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Toxicological significance of renal Bcrp: Another potential transporter in the elimination of mercuric ions from proximal tubular cells

Christy C. Bridges *, Rudolfs K. Zalups, Lucy Joshee

Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, GA, USA

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ABSTRACT

Secretion of inorganic mercury (Hg^{2+}) from proximal tubular cells into the tubular lumen has been shown to involve the multidrug resistance-associated protein 2 (Mrp2). Considering similarities in localization and substrate specificity between Mrp2 and the breast cancer resistance protein (Bcrp), we hypothesize that Bcrp may also play a role in the proximal tubular secretion of mercuric species. In order to test this hypothesis, the uptake of Hg^{2+} was examined initially using inside-out membrane vesicles containing Bcrp. The results of these studies suggest that Bcrp may be capable of transporting certain conjugates of Hg^{2+}. To further characterize the role of Bcrp in the handling of mercuric ions and in the induction of Hg^{2+}-induced nephropathy, Sprague–Dawley and Bcrp knockout (*bcrp*$^{-/-}$) rats were exposed intravenously to a non-nephrotoxic (0.5 μmol·kg^{-1}), a moderately nephrotoxic (1.5 μmol·kg^{-1}) or a significantly nephrotoxic (2.0 μmol·kg^{-1}) dose of $HgCl_2$. In general, the accumulation of Hg^{2+} was greater in organs of *bcrp*$^{-/-}$ rats than in Sprague–Dawley rats, suggesting that Bcrp may play a role in the export of Hg^{2+} from target cells. Within the kidney, cellular injury and necrosis was more severe in *bcrp*$^{-/-}$ rats than in controls. The pattern of necrosis, which was localized in the inner cortex and the outer stripe of the outer medulla, was significantly different from that observed in Mrp2-deficient animals. These findings suggest that Bcrp may be involved in the cellular export of select mercuric species and that its role in this export may differ from that of Mrp2.

Introduction

Inorganic mercury (Hg^{2+}) accumulates predominately in the kidneys, specifically in the epithelial cells lining the S1, S2 and S3 segments of proximal tubules (Rodier and Kates, 1988; Rodier et al., 1988; Zalups, 1991, 2000). Within biological systems, mercuric ions are thought to bind preferentially to thiol-containing molecules to form thiol *S*-conjugates of Hg^{2+} (Zalups, 2000; Bridges and Zalups, 2010). Thiol-*S*-conjugates of Hg^{2+} have been shown to be taken up by transport mechanisms present in luminal and basolateral membranes of proximal tubular epithelial cells (Zalups, 2000; Bridges and Zalups, 2010).

Once mercuric ions gain access to the intracellular compartment of target cells, they tend to be retained within the intracellular compartment due to complex binding reactions of these ions with protein- and non-protein thiols (Zalups, 2000; Clarkson et al., 2007). Mercuric ions are powerful electrophiles and thus, retention of these ions within cells may lead to serious deleterious effects in target cells. Indeed, exposure to moderate (1.5 μmol·kg^{-1} in rats) levels of Hg^{2+} can lead to acute renal tubular changes, which can be characterized by loss of membrane integrity, atrophy and subsequent death of the epithelial cells lining the proximal tubule. In cases of mild to moderate intoxication, cellular injury and death occur primarily in S2 segments located at the cortico-medullary junction and in S3 segments in the outer stripe of the outer medulla (OSOM) (Zalups and Diamond, 1987; Zalups et al., 1991; Zalups, 1997, 2000; Bridges and Zalups, 2010). In cases of severe nephropathy, cellular necrosis may be evident in other segments of the nephron, including S1 segments in the cortex.

A number of recent studies have identified specific mechanisms that are involved in the entry of mercuric species into proximal tubular epithelial cells (Zalups, 2000; Bridges and Zalups, 2010). However, little is known about the precise mechanisms involved in the export of mercuric ions from target cells. *In vivo* and *in vitro* studies have recently implicated the multidrug resistance-associated protein 2 (Mrp2), localized in the luminal membrane of proximal tubular cells (Schaub et al., 1999), in the export of certain mercuric species from within proximal tubular cells into the tubular lumen (Bridges et al., 2008a,b; Zalups and Bridges, 2009; Bridges and Zalups, 2010; Bridges et al., 2011). The results of these studies suggest that additional transport proteins may also be involved in the proximal tubular secretion of mercuric species into the tubular lumen. One potential candidate for this secretion is

Abbreviations: Bcrp, breast cancer resistance protein; Mrp2, multidrug-associated resistance protein 2; DMPS, 2,3 dimercapto-1-propane succinate; Hg^{2+}, inorganic mercury; OSOM, outer stripe of the outer medulla; Cys, cysteine; SD, Sprague–Dawley; *bcrp*$^{-/-}$, Bcrp knockout; *mrp2*$^{-/-}$, Mrp2 knockout.

* Corresponding author at: Mercer University School of Medicine, Department of Basic Medical Sciences, 1550 College St., Macon, GA 31207, USA. Fax: +1 478 301 5487.
E-mail address: bridges_cc@mercer.edu (C.C. Bridges).

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the breast cancer resistance protein (Bcrp; Abcg2). Like, Mrp2, Bcrp is an ATP-binding cassette protein that is localized in the apical membrane of proximal tubular epithelial cells (Huls et al., 2008) and it has been shown to be involved in the transport of a wide variety of drugs and xenobiotics (Leslie et al., 2005; Vlaming et al., 2009; Konig et al., 2013). Considering the similarities in localization and substrate specificity between Bcrp and Mrp2, we hypothesize that Bcrp may also play a role in the export of mercuric species from within proximal tubular epithelial cells. To test this hypothesis we 1) assessed the transport of mercuric species in inside-out membrane vesicles containing Bcrp; and 2) examined the disposition and nephrotoxicity of various doses of mercuric chloride ($HgCl_2$) in control and Bcrp knockout rats.

Methods

Animals. Male Bcrp (Abcg2) knockout rats (SD-*Abcg2*^{tm1sage}; *bcrp*$^{-/-}$) were obtained from Sage Labs (Huang et al., 2012; Zamek-Gliszczynski et al., 2012). Male Sprague–Dawley (SD) rats were used as controls and were obtained from Charles River Laboratories. Rats were housed in the Mercer University School of Medicine animal facility. Animals were provided a commercial laboratory diet (Teklad Global Soy Protein Free Extruded Rodent Diet, Harlan Laboratories) and water *ad libitum* throughout all aspects of the present study. All procedures involving animals were reviewed and approved by the Mercer University Institutional Animal Care and Use Committee. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Intravenous injections. SD and *bcrp*$^{-/-}$ rats, weighing 225–250 g, were injected intravenously (i.v.) with either a non-nephrotoxic (0.5 μmol·kg^{-1} · 2 mL^{-1} normal saline), a moderately nephrotoxic dose (1.5 μmol·kg^{-1} · 2 mL^{-1} normal saline) or a significantly nephrotoxic (2.0 μmol·kg^{-1} · 2 mL^{-1} normal saline) dose of $HgCl_2$ according to our previously published protocol (Bridges et al., 2008a,b). $HgCl_2$ is an inorganic salt to which humans and animals may be exposed. The injection solution contained radioactive mercury ([$^{203}Hg^{2+}$]) and was designed to deliver 1 μCi [$^{203}Hg^{2+}$] to each animal. [$^{203}Hg^{2+}$] was generated by neutron activation of mercuric oxide for four weeks at the University of Missouri Research Reactor (MURR) (Belanger et al., 2001; Bridges et al., 2008a).

At the time of injection, each animal was anesthetized with isoflurane and a small incision was made in the skin in the mid-ventral region of the thigh to expose the femoral vein and artery. A 0.5-μmol, 1.5-μmol or 2.0-μmol·kg^{-1} dose of $HgCl_2$ was administered into the vein. The wound was closed using two 9-mm stainless steel wound clips. Animals were then housed individually in metabolic cages. Forty-eight hours after injection with $HgCl_2$, animals were sacrificed and organs and tissues were harvested.

Collection of organs. At the time of euthanasia, animals were anesthetized with an intraperitoneal (i.p.) injection of ketamine (70 mg·kg^{-1}) and xylazine (30 mg·kg^{-1}). A 1-mL sample of blood was obtained from the inferior vena cava and set aside for determination of [$^{203}Hg^{2+}$] content. A separate sample of blood was placed in a Microtainer plasma separation tube in order to estimate content of [$^{203}Hg^{2+}$] in plasma and cellular fractions. The total volume of blood was estimated to be 6% of body weight (Lee and Blaufox, 1985).

The liver and kidneys were also removed from each rat. Each kidney was trimmed of fat and fascia, weighed, and cut in half along the mid-traverse plane. One-half of the right kidney was placed in fixative (40% formaldehyde, 50% glutaraldehyde in 96.7 mM NaH_2PO_4 and 67.5 mM NaOH) as preparation for histological analyses. The remaining half was frozen in liquid nitrogen for future RNA analyses. One-half of the left kidney was utilized for estimation of [$^{203}Hg^{2+}$] content. A 3-mm traverse slice was obtained from the remaining half and was used for dissection of renal zones (cortex, outer stripe of the outer medulla (OSOM), inner stripe of the outer medulla, and inner medulla). Each sample was weighed and placed in a separate tube for estimation of [$^{203}Hg^{2+}$]. The liver was weighed and a 1-g sample was removed for determination of [$^{203}Hg^{2+}$] content.

Urine and feces were collected in 24-h periods throughout the duration of the experiment. At the end of each 24-h collection period, a 1-mL sample of urine was weighed and placed in a tube for estimation of [$^{203}Hg^{2+}$] content. All of the feces excreted during each 24-h collection period were counted for estimation of [$^{203}Hg^{2+}$] content. The content of [$^{203}Hg^{2+}$] in each sample was determined by counting in a Wallac Wizard 3 automatic gamma counter (Perkin Elmer, Boston, MA) and the content of Hg^{2+} in each sample was estimated using standard computational methods.

Histological analyses. Kidneys were fixed in 40% formaldehyde, 50% glutaraldehyde in 96.7 mM NaH_2PO_4 and 67.5 mM NaOH for 48 h at 4 °C. Following fixation, kidneys were washed twice with normal saline and placed in 70% ethanol. Tissues were processed in a Tissue-Tek VIP processor using the following sequence: 95% ethanol for 30 min (twice); 100% ethanol for 30 min (twice); and 100% xylene (twice). Tissue was subsequently embedded in POLY/Fin paraffin (Fisher) and 5 μm sections were cut using a Leitz 1512 microtome and were mounted on glass slides. Sections were stained with hematoxylin and eosin (H & E) and were viewed using an Olympus I × 70 microscope. Images were captured with a Jenoptix Progress C12 digital camera.

Measurement of creatinine and blood urea nitrogen. Plasma creatinine and blood urea nitrogen (BUN) levels were assessed in order to estimate alterations in renal function. Following separation of plasma from cellular components of blood, an aliquot of plasma was stored at −20 °C. For determination of plasma creatinine, 30 μL of plasma was utilized and the concentration of creatinine was assessed using the QuantiChrome creatinine assay (BioAssay). Similarly, using a 5 μL sample of plasma, the concentration of BUN was determined using the QuantiChrome urea assay (BioAssay).

Vesicular transport assays. Inside-out membrane vesicles made from Sf9 cells containing mouse Bcrp were purchased from Solvo Biotechnology. Control membrane vesicles made from normal Sf9 cells were also purchased from Solvo Biotechnology. Bcrp transport activity was validated by measuring the uptake of 100 nM [^{3}-H]-estrone sulfate. Cysteine (Cys)-*S*-conjugates of Hg^{2+} were utilized for these experiments because there is substantial *in vivo* and *in vitro* evidence implicating this mercuric species in the luminal and basolateral uptake of Hg^{2+} by proximal tubular cells (Zalups, 2000; Bridges et al., 2004; Bridges and Zalups, 2010). Mercuric conjugates of 2,3-dimercapto-1-propane succinate (DMPS) were also examined since this species of Hg has been shown to be transported by the multidrug resistance-associated protein 2 (MRP2) as a means of eliminating mercuric ions from proximal tubular cells (Bridges et al., 2008a,b, 2011). Vesicular transport assays were carried out using a rapid filtration method according to a published protocol (Van Aubel et al., 1999; El-Sheikh et al., 2007; Bridges et al., 2013). Briefly, DMPS- and Cys-*S*-conjugates of Hg^{2+} were formed by mixing 5 μM (5 nmol·mL^{-1}) [^{203}Hg] with 12.5 μM (12.5 nmol·mL^{-1}) DMPS or Cys, respectively, in incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4) supplemented with 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase in the presence of 4 mM ATP or AMP. Incubation buffer containing mercuric conjugate or estrone sulfate was added to vesicle mixture (7.5 μg protein) and incubated for 30 s at 37 °C. Following incubation, ice-cold buffer containing 1 mM DMPS (to remove bound Hg) was added and each sample was filtered through a Multiscreen plate (0.45 μm; Millipore, Billarica, MA). Filters were removed and radioactivity contained on filter was determined using liquid scintillation spectroscopy.