct
Apoptosis
3-Bromotyrosine

ABSTRACT

The water disinfection byproduct bromate (BrO_3^-) produces cytotoxic and carcinogenic effects in rat kidneys. Our previous studies demonstrated that BrO_3^- caused sex-dependent differences in renal gene and protein expression in rats and the elimination of brominated organic carbon in their urine. The present study examined changes in renal cell apoptosis and protein expression in male and female F344 rats treated with BrO_3^- and associated these changes with accumulation of 3-bromotyrosine (3-BT)-modified proteins. Rats were treated with 0, 11.5, 46 and 308 mg/L BrO_3^- in drinking water for 28 days and renal sections were prepared and examined for apoptosis (TUNEL-staining), 8-oxo-deoxyguanosine (8-oxoG), 3-BT, osteopontin, Kim-1, clusterin, and p-21 expression. TUNEL-staining in renal proximal tubules increased in a dose-related manner beginning at 11.5 mg BrO_3^-/L in female rats and 46 mg/L in males. Increased 8-oxoG staining was observed at doses as low as 46 mg/L. Osteopontin expression also increased in a dose-related manner after treatment with 46 mg/L, in males only. In contrast, Kim-1 expression increased in a dose-related manner in both sexes, although to a greater extent in females at the highest dose. Clusterin and p21 expression also increased in a dose-related manner in both sexes. The expression of 3-BT-modified proteins only increased in male rats, following a pattern previously reported for accumulation of α-2_u-globulin. Increases in apoptosis in renal proximal tubules of male and female rats at the lowest doses suggest a common mode of action for renal carcinogenesis for the two sexes that is independent of α-2_u-globulin nephropathy.

Introduction

Bromate (BrO_3^-) is produced as a by-product of disinfection of drinking water with ozone. It has been shown to be carcinogenic to the kidney and thyroid of both male and female F344 rats and in the testicular mesothelium (DeAngelo et al., 1998; Kurokawa et al., 1990). BrO_3^- also induces renal tumors in hamsters and mice, but at much higher doses than required for rats (Gold et al., 2012). Further, there are substantive differences in the toxicodynamics of BrO_3^- in male and female rats (Bull et al., 2012; Kolisetty et al., in press; Umemura et al., 2004). These observations raise important questions about appropriate approaches for the assessment of cancer risk from BrO_3^- to humans consuming small amounts in ozonated drinking water.

We recently studied the kinetics of BrO_3^- absorption and distribution in F344 rats (Bull et al., 2012). BrO_3^- was rapidly reduced upon absorption, a process most likely mediated by plasma thiols. Most BrO_3^- was recovered in the urine as bromide (Br^-), but a small amount was eliminated in the urine as brominated organic carbon. Previous authors (Umemura et al., 2004) have shown that male rats treated with BrO_3^- develop α-2_u-globulin nephropathy. BrO_3^- was unique among oxidants in producing this pathology. Given this last observation and recognition that protein tyrosines are favored sites of bromination *in vivo* (Mita et al., 2004) we decided to determine

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http://dx.doi.org/10.1016/j.taap.2013.06.018

whether BrO_3^--treatment increased levels of 3-bromotyrosine-modified proteins in the kidney. α-2u-Globulin includes 7 tyrosines in its structure (Unterman et al., 1981) and is produced in large quantities by the liver of the male rat. Therefore, it was hypothesized that bromination of α-2_u-globulin may account for its accumulation in the male rat kidney and the resulting nephropathy.

In another recent study (Kolisetty et al., in press) we demonstrated substantial differences in gene expression in the renal cortex and protein expression in the renal proximal tubules of male and female F344 rats treated at concentrations of 96 and 308 mg BrO_3^-/L of drinking water for 28 days. This study found increases in the expression of the glycoprotein osteopontin in male, but not female rats. A similar difference between the sexes was observed in the expression of the KIM-1 gene. Other changes in expression were observed in both sexes, including the cell cycle-dependent kinase inhibitor p21 and clusterin. Osteopontin is a glycoprotein and a marker of renal tubular injury (Thukral et al., 2005; Xie et al., 2001), while clusterin is associated with clearance of cell debris and apoptosis (Jones and Jomary, 2002).

The objective of the present study was to examine patterns of protein expression at concentrations of 11.5, 46, and 308 mg BrO_3^-/L of drinking water that are separable from the effects of α-2_u-globulin nephropathy and to gather dose–response data using lowest doses that induced renal cancer (DeAngelo et al., 1998; Kurokawa et al., 1990) We report that proximal renal tubule cells of both male and female rats display increased rates of apoptosis and display changes in protein expression consistent with a secondary suppression of apoptosis. These data were considered to be more relevant to effects that might be observed in humans.

Materials and methods

Materials. Potassium bromate ($KBrO_3$) (99+ %) was purchased from ACROS organics (Fair Lawn, NJ, USA). Acetonitrile, xylene, ethanol (histological grade), ammonium hydroxide, Gill's counter stain, 30% hydrogen peroxide and tissue cassettes were purchased from Fisher Scientific (Hampton, NH, USA). Cocktail anesthesia was made with a mixture of ketamine hydrochloride (100 mg/mL) (Fort Dodge Animal Health, Fort Dodge, IA, USA), acepromazine maleate (10 mg/mL) (Boehringer-Ingelheim, Saint Joseph, MO, USA), and xylazine hydrochloride (20 mg/mL) (Lloyd Laboratories, Shenandoah, IA, USA) in the ratio of 3:2:1 v/v in our lab. Normal saline (0.9% w/v NaCl) solution was prepared by dissolving 9 g NaCl (Fisher Scientific) in 1 L deionized (DI) water. ABC and DAB immunohistostaining kits were purchased from Vector Labs (Burlingame, CA, USA). Carnoy's fluid was prepared by mixing 100% ethanol, chloroform and glacial acetic acid (Fisher Scientific) in a ratio of 6:3:1 (v/v). The 8-oxo-deoxyguanosine (8-oxoG) antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). Antibodies against p21 and clusterin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against kidney injury molecule (Kim-1) and osteopontin were purchased from R&D Scientific (Flanders, NJ, USA) and AbCam (Cambridge, MA, USA), respectively. The TUNEL apoptosis detection kit was purchased from GenScript (Piscataway, NJ, USA). The 3-bromotyrosine antibody was a gift of Dr. Richard Zangar, Pacific Northwest National Laboratories (Richland, WA, USA). Liquid nitrogen and nitrogen gas were purchased from Airgas (Athens, GA, USA).

Animal preparation. Aged matched male and female F344 rats weighing between 160 and 240 g were purchased from Charles River (Indianapolis, IN, USA). Animals were housed and maintained in accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol at The University of Georgia (UGA), Athens, GA, USA, and in accordance with the U.S. Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Animals were housed in pathogen-free cages within a light and temperature controlled isolated room and provided with autoclaved rodent chow Picolab® Rodent Diet 20, 5053 (PMI Nutrition International LLC, Brentwood, MO, USA), and autoclaved DI water *ad libitum*. Temperature and humidity were maintained at 72 °F and 40%, respectively. Animals were allowed to acclimate one week prior to BrO_3^- treatment. All treatments and protocols were approved by an IACUC at the UGA.

28 day drinking water study. Groups of 10 rats for each dose and sex were treated with 0, 11.5, 46, and 308 mg BrO_3^-/L drinking water (deionized) for 28 days. Water, food consumption and body weight were measured 3 times a week throughout the study. As in our previous study (Bull et al., 2012), the unit dose to female rats was 10–25% higher than in male rats at the two lowest doses (0, 1.08, 4.01 and 27.1 mg/kg day^{-1} in male rats and 0, 1.18, 4.68 and 29.6 mg/kg day^{-1} in female rats at 0, 11.5, 45, and 308 mg/L of drinking water). These were the same animals used for samples in the present study.

On day 28, body weights were recorded and the animals were euthanized by cervical dislocation. Following isolation of blood (closed cardiac puncture) and urine, whole body perfusion was performed using saline (0.9% NaCl) for tissue collection. Kidneys were collected, weighed and fixed in Carnoy's fluid for histopathology and immunohistochemistry.

Immunohistochemistry. Slides were deparaffinized in xylene 5 min each 2 times, followed by dehydration using gradients of histological grade ethyl alcohol (100%, 95% and 70%) for 6 min each. Slides were then rinsed in PBS. Later, slides were incubated in 30% H_2O_2 for 30 min; followed by incubation with 2% normal horse serum for 20 min to block nonspecific binding. Sections then were washed two times with PBS and incubated with primary antibodies against clusterin (10 μg/mL), Kim-1 (15 μg/mL) or osteopontin (10 μg/mL) for 24 h, or antibodies against 8-oxoG (9.75 μg/mL) for 18 h, or p21 (10 μg/mL) for 3 h, or 3-BT (10 μg/mL) at room temperature. Following incubation with the primary antibody, samples were washed three times in PBS for 5 min and incubated in PBS containing a universal secondary antibody and Elite Reagent (ABC Kit, Vector labs) for 30 min each, followed by incubation with DAB reagent and a counter stain (Gill's Haematoxylin). Slides were then bathed in ammonium hydroxide (10% v/v), dehydrated, and fluoromount was applied followed by cover slips. Visualization of staining was done using a Nikon AZ100 fluorescence microscope. Six pictures were taken randomly from each slide for each antibody per tissue. If the staining was nuclear, total nuclei as well as positive nuclei were counted. Results were expressed as % nuclei in a given field. If the staining was cytoplasmic, a score of 0–4 was given based on percent tubules stained in a given field. A score of 0 was given for no staining, 0.5 for ≤10%, 1 for 11–25%, 2 for 26–50%, 3 for 51–75%, and 4 for 76–100% staining in a given field.

TUNEL staining. Slides were incubated in xylene for 5 min each twice, followed by dehydration with a gradient of histological grade ethyl alcohol (100%, 95% and 70%) for 6 min each. Then slides were rehydrated and washed in PBS. Slides were placed in 0.1 M citrate buffer (pH 6) and microwaved for 1 min at 750 W, cooled with water and rinsed in PBS for 5 min. Later, slides were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min, and 100 μL DNase I Solution for 10 min (for the positive control slides only), and 50 μL TUNEL reaction mixture for 60 min, 50 μL streptavidin–HRP Solution for 30 min, followed by incubation with DAB reagent and a counter stain (Gill's). Slides were then bathed in ammonium hydroxide (10% v/v), dehydrated, and fluoromount was applied followed by cover slips. Visualization of staining was done using a Nikon AZ100 fluorescence microscope. Six pictures were taken randomly from each slide for each antibody per tissue. For nuclear staining, total nuclei as well as positive nuclei were counted. Results were expressed as % nuclei in a given field.