MRP2 and Acquired Tolerance to Inorganic Arsenic in the Kidney of Killfish (Fundulus heteroclitus)

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We used proximal tubules isolated from the killfish, Fundulus heteroclitus, to examine the effect of environmentally relevant, sublethal levels of arsenic on the function and expression of MRP2, an ABC transporter that transports xenobiotics into urine, including arsenic-glutathione conjugates. Exposure of fish to arsenic as sodium arsenite (4–14 days) increased both MRP2 expression in the apical membrane of proximal tubules and MRP2-mediated transport activity. The level of MRP2 mRNA was not affected, suggesting a posttranslational mechanism of action. Acute exposure of proximal tubules isolated from control fish to 75–375 ppb arsenic decreased mitochondrial function (inner membrane electrical potential). However, in tubules from fish that were preexposed to arsenic (4–14 days), no such effect on mitochondrial function was observed. Thus, chronic in vivo exposure to arsenic induces mechanisms that protect proximal tubules during subsequent arsenic exposure. Upregulation of MRP2 expression and activity is one likely contributing factor.

Key Words: adaptation; xenobiotic transport; Abcc2; environmental toxicant.

Arsenic, a toxic metalloid and carcinogen that occurs naturally in the environment, is also used clinically to treat acute promyelocytic leukemia and several other diseases (Abernathy et al., 1999; Rosen, 2002). Arsenic is present at significant concentrations in seawater and can be found in high concentrations in soils, surface waters, and well water as a result of both natural and anthropogenic sources. The current Environmental Protection Agency–regulated U.S. drinking water standard is 10 ppb. However, the EPA and the Canadian Council of Ministers of the Environment have suggested that the maximum safe arsenic concentration in seawater is 0.0175 and 12.5 ppb, respectively, concentrations that are often exceeded even in clean coastal waters (1–3 ppb) and definitely exceeded in polluted seawater (> 1000 ppb) (Boyle et al., 1973; Neff, 1997). Whereas humans acquire arsenic via food and water, fish are particularly vulnerable to arsenic in the environment because they also accumulate this toxicant through the gills (Bears et al., 2006).

The kidney plays an important role in regulating the excretion and bioavailability of xenobiotics and toxicants, including arsenic (Bodo et al., 2003; Miller et al., 2002; Terlouw et al., 2003). Excretion of many xenobiotics, metabolites, and toxicants into the urine is mediated in part by the multidrug resistance-associated protein 2 (MRP2/ABCC2), which is located on the apical membrane of proximal tubules (Bodo et al., 2003; Terlouw et al., 2003). MRP2 transports a wide range of anionic substrates, including lipophilic compounds, and organic anions, including arsenic-glutathione conjugates, and its activity and expression are modulated by hormones, protein kinases, nuclear receptors, and nephrotoxicants, including inorganic cadmium and mercury (Gutmann et al., 2000; Notenboom et al., 2004, 2005; Terlouw et al., 2001, 2002; Wever et al., 2007). Since arsenic-glutathione efflux from cells is driven in part by MRP2, the transporter protects against cytotoxicity. Thus, upregulation of MRP2 contributes to the development of arsenic tolerance (Lee et al., 2006; Liu et al., 2001). Although arsenic stimulates MRP2 expression in liver (Liu et al., 2001; Vernhet et al., 2001) and renal cell lines (HEK 293 cells) (Lee et al., 2006), the effects of arsenic on MRP2 expression and function in intact renal proximal tubules have not been investigated.

The euryhaline teleost, Fundulus heteroclitus (killfish), is a model organism that is utilized extensively to study the effects of environmental toxicants on cellular function (Bears et al., 2006; Moeller et al., 2003; Shaw et al., 2007; Stanton et al., 2006). Killfish and certain other teleosts have been particularly useful for the study of renal proximal tubular excretory function and its disruption by nephrotoxicants (Pritchard and Miller 1980; Terlouw et al., 2002). These comparative

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models offer the advantages of easy proximal tubule isolation and extended tissue viability. When used with fluorescent substrates and quantitative confocal microscopy, they provide an experimental system in which the mechanistic details of excretory transport can be studied in intact tubules. Such studies have shown that renal transport mechanisms for organic anions are remarkably conserved from fish to man (Terlouw et al., 2002). Indeed, intact proximal tubules from killifish have been used to elucidate the effects of xenobiotics and nephrotoxicants on MRP2 function (Gutmann et al., 2000; Noteboom et al., 2004, 2005; Terlouw et al., 2002). Here we used this comparative model to demonstrate that exposure of killifish to sublethal and environmentally relevant levels of arsenite in seawater increased MRP2 expression and function in renal proximal tubule. Transporter upregulation in vivo was accompanied by increased tolerance of tubules to arsenite in vitro.

**MATERIALS AND METHODS**

**Animals.** Studies were performed in compliance with Institutional Animal Care and Use guidelines approved by Mount Desert Island Biological Laboratory (MDIBL #A356-01) and Dartmouth Medical School (#A3259-01). Killifish were collected from Northeast Creek (Bar Harbor, ME) and held in aquaria containing running seawater at the MDIBL (Salisbury Cove, ME) for at least 2 weeks prior to use to ensure acclimation. Fish were exposed to arsenic in the seawater (100, 1000, and 8000 ppb of sodium arsenite) at levels previously demonstrated to be sublethal (Shaw et al., 2007), and controls were maintained in seawater alone.

**Tissue collection.** For experiments with renal tubules, fish were decapitated and the kidneys removed to a Petri dish containing ice-cold marine teleost saline containing in millimolars 140 NaCl, 2.5 KCl, 1.5 CaCl₂, 1.0 MgCl₂ and 20 Tris(hydroxy-methyl)-aminomethane (TRIS), pH 8.0. For tissue arsenic and mRNA analyses, fish were anesthetized in tricaine (MS-222: 0.1%), and tissue was collected for isolation of mRNA for quantitative-reverse transcription (Q-RT)-PCR or for analysis of arsenic levels by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (see below).

**Isolation of RNA and Q-RT-PCR.** Q-RT-PCR experiments were conducted to examine the effects of arsenic on MRP2 gene expression in the kidney and liver. To isolate total RNA, tissue was obtained as described above and immediately stored in RNAlater (Ambion, Austin, TX). Total RNA was isolated from 30 mg of tissue (from three animals to reduce animal to animal variation [Kendziorski et al., 2003]) per observation using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was treated with DNase (DNA-free, Ambion) to remove contaminating DNA. Total RNA was quantified using spectrophotometric OD260/280 measurements (NanoDrop, NanoDrop Technologies, Rockland, DE), and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Two-step RT-PCR was performed with 1 µg of total RNA (Retroscript Reverse Transcriptase, Ambion) and random decamers. Primers and probe for real-time PCR were synthesized using the Assays-by-Design service (Applied Biosystems, Foster City, CA). The sequence for killifish MRP2 was submitted for primer/probe design, and probe target was set to a predicted exon-exon splice junction. Sequence of the primers and probe (GACGCCGTTTCTTTTACACTGAA and MR22 forward: GAGTCAAGGTTTACACGTA and MRP2 reverse: CGTCAAGGGTTAAACGGTCTTT; MRP2 internal probe: CAGCGAAGAGAACTAG)

The internal probe was 6-FAM dye labeled with a minor groove-binding modification and nonfluorescent quencher on the 3’-end. The Assays-by-Design primers and probe premixed to a concentration of 18pM for each primer and 5pM for the probe (equivalent to a 20× mix) were combined with TaqMan Universal Master Mix (Applied Biosystems) and cDNA diluted in RNase-free H₂O in a 20-µl reaction and placed in a 96-well format spectrofluorometric thermal cycler (ABI Prism 7700 Sequence Detection System, Foster City, CA). Duplicate and/or triplicate reactions of each sample were incubated at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. In preliminary studies, Q-RT-PCR products were run on an LMP agarose gel to confirm product size, subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA), and submitted for sequence analysis to confirm identity of the products. Dilutions of MRP2 plasmid DNA prepared from the killifish Q-RT-PCR products were used to construct a standard curve. The standard curves showed a correlation coefficient close to 1 (R² ≥ 0.99) and were linear over a 6-log range. Equivalent amplification efficiencies of standard and target molecules were observed, and SYBR green melting curve dissociation analysis and sequencing revealed a single PCR product for each target. Raw data were analyzed; baseline and threshold values set and gene expression interpolated using the external standard curves. The cDNA generated during reverse transcription and used as template was quantified (NanoDrop), and data were calculated as gene expression (fg/ng cDNA).

**MRP2 transport assay.** To evaluate MRP2 transport activity, we measured the secretion of fluorescein-methotrexate (FL-MTX, Invitrogen), a fluorescent substrate of MRP2, into the lumen of isolated killifish renal proximal tubules. Briefly, clusters of renal proximal tubules were isolated in marine teleost saline solution as described previously (Gutmann et al., 2000; Maserereewu et al., 2000; Noteboom et al., 2004; Terlouw et al., 2002; Wever et al., 2007). Under a dissecting microscope, each cluster of tubules was teased apart with forceps and transferred to a chamber with a glass coverslip bottom. The chamber contained marine teleost saline with the fluorescent MRP2 substrate, FL-MTX (1µM). Tubules were incubated until a steady-state distribution of FL-MTX was achieved between the tubule lumen and cells (60 min [Maserereewu et al., 2000]). Subsequently, the chamber containing renal tubules was mounted on the stage of an Olympus Fluoview inverted confocal laser scanning microscope and viewed through a ×40 water immersion objective (numerical aperture 1.15). Excitation was provided by the 488-nm line of an argon ion laser. A 510-nm dichroic filter and a 515-nm long-pass emission filter were used. Neutral density filters and low laser intensity were used to avoid photobleaching. With the photomultiplier gain set to give an average luminal fluorescence intensity of 1500–3000 (on a scale of 0–4096), tissue autofluorescence was undetectable. Single proximal tubules with well-defined lumens and undamaged epithelium were selected for measurements. The plane of focus was adjusted to cut through the center of the tubular lumen and a confocal image was acquired by averaging four laser scans. The confocal image was viewed on a high-resolution monitor and saved to disk. Fluorescence intensities in cells and the lumen of proximal tubules were measured from stored images using ImageJ 1.30. Briefly, two or three adjacent cellular and luminal areas were selected from each tubule, and the average pixel intensity for each area was calculated. The values used for that tubule were the means of all selected areas after subtraction of the pixel intensity of the bathing medium, which was considered as background. Although there is a linear relationship between fluorescence intensity and FL-MTX concentration because of the uncertainties in relating cellular fluorescence to compound concentration in cells with complex geometry, results are reported as average measured pixel intensity rather than estimated dye concentration. All experiments were conducted at 18°C–20°C.

**ICP-MS analysis of tissue arsenic.** Gill, liver, kidney, and total body arsenic levels were measured by the Dartmouth Superfund Trace Element Analysis Core Facility using a cold vapor/hydride generation magnetic sector inductively coupled plasma-mass spectrometer (ICP-MS ELEMENT, Finnigan MAT) according to the methods of Klau & Blum (1999). Briefly, killifish were anesthetized as described above, rinsed with ultrapure water, and gill tissue was dissected, weighed, and stored frozen at −80°C. Gill, liver, kidney, and body tissue were digested under pressure using a sequential combination of nitric acid (optima grade) and hydrogen peroxide (optima grade). Arsenic levels are expressed as µmol/g of wet weight.
Measurement of mitochondrial function. Changes in mitochondrial inner membrane potential were monitored using MitoTracker Red CM-H2Xros (Invitrogen) as described previously (Notenboom et al., 2005; Stanton et al., 2006). This fluorescent probe is sequestered within the mitochondrial inner membrane as a function of electrical potential. Isolated proximal tubules were incubated for 1 h in marine teleost saline solution containing vehicle (H2O), arsenic (sodium arsenite, 75 or 375 ppb), or 1 μM sodium cyanide. All solutions contained 500nM MitoTracker Red CM-H2Xros. Subsequently, tubules were moved to Mitotracker Red-free solutions, and fluorescence was viewed using an Olympus Fluoview inverted confocal laser scanning microscope, and the 568-nm laser line. Images were collected and analyzed for cellular punctuate fluorescence as described previously (Notenboom et al., 2005; Stanton et al., 2006).

Immunohistochemistry. Killifish proximal tubules were isolated as described above and were processed in phosphate-buffered saline for immunostaining. This involved fixation with 2% (vol/vol) formaldehyde/0.1% (vol/vol) glutaraldehyde, permeabilization with 1% (vol/vol) Triton X-100; 90-min exposure to primary antibody (rabbit polyclonal antibody for Mrp2, k78, 1:50 [van Aubel et al., 1998]); and 60-min exposure to secondary antibody (Alexa488-labeled goat anti-rabbit IgG, 1:20; Sigma Mrp2 k78 was a generous gift of Dr Rosalinde Masereeuw, Nijmegen University, The Netherlands.). Antibody binding was detected with an Olympus Fluoview inverted confocal laser scanning microscope, and staining was quantified using ImageJ version 1.30 as described previously (Notenboom et al., 2005).

Analysis of data. Data are presented as mean ± SE. Statistical significance of experimental maneuvers was determined by the paired or unpaired Student’s t-test or ANOVA and the Tukey-Kramer multiple comparison test using GraphPad Instat (version 3.0a for Macintosh, GraphPad Software, San Diego, CA). A p value < 0.05 was considered significant.

RESULTS

Arsenic Increases MRP2 Transport Activity

Previous studies demonstrated that FL-MTX transport from cell to lumen of killifish proximal tubules is mediated by a teleost form of MRP2 located in the apical (brush border) plasma membrane (Gutmann et al., 2000; Masereeuw et al., 2000; Notenboom et al., 2004; Terlouw et al., 2002; Wever et al., 2007). Such transport is specific, concentrative, and dependent on cellular metabolism. A representative confocal image of control tubules after 60 min incubation in medium with 1μM FL-MTX is shown in Figure 1A. In agreement with previous studies, FL-MTX fluorescence distribution in these control tubules was lumen > cells > medium (Gutmann et al., 2000; Masereeuw et al., 2000; Notenboom et al., 2004; Terlouw et al., 2002; Wever et al., 2007).

Two in vivo dosing protocols were used to determine the effects of in vivo arsenic exposure on MRP2 transport activity in killifish tubules. In the first dosing protocol, naive killifish were exposed to 8000 ppb inorganic arsenic in seawater for 2 or 4 days, and FL-MTX transport was measured ex vivo in isolated proximal tubules as described in Materials and Methods. The representative confocal images shown in Figure 1 demonstrate that 4 days of arsenic exposure increased the accumulation of FL-MTX in the lumen. Quantitation of luminal and cellular fluorescence in a population of tubules showed significantly increased luminal fluorescence with no change in cellular fluorescence (Fig. 2). As a result, the lumen-to-cell fluorescence ratio for tubules from fish exposed to arsenic for 4 days increased significantly. Two-day exposure to 8000 ppb arsenic had no effect on renal tubular transport (Fig. 2). These data demonstrate that exposure to arsenic for 4 days enhances MRP2 xenobiotic transport activity in killifish renal proximal tubule.

In the studies described above, the concentration of arsenic in the water exceeded levels observed in contaminated environments. Thus, additional studies were conducted to examine the effects of lower, more environmentally relevant, levels of arsenic on MRP2 function (Boyle et al., 1973; Neff, 1997). Accordingly, fish were exposed to 100 or 1000 ppb arsenic in the water, but for a longer time (14 days), and MRP2 activity was measured in isolated proximal tubules as described above. Although 100 ppb arsenic had no effect on the transport of FL-MTX, 1000 ppb arsenic enhanced MRP2 transporter activity roughly to the same extent as a 4-day exposure to 8000 ppb arsenic (Fig. 3). Thus, exposure of fish to environmentally relevant levels of arsenic enhances MRP2 activity.

Arsenic Increases MRP2 Protein Abundance

To determine whether the increase in MRP2 transport activity found with arsenic exposure was associated with an increase in MRP2 protein levels, immunostaining studies were conducted on proximal tubules from fish exposed to 8000 ppb

FIG. 1. Representative confocal images demonstrating that chronic arsenic exposure (8000 ppb for 4 days) enhanced FL-MTX secretion into the lumens of killifish renal proximal tubule. (A) Control. (B) Arsenic treated. Arrows indicate lumen of proximal tubule. Asterisks indicate proximal tubule cell.
arsenic for 4 days using an antibody to mammalian MRP2. The representative confocal images shown in Figure 4 demonstrate increased luminal immunoreactivity in tubules from arsenic-exposed fish. Quantitation of luminal membrane immunofluorescence showed that MRP2 protein abundance increased by 86% (membrane fluorescence intensities in 9–13 tubules from each of four fish were 1449 ± 102 units for controls and 2674 ± 271 units for arsenic-exposed fish, \( p < 0.01 \)). This increase is comparable to the 87% increase in FL-MTX transport activity shown above. Unfortunately, these results cannot be confirmed by Western blot analysis because of the small amount of plasma membrane protein isolated from killifish proximal tubules (Notenboom et al., 2005). Thus, these observations suggest that enhanced FL-MTX transport is due primarily to an increase in MRP2 transporter abundance in the proximal tubule apical membrane.

**Arsenic Does Not Increase MRP2 mRNA Expression**

To determine whether increased MRP2 protein expression was the result of upregulation of MRP2 gene expression, MRP2 mRNA levels were measured using Q-RT-PCR. Figure 5 shows that exposing killfish to 8000 ppb arsenic for 6, 12, 24, 48, or 96 h did not alter MRP2 mRNA expression in kidney tubules. By contrast, arsenic (8000 ppb for 48 and 72 h) increased MRP2 mRNA expression in liver (Fig. 5). Taken together with the observation that arsenic increases MRP2 protein abundance, the Q-RT-PCR data suggest that arsenic regulates MRP2 abundance in killfish kidney by a posttranslational mechanism.

**Arsenic Tolerance in the Kidney**

Acquired tolerance to arsenic can be due to many factors including, but not limited to, elevated expression of γ-glutamylcystein synthetase and glutathione S-transferase, and increased cellular efflux of arsenic by MRP2 (Terlouw et al., 2003). To determine whether proximal tubules acquired tolerance to arsenic due to upregulation of MRP2, we examined the effects of acute arsenic exposure on mitochondrial integrity (inner-membrane electrical potential) in tubules isolated from control and arsenic-pretreated fish. Mitochondrial integrity in individual tubules was evaluated using MitoTracker Red and confocal microscopy as described in Materials and Methods. The rationale for these experiments is twofold. First, assessment of mitochondrial function using MitoTracker Red is one of a very few methods available to study cellular function in single killfish renal tubules. Second, mitochondrial integrity is related to arsenic toxicity. As in the transport studies, two in vivo dosing protocols were used. In proximal tubules, isolated from fish not preexposed to arsenic acute exposure (1 h) to 75 or 375 ppb arsenic ex vivo reduced mitochondrial function in a concentration-dependent manner (Fig. 6). With 375 ppb arsenic, MitoTracker Red fluorescence was not different from the positive control, 1 mM cyanide. Thus, as anticipated, acute exposure to arsenic profoundly reduced mitochondrial function in tubules isolated from fish not preexposed to arsenic. Preexposure of fish to sublethal levels of arsenic in seawater (8000 ppb for 4 days) had no effect on mitochondrial function in tubules as measured by MitoTracker Red fluorescence (Fig. 6; As + Veh). Moreover, preexposure to arsenic (8000 ppb for 4 days) eliminated the inhibition in mitochondrial function produced by the acute exposure to arsenic. That is, when fish were preexposed to 8000 ppb arsenic for 4 days, subsequent acute exposure of isolated tubules to 75 or 375 ppb arsenic was without effect on mitochondrial function (Fig. 6). Thus, by this endpoint, arsenic preexposure in vivo increased arsenic tolerance of renal tubules.

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arsenic on mitochondrial function (Boyle et al., 1973; Neff, 1997). Accordingly, fish were exposed to 100 or 1000 ppb arsenic in the water, but for a longer time (14 days), and mitochondrial function was measured in isolated proximal tubules as described above. Preexposure of fish to sublethal levels of arsenic in seawater (100 or 1000 ppb for 14 days) had no effect on mitochondrial function in tubules as measured by MitoTracker Red fluorescence (Fig. 7; As + Veh). Moreover, preexposure to arsenic (100 and 1000 ppb for 4 days) eliminated the inhibition in mitochondrial function produced by the acute exposure to arsenic. That is, when fish were preexposed to 100 or 1000 ppb arsenic for 14 days, subsequent acute exposure of isolated tubules to 375 ppb arsenic was without effect on mitochondrial function (Fig. 7). Thus, by this end point, long-term arsenic exposure in vivo increased arsenic tolerance of renal tubules.

**Tissue Arsenic Levels**

Arsenic levels were measured in the gill, liver, and body of control and arsenic-exposed killifish by high-resolution ICP-MS.

After 4 days of exposure to 8000 ppb arsenic in the water, tissue arsenic levels were more than an order of magnitude higher than untreated controls. In body tissue, gill tissue, liver, and kidney, arsenic levels ranged from 0.056 to 0.141 μmol/g of tissue (Fig. 8) with kidney levels being highest. Although the concentration of arsenic in the water (8000 ppb) was higher than observed in polluted seawater, we believe that tissue arsenic data reported can be considered as environmentally relevant because they are lower than those found in wild killifish collected from polluted environments (see Discussion).

In fish exposed to more environmentally relevant levels of arsenic, only total body levels could be measured accurately. Fish were exposed to 100 or 1000 ppb arsenic for 1, 3, 7, and 14 days (Fig. 9). For fish exposed to 100 ppb arsenic, total body arsenic increased after 1 day but did not increase further after 3, 7, or 14 days. In contrast, for fish exposed to 1000 ppb, total body arsenic levels increased with exposure time so that after 14 days arsenic levels were at least 10 times those in controls.
DISCUSSION

The major new findings in this report are that renal proximal tubules from killifish exposed to sublethal levels of arsenic in vivo exhibit (1) increased expression and transport activity of MRP2, a xenobiotic efflux pump, and (2) reduced sensitivity to acute arsenic toxicity in vitro. Even at the high level used (8000 ppb for 4 days), the effects of arsenic found here were specific. As shown previously (Shaw et al., 2007), this level of exposure had no effect on actin or HSP70 expression as determined by Western blot analysis, and arsenic did not affect Na⁺-K⁺-ATPase or NKCC1 mRNA expression or protein abundance in gill tissue. In addition, mitochondrial function (inner membrane electrical potential) in proximal tubules from arsenic pre-exposed killifish was not different from tubules isolated from control fish (this study). Thus, the effects of arsenic on killifish proximal tubule do not appear to involve a general cellular stress response or nonspecific toxicity.

Several lines of evidence demonstrate that the concentrations of arsenic used in the present study were environmentally relevant. Although 8000 ppb is certainly higher than that found in even the most polluted environments, resulting tissue levels of arsenic in gill, liver, kidney, and body in the present study (0.056–0.141 μmol/g of tissue) were lower than in killifish collected from arsenic contaminated water in California (~0.41 μmol/g) (Moeller et al., 2003) or in livers of flathead sole collected off the coast of Alaska (0.1945–0.5516 μmol/g) (Meader et al., 2004). Clearly, while higher external levels may be required experimentally in short-term studies to achieve biological responses, it is important to compare tissue levels rather than external levels when evaluating effects. On the other hand, exposure to 1000 ppb arsenic for 14 days, an environmentally relevant exposure, increased whole-body arsenic levels to 0.019 μmol/g of tissue, a value 20-fold less than observed in wild killifish. Indeed, the biological response to this lower level of exposure was similar to that seen with 8000 ppb arsenic (i.e., enhanced MRP2-mediated transport of FL-MTX and increased arsenic tolerance in intact killifish renal tubules).

Interestingly, at 100 ppb, the body levels of total arsenic initially increased but then remained constant at subsequent time points, suggesting a homeostatic mechanism that is able to balance uptake and excretion at this dose but which is exceeded at the 1000-ppb concentration leading to progressive accumulation of arsenic body burdens.

FIG. 7. Summary of MitoTracker Red experiments to examine the effects of arsenic on mitochondrion function in isolated proximal tubules. Fish were preexposed to 100 ppb arsenic (As 100 ppb), 1000 ppb arsenic (As 1000 ppb), or vehicle (control) in seawater for 14 days. Subsequently, tubules were isolated and exposed for 1 h to 0 arsenic (Veh), 75 ppb arsenic, 375 ppb arsenic, or NaCN (1 μM) in a medium containing MitoTracker Red. After 1 h, tubules were removed to a MitoTracker Red-free medium, and intracellular fluorescence was monitored using confocal microscopy as described in Materials and Methods. n = 11–22/tubules/group. ***p < 0.05 versus control + Veh, As 100 ppb + Veh, As 100 ppb + As (375 ppb), As 1000 ppb + Veh, and As 1000 ppb + As (375 ppb).

FIG. 8. Arsenic levels in whole body, gill, liver, and kidney. Fish were exposed to arsenic (8000 ppb) for 4 days and intracellular [As] was measured by ICP-MS as described in the Materials and Methods. Hatched bars, control. Filled bars, fish exposed to arsenic. n = 4/group. *p < 0.05 versus control in the same tissue. **p < 0.01 versus control in the same tissue.

FIG. 9. Arsenic levels in whole body as a function of arsenic concentration in the water and time. Fish were exposed to arsenic (100 or 1000 ppb) in the water for 1, 3, 7, or 14 days and intracellular [As] was measured by ICP-MS as described in the Materials and Methods. Hatched bars, 100 ppb. Filled bars, 1000 ppb. n = 4/group. *p < 0.01 versus control at each exposure level (0; no arsenic exposure).
MRP2 Upregulation

Both arsenic dosing protocols used in the present study increased renal tubule MRP2 protein expression and transport activity. MRP2 regulates the bioavailability of xenobiotics and contributes to xenobiotic defense mechanisms in barrier and excretory tissues. MRP2 limits xenobiotic absorption across the gut and xenobiotic entry into the central nervous system (Bodo et al., 2003; Miller et al., 2002). MRP2 is also present in liver and kidney, organs that are essential for the excretion of xenobiotics, xenobiotic metabolites, and endogenous waste products (Bodo et al., 2003; Kala et al., 2000; Robertson and Rankin, 2006; Terlouw et al., 2002; Trauner et al., 1997; Vernhet et al., 2001). Thus, upregulation of kidney MRP2 by arsenic suggests that arsenic will profoundly affect the excretion, and therefore, bioavailability of a number of organic anions that are substrates for MRP2. These may include toxicants and therapeutic drugs.

The present Q-PCR studies demonstrated that the arsenic-induced increase in MRP2 expression and activity in killifish proximal tubules were not accompanied by a detectable increase in MRP2 mRNA. This suggests a posttranslational mechanism of arsenic action. Arsenic exposure has been shown to increase MRP2 expression in a kidney cell line (HEK 293) (Lee et al., 2006), and gentamicin also increases MRP2 activity and abundance in transfected MDCK cells by a posttranslational mechanism (Notenboom et al., 2006). Although arsenic transcriptionally regulates MRP2 in liver (Kala et al., 2000; Liu et al., 2001; Vernhet et al., 2001), arsenic is also known to posttranslationally regulate MRP2 abundance (Jones et al., 2005; Liu et al., 2001). It remains to be determined by what mechanism MRP2 expression is increased in our killifish tubules but it is intriguing that MRP2 regulation in response to arsenic appears to be fundamentally different in liver and kidney of the same animals.

Mitochondrial Protection

The present results show that acute exposure of isolated renal tubules to low concentrations of arsenic (75 and 375 ppb [1 and 5μM] for 1 h) reduced mitochondrial function. This finding is in agreement with experiments using HK-2 cells, a human kidney cell line (Peraza et al., 2003, 2006). In those cells, exposure to subcytotoxic levels of arsenic (<10μM [750 ppb] for 3–24 h) reduced mitochondrial integrity, measured using MitoTracker Red. Importantly, the present experiments also show that prolonged (14 day) pretreatment of fish with arsenic induced arsenic tolerance when tubules were tested in vitro. That is, tubules from fish that were exposed to arsenic for 14 days no longer lost mitochondrial function in response to an acute challenge with arsenic in vitro. Note that this occurred even in the fish exposed to 100 ppb arsenic, a dose that did not significantly affect MRP2-mediated FL-MTX transport. We suspect that this acquired arsenic tolerance is due to a concerted upregulation of phase I and phase II enzymes that biotransform and detoxify arsenic and, likely upregulation of MRP2, which exports arsenic and its conjugates from cells. The net effect of these changes would be reduced intracellular arsenic levels. Thus, it is likely that increased MRP2 activity contributed in part to arsenic tolerance in killifish proximal tubules.

In conclusion, we report that in vivo exposure of killifish to environmentally relevant levels of arsenic in water increase MRP2 abundance and transport activity in isolated proximal tubules and that this effect is posttranslational. Moreover, we report that proximal tubules from arsenic-exposed fish have acquired tolerance to acute arsenic toxicity, likely in part due to the upregulation of MRP2 expression and activity. These data have important implications for effects of arsenic on xenobiotic excretion and bioavailability.

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