Renal Na-Si cotransporter NaSi-1 is inhibited by heavy metals

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Markovich, Daniel, and David Knight. Renal Na-Si cotransporter NaSi-1 is inhibited by heavy metals. Am. J. Physiol. 274 (Renal Physiol. 43): F283–F289, 1998.—Heavy metal intoxication leads to a number of reabsorptive and secretory defects in renal transport systems. We have studied the effects of several heavy metals on the expression of the renal Na-Si cotransporter NaSi-1. NaSi-1 cRNA was injected into Xenopus oocytes, and Na-Si cotransporter activity was measured in the presence of mercury, lead, cadmium, or chromium. Mercury strongly inhibited NaSi-1 transport reversibly by decreasing both maximal velocity ($V_{\text{max}}$) and Michaelis constant ($K_m$) for inorganic sulfate (S). Lead inhibited NaSi-1 transport reversibly by decreasing $V_{\text{max}}$ but not $K_m$ for S. Cadmium showed weak reversible inhibition of NaSi-1 transport by decreasing only NaSi-1 $V_{\text{max}}$. Chromium strongly inhibited NaSi-1 cotransport reversibly by decreasing $K_m$ for S; by sevenfold, most probably by binding to the S site, due to the strong structural similarity between the CrO$^{4-}$ and SO$^{4-}$ substrates. In conclusion, this study presents an initial report demonstrating heavy metals inhibit renal brush border Na-Si cotransport via the NaSi-1 protein through various mechanisms and that this blockade may be responsible for sulfaturia following heavy metal intoxication.

sodium-sulfate cotransport; brush-border membrane; Xenopus laevis oocytes; nephrotoxicity

THE MAMMALIAN KIDNEY is a primary organ involved in heavy metal excretion and accumulation. Chronic heavy metal intoxication of the kidney can lead to a number of reabsorptive and secretory defects. Inhibition of tubular reabsorption and secretion by heavy metals leads to proteinuria and polyuria, as well as conditions including glucosuria, aminoacidurias, calciurias, phosphaturia, and sulfaturia (16, 20, 22). Serum inorganic sulfate (S) concentrations are controlled to a large extent by the regulation of S reabsorption in the renal proximal tubule (3, 21). The cloning of the NaSi-1 cDNA (15), encoding the rat renal brush-border membrane Na-Si cotransporter NaSi-1 protein by blocking the expression of the cloned amino acid transport by decreasing the maximal rate of uptake for S, Cadmium showed weak reversible inhibition of NaSi-1 transport by decreasing only NaSi-1 $V_{\text{max}}$. Chromium strongly inhibited NaSi-1 cotransport reversibly by decreasing $K_m$ for S; by sevenfold, most probably by binding to the S site, due to the strong structural similarity between the CrO$^{4-}$ and SO$^{4-}$ substrates. In conclusion, this study presents an initial report demonstrating heavy metals inhibit renal brush border Na-Si cotransport via the NaSi-1 protein through various mechanisms and that this blockade may be responsible for sulfaturia following heavy metal intoxication.

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oocytes and injections. Female X. laevis toads were obtained from African Xenopus Facility (Noordhoek, South Africa). Small clumps of oocytes (total ~500–1,500 oocytes) were treated for 60–90 min in collagenase type 4 (Worthington Biochemical, 2 mg/ml) in calcium-free OR II solution [in mM: 82.5 NaCl, 2 KCl, 1 MgCl$_2$, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-tris(hydroxymethyl)aminomethane (HEPES-Tris), pH 7.5]. Oocytes were then washed thoroughly five times with OR II solution and five times with modified Barth’s solution (in mM: 88 NaCl, 1 KCl, 0.82 MgSO$_4$, 0.4 CaCl$_2$, 0.33 Ca(NO$_3$)$_2$, 2.4 NaHCO$_3$, 10 HEPES-Tris, pH 7.4, and 20 mg/l gentamicin sulfate). The oocytes were sorted for morphologically intact, healthy-looking, stage V–VI oocytes, incubated in MBS at 17°C, and injected with either 50 nl water (control) or 1 ng cRNA/oocyte derived from the NaSi-1 (15) and NaPi-3 (13) cDNAs, using a Nanoject automatic oocyte injector (Drummond Scientific, Broomall, PA). Oocytes were then kept at 17°C in MBS for 1–4 days, with daily changes of MBS solution.

In vitro transcription. NaSi-1 and NaPi-3 cRNA were synthesized in vitro, as described previously (13–15). Briefly, the transcription mixture (transcription buffer, 1X [40 mM Tris-HCl, pH 7.9, 2 mM spermine and 6 mM MgCl$_2$], 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM m7G(5’ppp)5’G, 0.1 mM GTP, 10 mM dithiothreitol, 50 units ribonuclease (RNase) inhibitor, and 50 units T7 RNA polymerase) was added to 1 µg of Not I linearized pSPORT-1 plasmid DNA. The reaction was incubated at 37°C for 1 h, followed by 50 units of RNase inhibitor, and 10 units of deoxyribonuclease I. RNase free was added to the samples for a further 15 min at 37°C. cRNA was then extracted twice with phenol-chloroform-isooamyl alcohol (25:24:1) and precipitated with 1 vol of ammonium acetate (7.5 M) and 2.5 vol of ethanol. cRNA was resuspended in 15 µl of water and used directly for injection.

Oocyte uptakes. Uptakes were performed as described previously (13–15, 19). In brief, oocytes (10 oocytes/individual data point) were first washed for 1–2 min in solution A (in mM: 100 choline chloride, 2 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES-Tris, pH 7.5). This solution was then replaced by 100 µl of solution B (in mM: 100 NaCl, 2 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES-Tris, pH 7.5) and supplemented with the desired concentration of cold substrate (K$_2$SO$_4$ or K$_2$HPO$_4$/KH$_2$PO$_4$; see Figs. 1–5) and labeled substrate Na$^{235}$SO$_4$ or H$^{33}$PO$_4$ (NEN) at the specific activity of 20 µCi/ml in the presence or
absence of heavy metals HgCl2, Pb(NO3)2, CdCl2, or CrO3 at the desired concentrations (see Figs. 1–5). Oocytes were incubated at room temperature 25°C for various times (1–60 min). Because of the nonlinearity of NaSi-1-induced transport activity up to 10 min, all transport assays (except in Figs. 2B–5B) were performed at uptake times ≥30 min. After incubation, the uptake solution was removed, and the oocytes were washed three times with 3 ml of ice-cold stop solution (solution A). Each single oocyte was then placed into a scintillation vial, dissolved in 250 µl of 1% sodium dodecyl sulfate, followed by the addition of 2 ml of scintillation fluid (Emulsifier Safe, Canberra Packard), and counted (2 min/oocyte), using liquid scintillation spectrometry. Reversibility of heavy metals was performed by preincubating oocytes with the heavy metals HgCl2 (0.1 mM), Pb(NO3)2 (0.1 mM), CdCl2 (0.5 mM), or CrO3 (0.5 mM), independently, for 30 min at room temperature. Controls were subjected to solution A at room temperature for 30 min. Heavy metals were then removed, and oocytes were rinsed three times with (control) solution A at room temperature, then subjected to a standard 35SO42—uptake with 0.1 mM K2SO4 (substrate concentration) at room temperature for 30 min (as described above).

Data presentation and statistics. All experiments were repeated at least three times with different batches of oocytes. Each single point on the graphs is derived from a mean of 7–10 oocytes ± SE. Error bars not visible on graphs are smaller than the symbol used for that point. Statistical significance was tested using the paired student t-test, with P < 0.05 considered significant and any value greater considered nonsignificant (NS). The Michaelis-Menten equation was used to calculate Km and Vmax, using nonlinear regression.

RESULTS

Rat renal NaSi-1 (15) cRNA, when injected into X. laevis oocytes, leads to a strong (>50-fold) stimulation of Na-Si cotransport (measured by 35S uptake), compared with water injected oocytes (Fig. 1A). This stimulation was observed to be strongly inhibited by the heavy metals mercury (Hg2+) and lead (Pb2+) but not significantly by cadmium (Cd2+) and to a significant degree by the trace metal chromium (Cr6+), each at 0.1 mM final concentration, with the order of potency of NaSi-1 inhibition in descending order: Hg2+ > Pb2+ > Cr6+ > Cd2+ (Fig. 1). A similar order of potency of inhibition by these heavy metals was observed with the human renal NaPi-3 (13) cotransporter, when expressed in Xenopus oocytes, followed by 32P uptake measurement in the presence of these same heavy metals (data not shown).

To determine whether the heavy metals bound permanently to the NaSi-1 protein (and thus blocked transport activity), we tested the reversibility of these heavy metals to the NaSi-1 transporter by performing oocyte washout experiments (Fig. 1B). The effect of Hg2+ on NaSi-1 cotransport was not reversible, with the expressed NaSi-1 activity in oocytes being only 14.1 ± 1.7% on washout, whereas the effect of Pb2+, Cd2+, and Cr6+ on NaSi-1 cotransport was fully reversible (Fig. 1B).

Mercury interaction with the NaSi-1 transporter. To further characterize the inhibition of Hg2+ on NaSi-1 transport, we performed a series of transport kinetics in NaSi-1 cRNA-injected Xenopus oocytes. Hg2+ inhibition of NaSi-1 transport was both dose and time dependent (Fig. 2A and B). Inhibition by Hg2+ was already observed at a concentration of 100 nM, and almost complete inhibition was observed at 100 µM (Fig. 2A). Half maximal inhibition (K1) of Na-Si cotransport by Hg2+ was determined as 7.1 ± 0.3 µM. Hg2+ inhibition of NaSi-1 transport was observed as early as 1 min (by 100 µM Hg2+) and continued in a time-dependent fashion up to 60 min (Fig. 2B); however, the slope did flatten after 10 min. NaSi-1-induced transport was not linear for the first 10 min (inset, Fig. 2B); thus all other assays were performed in the linear phase (30-min uptakes). The effect of Hg2+ not only
decreased the maximal transport capacity ($V_{\text{max}}$) 
\[38.00 \pm 1.12 \text{ pmol } \text{Si} \cdot \text{oocyte}^{-1} \cdot \text{min}^{-1} \text{ (control) vs. 38.00} \pm 1.12 \text{ pmol } \text{Si} \cdot \text{oocyte}^{-1} \cdot \text{min}^{-1} \text{ (100 } \mu \text{M } \text{Hg}^{2+}) \]; $P < 0.01$ but also the apparent affinity ($K_m$) of the NaSi-1 transporter for Si 
\[0.35 \pm 0.12 \text{ (control) vs. 0.85} \pm 0.08 \text{ mM (100 } \mu \text{M } \text{Hg}^{2+}) \]; $P < 0.05$; Fig. 2C).

Lead interaction with the NaSi-1 transporter. Pb$^{2+}$ inhibition of NaSi-1 transport was also dose and time dependent (Fig. 3A and B). Significant inhibition was observed by 100 nM Pb$^{2+}$ (and linear up to 100 µM; Fig. 3A), as early as 1 min (by 100 µM Pb$^{2+}$), and increased with time up to 30 min, after which it remained constant up to 60 min (Fig. 3B). $K_i$ of Na-Si cotransport by Pb$^{2+}$ was determined as 21.3 ± 1.8 µM. The effect of Pb$^{2+}$ only decreased $V_{\text{max}}$ 
\[49.74 \pm 3.95 \text{ (control) vs. 32.84} \pm 3.09 \text{ pmol } \text{Si} \cdot \text{oocyte}^{-1} \cdot \text{min}^{-1} \text{ (100 } \mu \text{M } \text{Pb}^{2+}) \];
DISCUSSION

Since the kidney is one of the primary organs involved in excretion of metals, it is also the site for heavy metal intoxication. To date, very few studies have looked at the interaction of heavy metals on specific renal transporters. This is the first study that examines the interaction of heavy metals with the proximal tubular brush border Na-Si cotransporter, NaSi-1. As we have previously demonstrated for the amino acid transporter, rBAT (24), and as was recently shown for the human Na-Pi cotransporter, NaPi-3 (23), heavy metals have the ability to inhibit the function of cloned renal transporters by mechanisms yet to be determined. In this study, we show that the Na-Si cotransporter NaSi-1 is inhibited by heavy metals Hg2+, Pb2+, Cd2+, and the trace metal Cr6+.

Hg2+ showed a very strong inhibition of NaSi-1-induced Na-Si cotransport in Xenopus oocytes (Km(Hg) = 7.1 ± 0.3 µM) by reducing both the Vmax and Km for Si: the reduction in NaSi-1 Km for Si was over twofold by Hg2+, suggesting that the metal may be interfering with Na-Si cotransport by competitive inhibition. The Hg2+ inhibition of NaSi-1 cotransporter activity was not reversible, as previously described for the interaction of Hg2+ with both the NaPi-3 (23) and rBAT (24) transporters. NaPi-3 was also strongly inhibited by Hg2+ (data not shown; Ref. 23); however, only its Vmax was altered, with no apparent change in Km for P; (23). This may suggest that indeed Hg2+ interaction with the NaSi-1 transporter is different than its interaction with the NaPi-3 transporter, in that Hg2+ could be competing for the S; binding site on NaSi-1 and not for the P; site on NaPi-3. At this stage, this is only speculation, since the S; binding site on NaSi-1 protein has not yet been determined; however, we have recent evidence suggesting that it is not located within the first four transmembrane domains of the NaSi-1 protein (Pajor and Markovich; unpublished observations). A second...
mechanism by which Hg$^{2+}$ may be inhibiting NaSi-1 transport is by oxidation: the NaSi-1 protein has several intracellularly located cysteine residues (at positions 318, 329, and 449; predicted by the hydropathy plot (Ref. 15)), which may have their thiol groups oxidized by the metal Hg$^{2+}$, as postulated for the mechanism of Hg$^{2+}$ inhibition of the NaPi-3 (23) and rBAT (24) transporters. In addition, since Hg$^{2+}$ has been shown to interact with intracellular sulfhydryl groups (7, 8), this mechanism may also be responsible for inhibition of NaSi-1 transport, as postulated for rBAT (24). There may be a further possibility: since Hg$^{2+}$ has been shown to block Na$^{+}$-K$^{+}$-adenosinetriphosphatase (Na$^{+}$-K$^{+}$-ATPase) activity by ligand binding (2), it could be that Hg$^{2+}$ is indirectly inhibiting NaSi-1 transport by blocking the activity of the endogenous Na$^{+}$-K$^{+}$-ATPase pump and, as a consequence, inhibiting Na/Si uptake into the oocyte. This type of inhibition was shown previously for the Na-Pi cotransporter, which was blocked indirectly via the inhibition of the Na$^{+}$-K$^{+}$-ATPase by hydrogen peroxide in LLC-PK$_{1}$ cells (1).

Lead interaction with the NaSi-1 transporter was different than Hg$^{2+}$. Despite its strong inhibition ($K_{i}$ of 21.3 ± 1.8 µM), only its $V_{\text{max}}$ was altered, with no apparent change in $K_{m}$ for $S_{i}$, suggesting that the inhibition may be via a noncompetitive mechanism or allosteric fashion. This effect was analogous to the inhibitory effect of lead on rBAT transport (24) but was in contrast to its effect on NaPi-3 transport, in which Pb$^{2+}$ decreased both $V_{\text{max}}$ and $K_{m}$ for $P_{i}$ on the NaPi-3 transporter (23). The inhibition of NaSi-1 cotransporter activity by Pb$^{2+}$ was fully reversible, as previously demonstrated with the NaPi-3 (23) and rBAT (24) transporters. This would suggest that Pb$^{2+}$ may be interacting with NaSi-1 at an extracellular site. As with Hg$^{2+}$, Pb$^{2+}$ has been shown to interact with sulfhydryl groups of proteins (22), so this may be its mode of inhibition on NaSi-1 transport.

Cadmium inhibition of NaSi-1 cotransport in rats treated chronically (14 days) with Cd$^{2+}$ was not significantly different than the control condition. Only a decrease in $V_{\text{max}}$ was observed (using 0.5 mM CdCl$_{2}$), suggesting that its interaction with NaSi-1 may be via a noncompetitive mechanism, as postulated for the interaction with Pb$^{2+}$. Cadmium inhibition of NaSi-1 cotransporter activity was fully reversible, as previously shown for Cd$^{2+}$ with the NaPi-3 transporter (23). As with Hg$^{2+}$ and Pb$^{2+}$, Cd$^{2+}$ has been shown to interact with sulfhydryl groups (20), and this may be the mechanism of inhibition of NaSi-1 transport. A recent study looking at brush-border membrane Na-Si cotransport in rats treated chronically (14 days) with Cd$^{2+}$ showed no inhibition of Na-Si cotransport but strongly impaired Na-Pi cotransport compared with
control rats (9). The lack of Cd\(^{2+}\) inhibition on Na-Si cotransport in that study (in contrast to our present study) may be due to the different approach used to study the interaction between Cd\(^{2+}\) and Na-Si cotransport. Our study looked at the interaction of Cd\(^{2+}\) on NaSi-1 protein expression in Xenopus oocytes, whereas the other study measured \(^{35}\)S uptake in renal cortical brush-border membrane vesicles (BBMV) isolated from control rats and rats chronically treated with Cd\(^{2+}\) (9). The lack of an effect on \(^{35}\)S uptake in BBMV from Cd\(^{2+}\)-treated rats (compared with controls) may suggest that long-term exposure of Cd\(^{2+}\) may not lead to alterations in the overall number (or function) of sulfate transporters in the proximal tubules of rats. In our study, cadmium has a distinct effect on NaSi-1 protein expression when measured in a heterologous expression system (Xenopus oocytes), and this effect maybe more pronounced or more accurately quantitated when studying an individual protein (NaSi-1) than when studying a population of transporters present in renal cortical BBMV (9).

Cadmium showed no inhibition of rBAT amino acid transport in Xenopus oocytes (24), whereas it showed a dose-dependent inhibition of NaPi-3 transport (with maximal inhibition of P\(^{-}\)-induced current of 27.5 ± 2.6% with 1 mM Cd\(^{2+}\) (Ref. 23)). With radiotracer uptake studies, our experiments show that 0.1 mM CdCl\(_2\) produces ~10% inhibition of both NaSi-1 and NaPi-3 (data not shown)-induced transport activities, and 0.5 mM CdCl\(_2\) shows a 30 ± 3% inhibition of NaSi-1 transport. This suggests that NaSi-1 transporter is inhibited by Cd\(^{2+}\) to a similar degree as the NaPi-3 transporter (data not shown; Ref. 23), most probably by a noncompetitive interaction. This is of particular importance, since Cd\(^{2+}\) is an occupational and environmental hazard having strong nephrogenic actions (20).

Chromium inhibition of the NaSi-1 transporter reduced both its \(V_{\text{m}}\) and \(K_m\) for \(S_1\); the reduction in NaSi-1 \(K_m\) for \(S_1\) was nearly sevenfold by Cr\(^{6+}\), suggesting that it may be strongly competing for the \(S_1\) binding site on the NaSi-1 protein. This is in close agreement with the \(K_m\) of Cr\(^{6+}\) on Na-Si cotransport being very close to the \(K_m\) value for NaSi-1 interaction with \(S_1\). As with Pb\(^{2+}\) and Cd\(^{2+}\), the inhibition of NaSi-1 cotransporter by Cr\(^{6+}\) was fully reversible on washout, suggesting that the interaction is at an extracellular binding site on NaSi-1 protein. Chromium (VI) oxide forms an oxyanion (CrO\(_4^{2-}\)) that has been reported to mimic the sulfate (SO\(_4^{2-}\)) anion, which is believed to cross plasma membranes using S\(_1\) transport systems (5, 12, 25). We have shown that chromium oxide can strongly inhibit NaSi-1 transport in Xenopus oocytes and believe that its mechanism of inhibition is by competitive binding for the \(S_1\) binding site on NaSi-1. This is of very significant importance, since chromium is an essential trace metal necessary for certain physiological functions, e.g., glucose metabolism (17). Overexposure to chromium has led to (among other things) renal tubular necrosis (11), and thus it is of special importance in determining the transport systems involved in its uptake in kidneys. NaSi-1 may play a key role in renal chromium intoxication, as well as in the degeneration of tubular function by other metals, e.g., mercury, lead, and cadmium, as analyzed in this study.

In summary, we have shown that mercury, lead, cadmium, and chromium can all inhibit NaSi-1-induced Na-Si cotransport. This is of pathophysiologically relevance, since the renal NaSi-1 transporter is essential for maintaining sulfate homeostasis and serum S\(_1\) levels. These metals are known to produce cell injury in the kidneys (and other organs) and may have a significant involvement during nephrotoxicity, which may be due to accumulation of the metals after systemic application (18, 26). Heavy metals have also been shown to impair the active transport of glucose and other substrates in the intestine (10). Since the NaSi-1 transporter is also localized in the gut and plays an important role in sulfate absorption in the small intestine (15, 19), its ability to transport sulfate across intestinal enterocytes would also be impaired by the heavy metals tested above. This is the first study showing heavy metal inhibition of the cloned NaSi-1 protein, involved in renal and small intestinal Na\(^{+}\) (re)absorption, which, as a consequence, may be responsible for the sulfaturia or Fanconi-type syndrome following heavy metal intoxication.

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