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Chronic, sublethal nickel acclimation alters the diffusive properties of renal brush border membrane vesicles (BBMVs) prepared from the freshwater rainbow trout

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Abstract

Brush border membrane vesicles (BBMVs) were prepared from the kidneys of rainbow trout exposed acutely (72 h; 13,380 μg Ni L^{-1}), chronically (11 months; 289 μg Ni L^{-1}), or chronically and acutely, to waterborne nickel (Ni). Uptake of ^{63}Ni into renal BBMVs was temperature-dependent and fitted a two component kinetic model composed of a saturable, Michaelis–Menten component prominent at lower Ni concentrations, and a moderate linear diffusive component apparent at higher Ni concentrations. Chronic Ni exposure reduced the permeability of the BBM to Ni, evidenced by a significantly reduced slope of the linear diffusive component of BBMV uptake. Efflux of Ni from ^{63}Ni-loaded renal BBMVs was not significantly altered by acute Ni challenge. Both Ca^{2+} and Mg^{2+} inhibited uptake of Ni into renal BBMVs when present at a molar ratio to Ni of 1000:1. Mg^{2+}-induced inhibition, however, was concentration-dependent and significant in BBMVs prepared from chronically Ni-acclimated fish at far lower molar ratios of 100 and 10 to 1. The data suggest that subtle, long-term modulation of membrane structure and function in the rainbow trout may be a compensatory response to chronic waterborne Ni exposure. Additionally, the data challenge the assumptions of constancy of the physiological parameters underlying physiologically based approaches to modeling metal toxicity. Such approaches are currently employed to derive water quality criteria for some metals.

Keywords: Ni; Waterborne exposure; Acute; Chronic acclimation; Brush border membrane vesicles; BBMVs; Transport; Diffusion

1. Introduction

Current approaches to creating water quality guidelines for metals in aquatic systems take into account the physiological mechanisms of metal toxicity in key aquatic species. One such approach is the physiologically based Biotic Ligand Model (BLM; DiToro et al., 2001), which strives to supplement non-biological freshwater parameters such as water hardness, traditionally used to derive water quality guidelines and risk assessments (USEPA, 1986), with geochemically relevant parameters (specific ions) and true biological parameters (De Schamphelaere et al., 2002). One of the more difficult aspects of investigating the physiological mechanisms of metal toxicity in aquatic animals is accounting for subtle physiological changes accompanying chronic, low-level metal exposures via either dietary or waterborne routes. This is particularly relevant in the case of trace metals whose essentiality raises the dual issue of toxicity and deficiency.

Nickel (Ni) is a trace metal whose essentiality is certain in microorganisms (Mulrooney and Hausinger, 2003), and highly probable in higher animals (Nielsen et al., 1975a,b; Eisler, 1998). One of the arguments for essentiality is the consistent, well-regulated background level of Ni in all animal tissues measured to date (see Eisler, 1998 for a review of Ni essentiality). Accordingly, a top aquatic predator such as the rainbow trout (*Oncorhynchus mykiss*) takes up Ni both from the diet and from freshwaters, of which Ni is either a consistent trace component due to natural weathering processes, or a toxicant due to anthropogenic processes.

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In two previous reports on rainbow trout, we have documented subtle physiological impacts of chronic, sublethal exposure to waterborne Ni at concentrations typically seen in aquatic ecosystems subjected to industrial, anthropogenic Ni loading. At the whole organism level, the cost of chronic Ni acclimation was manifested in a reduced aerobic swimming capacity during strenuous exercise (Pane et al., 2004), while at the organ level, renal function during subsequent acute Ni challenge was actually improved by chronic, sublethal Ni acclimation (Pane et al., 2005a).

Our primary goal with this current report was to investigate possible costs of, or compensatory responses to, chronic waterborne Ni acclimation at the cellular level. In the rainbow trout, it has been shown that the kinetic properties of key ion transporters in the gill change over the course of chronic, sublethal exposures to metals in water (Niyogi and Wood, 2003). Since Ni readily enters the blood plasma across the gill, we hypothesized that an eleven month sublethal exposure to waterborne Ni would similarly result in altered kinetic properties of Ni transport in internal tissues. Our model system of choice was brush border membrane vesicles (BBMVs) isolated from the kidney of the rainbow trout. The renal brush border membrane (BBM) is a dynamic epithelium, playing a key role in both urine-to-blood plasma reabsorption of essential ions and metabolites, as well as blood-to-urine secretion of waste products and xenobiotics. Preparation of BBMVs allow for controlled in vitro investigation of transport (Murer and Kinne, 1980; Berteloot and Semenza, 1990) across isolated and vesiculated renal BBM fragments. Comparison of cellular transport processes from animals (i.e. the rainbow trout) in different physiological states (i.e. under chronic Ni stress) also provides insight into the regulation of transport (Hopfer, 1978).

The isolation methodology and the experiments characterizing the BBMV preparation are identical to those of a companion paper, Pane et al. (in press). This companion paper characterizes in vitro Ni transport into renal BBMVs prepared only from naïve fish. The current report uses similar experimental methodology to investigate the membrane-level effects of chronic Ni acclimation. Accordingly, all of the control data presented herein are from the companion paper, Pane et al. (in press). Additionally, in this paper, acute (3 d) high-level Ni exposures were used either alone, or in combination with chronic (11 months) sublethal exposure, to characterize Ni transport across renal BBMs stressed by acute Ni exposure.

2. Materials and methods

2.1. Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss* Walbaum) (200–1500 g) of both sexes were obtained from Humber Springs Trout Farm, Orangeville, ON, Canada. Prior to chronic Ni exposure, fish were maintained in the laboratory for approximately one month in 500 L fiberglass tanks served with aerated, flowing, dechlorinated Hamilton tap water from Lake Ontario. Water composition was (in mM) Ca^{2+}=1.0, Mg^{2+}=0.2, Na^{+}=0.6, Cl^{-}=0.8, SO_4^{2-}=0.25, titratable alkalinity to pH 4.0=1.9. Background Ni=20–30 nM, dissolved organic carbon (DOC)=3 mg L^{-1}, total hardness (as $CaCO_3$)=140 mg L^{-1}, pH=7.9–8.0, and temperature=12–14 °C.

2.2. Ni exposure regimes

Fish were split into two groups, one of which (n=25) was chronically acclimated to low-level Ni for 11 months, along with a chronic control group (n=25), served with Hamilton tap water for the 11 month period. Throughout the chronic exposure period, fish were fed 1% of their body weight every other day with commercial trout pellets. The composition of the food was 40% crude protein, 11% crude fat, 3.5% crude fiber, 1.0% calcium, 0.85% phosphorus, 0.45% sodium, and 65 μmol Ni kg^{-1} dry mass.

Ni was delivered by gravity-feed from a concentrated stock of $NiSO_4 \cdot 6H_2O$. Water samples were taken every other day, 0.45 μM filtered and analyzed for [Ni] by graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA, Varian, Australia) against certified atomic absorption standards (Fisher Scientific). The chronic exposure concentration was 289 μg L^{-1}, or less than 1% of the 96-h LC_{50} (median lethal concentration) for adult trout (Segner et al., 1994).

Following 11 months of chronic exposure, both groups of fish were acutely exposed for 72 h to either 0 (control) or 13,380 μg L^{-1} (~30% of the 96-h LC_{50}) as $NiSO_4 \cdot 6H_2O$ delivered by gravity-feed as described above. This resulted in four treatments labeled throughout as UC (unacclimated, control exposure), UN (unacclimated, acutely Ni-exposed), AC (chronically acclimated, control exposed), and AN (chronically acclimated, acutely Ni-exposed).

2.3. Preparation of BBMVs

Isolation of brush border membrane vesicles (BBMVs) from rainbow trout kidneys followed closely the protocol of Freire et al. (1996), as modified from Schwartz et al. (1974), and described in greater detail in a companion paper, Pane et al. (in press). All procedures were performed on ice or at 4 °C. BBMVs were prepared from individual fish from each Ni treatment, with one vesicle preparation per fish. Following euthanization by a blow to the head, approximately 1 g of kidney tissue was excised and quickly rinsed in ice-cold Ringer solution containing (in mM) 15 tris(hydroxymethyl)aminomethane (Tris)–HCl, 1.5 $CaCl_2$, 135 NaCl, 2.5 KCl, 1 $MgCl_2$, and 0.5 NaH_2PO_4. The solution pH was adjusted to 7.85 with 0.1 M Tris Base, and the osmotic pressure was 293 mOsm kg^{-1}. Kidney pieces were then frozen in liquid nitrogen and used for BBMV preparations within 24 h.

Frozen kidneys were minced in 10 mL of ice-cold homogenization buffer (250 mM sucrose, 10 mM triethanolamine–HCl, and 0.1 mM phenylmethanesulfonylflouride (PMSF). The buffer solution pH was adjusted to 7.60 with 0.1 M NaOH, and the osmotic pressure was 294 mOsm kg^{-1}). Minced kidney was then manually homogenized in a glass dounce homogenizer (Kontes), strained through cheesecloth, and brought up to 35 mL with homogenization buffer. The

homogenate was then centrifuged at 700 ×*g* for 10 min (Sorvall RC-5B) yielding supernatant SN1 which was aspirated, transferred to a fresh centrifuge tube, and brought up to 35 mL with homogenization buffer. The dark, viscous pellet was collected and added to a separate "pool fraction" for later analysis of enzyme activity.

A series of three identical centrifugations (16,000 ×*g* for 20 min) followed, with the first solution to be centrifuged created by addition of homogenization buffer to SN1 as described above. Each of these three centrifugations at 16,000 ×*g* produced a two-toned pellet consisting of a dark central pellet surrounded by a fluffy, lighter-colored, outer ring. The dark central core of each two-toned pellet was harvested and added to the pool fraction. The fluffy outer ring of each pellet was used to prepare the proceeding solution for centrifugation by scraping the fluffy outer ring, resuspending it in 10 mL of homogenization buffer, gently homogenizating with a glass dounce homogenizer and rod ("tight pestle"; Kontes), and topping off to 35 mL with homogenization buffer. The supernatants (SN2, SN3, and SN4) of each spin at 16,000 ×*g* were aspirated, brought up to 35 mL with homogenization buffer, and reserved for ultracentrifugation.

The three supernatants from the 16,000 ×*g* spins (SN2, SN3, and SN4), and the final solution prepared from the fluffy outer ring as described above were then subjected to ultracentrifugation at 100,000 ×*g* for 75 min in a Beckman L8-70M ultracentrifuge equipped with an SW 28 rotor. The supernatants created by ultracentrifugation were added to the pool fraction. The ultracentrifugation pellet was also two-toned, made up of a lighter, fluffy outer ring and a dark, firm central core. The central core pellet was substantially less enriched in the basolateral marker enzyme than the fluffy outer pellet (see Pane et al., in press for details). To ensure BBMVs with the greatest ratio of apical to basolateral enrichment, the central pellet was scraped and resuspended in ~0.6 mL of vesicle buffer containing (in mM) 60 mannitol, 100 NaCl, 50 KCl, 10 *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES). The solution pH was adjusted to 7.40 with 0.1 M Tris Base, and the osmotic pressure was 345 mOsm kg^{-1}. The fluffy pellet was returned to the pool fraction.

The suspension of the ultracentrifugation central pellet in vesicle buffer was passed 50 times through a 23-gauge needle (Glover et al., 2003; Klaren et al., 1993), and the protein concentration of the resulting vesicle solution was measured using the method of Bradford (1976). Vesicle solutions were then diluted to a protein concentration of 0.9 mg mL^{-1} with vesicle buffer. Uptake experiments were conducted over a 6- to 8-h period immediately following isolation of BBMVs, during which time vesicle preparations were stored on ice and aliquotted as needed. There was no discernable deterioration of the vesicle preparation over the experimental period.

2.4. Uptake experiments

Uptake of Ni into renal BBMVs was analyzed by the rapid filtration method of Hopfer et al. (1973). Aliquots of vesicle solution (25 μL) were added to 140 μL of assay buffer in 1.5 mL bullet tubes equilibrated in a water bath to the acclimation temperature of the fish (12 °C) (except in the case of the Q_{10} experiments, where 4 °C incubations were used). The assay buffer was simply the vesicle buffer plus ~37 KBq mL^{-1} ^{63}Ni ($NiCl_2$; Perkin Elmer), and other compounds added, appropriate to each experiment. Vesicles were added to the radiolabeled assay buffer (time 0), the reaction tube was immediately vortexed, and then returned to the 12 °C water bath. Unless otherwise noted, incubation time was 25 s at 12 °C, then uptake was terminated by addition of 42 μL aliquots in triplicate to 0.45 μM methylcellulose membrane filters (ME25; Schleicher and Schuell) in a rapid filtration manifold (Millipore). The filters were presoaked at 4 °C to saturate Ni-binding sites with a stop/wash buffer containing (in mM) 20 $NiCl_2$, 100 NaCl, 50 KCl, 20 HEPES. The solution pH was adjusted to pH 7.40 with 0.1 M Tris Base, and the osmotic pressure was 333 mOsm kg^{-1}. Filters were washed twice with 2 mL of ice-cold stop/wash buffer, then acid digested in 20 mL glass scintillation vials with 2 mL of 1 N HNO_3 (Trace metal grade; Fisher Scientific) for 24 h. Ten mL of Ultima-Gold fluor (Packard BioScience) was then added to the scintillation vial and the filter digested for an additional 48 h. Liquid scintillation counting (1217 Rackbeta; LKB Wallac), with quench correction by the method of external standard ratios, was used to determine ^{63}Ni radioactivity.

The kinetics of Ni uptake by renal BBMVs prepared from kidneys of fish exposed to 4 different Ni regimes were analyzed by measuring Ni uptake rate across a range of extravesicular Ni concentrations achieved by addition of unlabeled $NiSO_4 \cdot 6H_2O$. Nominal (and actual, as measured by GFAAS) concentrations were 1 (3.5), 10 (13.8), 100 (106.4), 500 (467.6), and 1000 (976.6) μM.

The temperature-dependence of Ni uptake was investigated by uptake experiments at 4 and 12 °C, at the three lowest extravesicular Ni concentrations (nominally 1, 10, and 100 μM). Incubations were 30 s at 4 °C and 25 s at 12 °C.

2.5. Efflux of Ni from BBMVs

Only fish from the UC and UN treatments were used for efflux experiments due to sample limitation. Vesicles (25 μL aliquots) were incubated for 10 min with 140 μL of radiolabeled assay buffer at 1 μM extravesicular Ni to equilibrate vesicles with ^{63}Ni. A 35 μL aliquot of this reaction solution was then added to 350 μL of (Ni-free) vesicle buffer and efflux of Ni from vesicles was tracked over 5 min by periodic rapid filtration of 25 μL aliquots in triplicate.

2.6. Cation competition experiments

To assess the potential inhibitory effects of Mg^{2+} and Ca^{2+} on Ni uptake by renal BBMVs, and the possible modifying effect of Ni exposure history, Mg^{2+} and Ca^{2+} were added from concentrated stocks of sulfate salts at concentrations of 10, 100, 1000, and 10,000 μM to assay solutions containing 10 μM extravesicular Ni. Uptake experiments were performed as described above. Due to sample limitation, Ca^{2+} competition experiments were run only with UC and UN fish.

2.7. Enzyme activity

The starting renal homogenate activities of alkaline phosphatase (ALP; EC 3.1.3.1), Na-K-ATPase (EC 3.6.3.9), and succinate dehydrogenase (SDH; EC 1.3.99.1) were compared among the 4 Ni treatments to assess the impact of Ni exposure on these key cellular enzymes. ALP activity was measured according to the protocol of Sigma Technical Bulletin 104; Na-K-ATPase activity was measured according to the protocol of McCormick (1993), and SDH activity was measured according to the protocol of Flik et al. (1983). Enzyme activities were measured after 1 min incubations of the homogenate (see above) with 0.2% Triton-X at room temperature to unmask enzyme activity by membrane solubilization. Activities were normalized for protein concentration as measured by the method of Bradford (1976). Enzyme assays in the starting homogenates were part of a larger evaluation of BBMV purity and degree of apical enrichment as presented elsewhere (Pane et al., in press).

2.8. Calculations and statistics

Uptake of Ni into renal BBMVs was calculated according to the formula:

$$\text{Uptake} = \left(\frac{\text{cpm}}{\text{SA}}\right)/[\text{Pr}] \quad (1)$$

where cpm is the average cpm of 3 filters for each reaction, SA is the measured specific activity of the assay solution, and [Pr] is the calculated protein concentration. All filter cpm data were background corrected for non-specific Ni binding to the filters determined under each set of experimental conditions. From the cpm mL^{-1} of the appropriate assay solution and the total Ni concentration (nmol ml^{-1}), as measured by GFAAS, the SA (cpm $nmol^{-1}$) was calculated. Uptake rate in nmol mg $protein^{-1}$ min^{-1} was derived by dividing uptake by the incubation time (typically 25 s). All kinetic parameters were calculated directly from the lines of best fit generated within Sigma Plot (SPSS Inc.).

Q_{10} values to assess temperature-dependence were calculated at each Ni concentration for each of the 4 Ni treatments from the uptake rates at 4 and 12 °C according to the formula:

$$Q_{10} = (k_2 - k_1)^{10/(T_2 - T_1)} \quad (2)$$

where k refers to the uptake rate and T refers to the incubation temperature.

Ni efflux data were arcsine transformed and fit to a model of exponential decay, from which a half-time ($T_{1/2}$) was calculated for each Ni treatment. Data are presented as the percent of original ^{63}Ni (cpm) remaining per microgram of vesicular protein. The two regression equations describing efflux in Fig. 3 were tested for significant differences according to the method of Zar (1984).

All data passed the Shapiro–Wilk test for normality and equality of variance tests (F-test for $k=2$; Levene test for $k>2$). Data were then tested for significant differences using either a two-tailed Student's t-test ($k=2$ treatments), or a one-way ANOVA ($k>2$ treatments). When warranted (i.e. F value indicated $P<0.05$), means were compared with either a Bonferroni post hoc test or a Dunnett's post hoc test if there was an appropriate time-zero control treatment. Kinetic parameters were compared among the 4 Ni treatments by the method of Motulsky (1998). Data are expressed as mean ± 1 SE; n = number of individual BBMV preparations from individual fish.

3. Results

3.1. Renal BBMV Ni uptake

Renal BBMVs prepared from freshwater rainbow trout were characterized in detail in a companion paper (Pane et al., in press). Briefly, BBMVs were preferentially enriched in an apical enzyme marker and oriented right-side-out. The half-time to saturation of Ni uptake was found to be about 20 s, and transmembrane uptake and membrane binding were both confirmed (Pane et al., in press).

Ni uptake into renal BBMVs measured across a range of [Ni] from 1 to 1000 μM was fitted to a two component kinetic model and compared across the various Ni treatments. The two components were saturable, Michaelis–Menten kinetics prevailing at lower [Ni], and a linear diffusive component prevailing at higher [Ni] (Fig. 1). The plot of Ni uptake rate as a function of [Ni] was best described by the equation:

$$\text{Uptake rate} = \frac{(J_{\max})[\text{Ni}]}{[\text{Ni}] + K_m} + m[\text{Ni}] \quad (3)$$

where J_{max} is the maximal rate of transport, K_m is the concentration of Ni at which transport is half-maximal, and m is the slope of the linear diffusive component of Ni uptake.

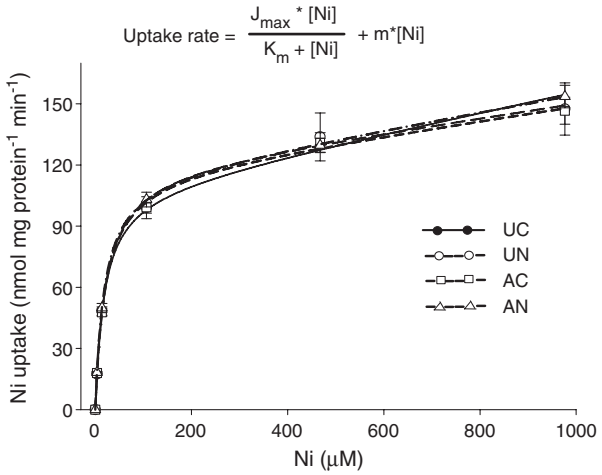

Fig. 1. Ni uptake rate (nmol mg $protein^{-1}$ min^{-1}) into renal BBMVs as a function of extravesicular Ni concentration and Ni exposure history. Extravesicular Ni concentrations were measured, not nominal. UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed; AC = chronically acclimated, control exposed; AN = chronically acclimated, acutely Ni-exposed. Incubations were for 25 s at 12 °C. Data are presented as mean ± SE (n = 3–4).

Table 1
Kinetic parameters for Ni uptake into renal BBMVs based on Ni exposure history

	J_{max}	K_m	m	R^2
UC	108.3±3.7	18.0±1.9	0.049±0.005	1
UN	119.0±6.0	21.3±3.3	0.034±0.008	0.999
AC	117.2±4.1	20.7±2.2	0.034±0.005*	1
AN	115.4±0.7	18.5±0.3	0.041±0.001	1

UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed; AC = chronically acclimated, control exposed; AN = chronically acclimated, acutely Ni-exposed. Incubations were for 25 s at 12 °C. Kinetic parameters were calculated directly from the lines of best fit generated by Sigma Plot 9.0 (SPSS Inc.). Asterisk "*" indicates significantly different from UC treatment. Data are presented as mean±SE (n=3–4).
Data were fitted to the two component equation: Uptake rate $= \frac{(J_{max})[Ni]}{[Ni]+K_m} + m[Ni]$ where J_{max} = maximal rate of transport (nmol Ni mg protein^{-1} min^{-1}), K_m = affinity constant (μM), and m = slope of the linear diffusive component (nmol Ni mg protein^{-1} min^{-1} per μM of Ni).

Table 1 gives the kinetic parameters calculated for each of the 4 Ni treatments. Maximal rates of transport (J_{max}) ranged from 108 to 119 nmol mg protein^{-1} min^{-1}, and were not significantly different among Ni treatments. Affinity constants (K_m) ranged from 18 to 21 μM and also were not significantly different among Ni treatments. The slopes of the linear portion of the kinetic model (m), however, were not the same for each Ni treatment. For chronically acclimated, control exposed (AC) fish, the slope of the linear diffusive component was significantly lower than that of the UC fish (Table 1).

Temperature-dependent Ni uptake into renal BBMVs was confirmed by investigating the temperature-dependence of Ni uptake over the range of 4 to 12 °C. Q_{10} values for each of the four Ni treatments are plotted at extravesicular Ni concentrations of 1, 10, and 100 μM Ni (Fig. 2). Q_{10} values at 1 μM were on the order of 3 to 4, and at 10 μM were around 2.8 to 3.1, while uptake at 100 μM Ni was less temperature-dependent, with Q_{10} values of approximately 1.5 (Fig. 2). At 1 μM Ni, uptake in the AC treatment was significantly more temperature sensitive (higher Q_{10} value) than uptake in the other Ni treatments. Additionally, only in the AC treatment was uptake at 1 μM more temperature sensitive than uptake at 10 μM extravesicular Ni (Fig. 2). For the other three Ni treatments, Q_{10} values at 1 μM were significantly different only from Q_{10} values at 100 μM.

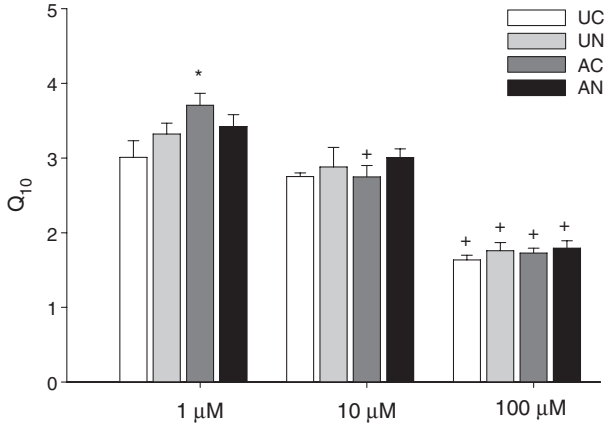

Fig. 2. Temperature-dependence of Ni uptake rate (nmol mg protein^{-1} min^{-1}) into renal BBMVs at varying extravesicular Ni concentrations. Incubations were for 25 s at 12 °C and 30 s at 4 °C. UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed; AC = chronically acclimated, control exposed; AN = chronically acclimated, acutely Ni-exposed. Q_{10} values were calculated as described in the text using Ni uptake rate at 4 and 12 °C for each fish in each treatment at each extravesicular Ni concentration (1, 10, and 100 μM). Asterisk "*" indicates a Q_{10} value significantly different than that of the UC treatment within any [Ni]. "+" indicates Q_{10} values significantly different from those at 1 μM Ni within any treatment. Data are presented as mean±SE (n=4–5).

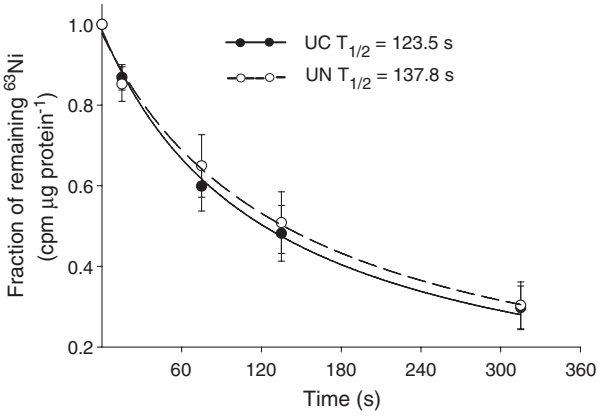

Fig. 3. Time course of Ni efflux (% remaining of original cpm μg protein^{-1}) from pre-loaded renal BBMVs at 12 °C. UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed. Data are presented as mean±SE (n=5–6). To calculate the $T_{1/2}$ values, efflux data were arcsine transformed and fitted to an exponential decay model.

3.2. Efflux of Ni from renal BBMVs

After loading of renal BBMVs with radiolabeled Ni, the time course of Ni efflux from BBMVs was measured by transfer of radiolabeled vesicles into Ni-free assay solution (Fig. 3). From

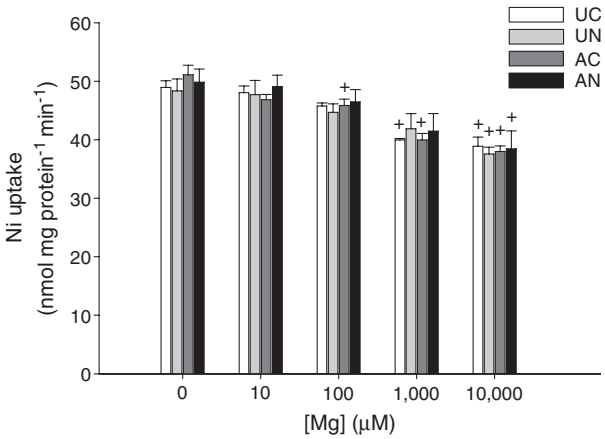

Fig. 4. Competing influence of Mg^{2+} (as $MgSO_4$) on Ni uptake rates (nmol mg protein^{-1} min^{-1}) into renal BBMVs. Incubations were for 25 s at 12 °C at an extravesicular concentration of 10 μM Ni. UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed; AC = chronically acclimated, control exposed; AN = chronically acclimated, acutely Ni-exposed. "+" indicates significantly different from Mg^{2+}-free treatment within any Ni treatment. Uptake rates at any given extravesicular [Mg^{2+}] were not significantly different among Ni treatments. Data are presented as mean±SE (n=3–4).

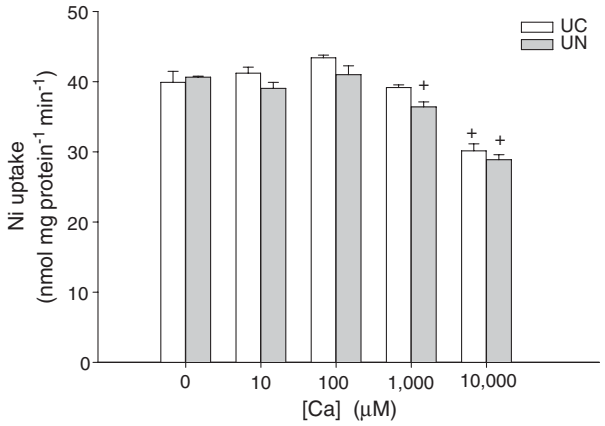

Fig. 5. Competing influence of Ca^{2+}(as $CaSO_4$) on Ni uptake rates (nmol mg $protein^{-1}$ min^{-1}) into renal BBMVs. Incubations were for 25 s at 12 °C at an extravesicular concentration of 10 μM Ni. UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed. "+" indicates significantly different from Ca^{2+}-free treatment within any Ni treatment. Uptake rates at any given extravesicular [Ca^{2+}] were not significantly different among Ni treatments. Data are presented as mean±SE (n=4).

arcsine transformed efflux data, $T_{1/2}$ was calculated as 123.5 s for UC fish and 137.8 s for UN fish (Fig. 3), and these values were not significantly different.

3.3. Cation competition experiments

Inhibition of Ni uptake rate by magnesium, as $MgSO_4$, was concentration-dependent, being significantly reduced at 10,000 μM Mg^{2+}in all Ni treatments (Fig. 4). As all cation competition experiments were run at a [Ni] of 10 μM, significant Mg-induced inhibition of Ni uptake rate occurred in all treatments at a 1000:1 Mg:Ni molar ratio. In treatments not involving acute Ni exposure (i.e. UC and AC), Ni uptake rate was also significantly inhibited at 1000 μM Mg^{2+} (UC and AC), and at 100 μM Mg^{2+} (AC only).

Calcium-induced inhibition of Ni uptake rate by renal BBMVs was not concentration-dependent, and was significant in both UC and UN fish at the highest Ca^{2+} concentration used, 10,000 μM (Fig. 5). In UN fish, however, significant inhibition was also observed at an extravesicular Ca^{2+} concentration of 1000 μM.

3.4. Enzyme activities

Characterization of the membrane composition of BBMVs (see Pane et al., in press) necessitated measurement of the activities of three key cellular enzymes (ALP, Na-K-ATPase, and SDH) in the starting kidney homogenate. Table 2, however, shows no significant effect of either acute, chronic, or combined chronic and acute Ni exposure on the renal homogenate activities of these selected enzymes.

4. Discussion

4.1. Changes in the renal BBM permeability to Ni

Several lines of evidence suggest that chronic, sublethal Ni acclimation results in a tighter renal brush border membrane with a reduced permeability to Ni. While there were no significant differences among the kinetic parameters of the saturable, Michaelis–Menten component of renal BBMV Ni uptake when compared among the four Ni treatments, the linear diffusive component of Ni passage across the BBM, prevalent at higher extravesicular Ni concentrations, was significantly reduced in chronically acclimated, control exposed (AC) fish (Fig. 1; Table 1). Such an acclimation response of reduced Ni permeability would favorably serve to reduce diffusive urine-to-blood reabsorption of Ni across the renal BBM during chronic Ni loading. In support of our in vitro data, in vivo clearance studies have shown that rainbow trout chronically acclimated to Ni have significantly higher clearance ratios for Ni (i.e. lower urine-to-blood reabsorption; Pane et al., 2005a).

The diffusive component of renal BBMV Ni transport was most apparent at extravesicular Ni concentrations greater than 100 μM (Figs. 1 and 2). Though background urine Ni concentrations in the rainbow trout are only about 0.3 μM, we have observed extensive urine (and plasma) Ni loading during acute and chronic Ni exposures (Pane et al., 2004, 2005a). Ni levels in the plasma exceeded 100 μM during chronic exposure (Pane et al., 2004), and though neither plasma nor urine [Ni] was measured in the present study, urine Ni concentrations of 35 μM were observed after only 5 weeks of acclimation (Pane et al., 2005a) to a water Ni concentration very similar to that used in the present study for an 11 month acclimation.

The mechanism by which the permeability of the BBM is reduced by chronic Ni acclimation is unknown, though it is interesting to speculate whether reduced permeability is simply a function of increased Ni associated with the BBM, consistent with the approximate 10-fold increase in total kidney Ni burden following chronic Ni acclimation (Pane et al., 2004). This hypothesis is based on the chemical similarity of Ni and Mg (e.g. Snavely et al., 1991; Smith et al., 1995) and the fact that

Table 2
Comparison of renal enzyme activities across four Ni treatments

Enzyme	Units	UC	UN	AC	AN
Alkaline phosphatase	IU mg $protein^{-1}$ h^{-1}	136.6±12.5 (12)	115.3±11.4 (14)	101.1 ±15.5 (5)	110.7±7.8 (5)
Na-K-ATPase	μmol ADP mg $protein^{-1}$ h^{-1}	4.54±0.41 (13)	4.24±0.52 (14)	4.34±0.73 (5)	4.48±0.57 (5)
Succinate dehydrogenase	A_{490} mg $protein^{-1}$ h^{-1}	0.17±0.04 (4)	0.11±0.02 (5)	NA	NA

Activities were measured in the starting kidney homogenate (see text for details). UC = unacclimated, control exposed; UN = unacclimated, acutely Ni-exposed; AC = chronically acclimated, control exposed; AN = chronically acclimated, acutely Ni-exposed. Data are presented as mean±SE (n=3–4). "NA" means activities were not measured. There were no significant differences among treatments.

increased levels of Mg associated with epithelia decrease membrane permeability (Tidball, 1964).

Theoretically, chronically induced reductions in the permeability of the renal BBM to Ni passage would also decrease the rate of efflux from Ni-loaded BBMVs prepared from AC fish. Fig. 2 shows that efflux from BBMVs prepared from UN fish was not significantly different from that of UC fish, supporting the conclusion that subtle changes in membrane permeability are not induced by acute Ni stress. Unfortunately, due to sample limitation, efflux from BBMVs prepared from AC and AN fish could not be measured. If it is the case that efflux from BBMVs relies substantially on a diffusive component, we would hypothesize that efflux from renal BBMVs prepared from AC fish to show a significantly longer $T_{1/2}$.

4.2. Cation competition

Ni interacts antagonistically with both Ca^{2+} and Mg^{2+} (see Eisler, 1998 for a review). Specifically, Ni and Mg are very similar chemically, acting as specific antagonists of one another in a whole host of systems including bacteria (Kaltwasser and Frings, 1980; Smith et al., 1995), mold (Adiga et al., 1962), yeast (Ross, 1995), invertebrates (Pane et al., 2003), amphibians (Brommundt and Kavaler, 1987), fish (Pane et al., 2005a, in press), and mammals (Enyedi et al., 1982; Kasprzak and Poirier, 1985; Kasprzak et al., 1986; Miki et al., 1987; Sunderman, 1989; Costa, 1991; Costa et al., 1993).

In the companion BBMV paper (Pane et al., in press), data were presented suggesting that a portion of Ni transport into renal BBMVs occurs non-specifically via a high-capacity Mg^{2+} transport system. The control data (UC fish) of Fig. 4 show that Ni uptake into renal BBMVs is inhibited in a concentration-dependent manner by increasing levels of Mg^{2+} in the extravesicular assay buffer. At 1000 μM Mg^{2+}, closely representative of both plasma and urine concentrations in freshwater rainbow trout, Ni uptake into renal BBMVs is significantly inhibited (Fig. 4). Conversely, the control data (UC fish) of Fig. 5 show that Ca^{2+} inhibition of renal BBMV Ni uptake is not concentration-dependent, and that the same physiologically relevant concentration of 1000 μM Ca^{2+} has no effect on Ni uptake into renal BBMVs. Note that inhibition by Mg^{2+} and Ca^{2+} at grossly supraphysiological concentrations of 10,000 μM are presumed to stem from alterations to the BBM itself, in addition to specific antagonism of Ni transport (Pane et al., in press).

Comparison of Ni uptake in the presence of competing levels of Mg^{2+}, and across various Ni treatments (Fig. 4), revealed a concentration-dependent difference in Ni/Mg interaction in AC fish, suggesting that chronic Ni acclimation altered the way in which the renal BBM handles Ni and Mg^{2+}. The mechanisms underlying such a putative effect are unknown, though it is interesting to note that chronic Ni stress has been shown to protect against Mg^{2+} loss in rainbow trout when fish were subsequently acutely challenged by Ni exposure (Pane et al., 2005a). These in vivo results complement the present in vitro results in suggesting that chronic Ni acclimation affects the handling of both Ni and Mg^{2+}. Additionally, these combined results suggest that Mg^{2+} and Ni compete, to some extent, for the same transport system in the renal BBM.

It is also of interest to note that only AC fish showed differential temperature-dependence of Ni uptake at an extravesicular Ni concentration as low as 10 μM (Fig. 2), indicating that chronic Ni acclimation also affects the physical properties of the renal BBM.

4.3. The pathophysiology of chronic Ni exposure

Despite previous in vivo clearance studies suggesting that the overall reabsorptive capacity of the rainbow trout kidney was compromised by acute Ni challenge (Pane et al., 2005a), we found no evidence that either acute, chronic, or combined Ni exposure impacted the activity of three key cellular enzymes from the trout kidney (Table 2). In particular, the activity of Na-K-ATPase in the trout kidney, an enzyme critical to the establishment of transport gradients, was unaffected by Ni exposure.

With respect to subtle alterations of membrane function, however, our current findings of reduced permeability of the renal BBM to Ni are reminiscent of previous findings that chronic Ni acclimation resulted in a reduced maximal capacity of gas exchange in exercised rainbow trout (Pane et al., 2004). Morphometrically, chronic, Ni-induced reductions in the gas exchange capacity of the rainbow trout gill were attributed in part to a swelling of the branchial epithelium resulting in a greater barrier to gas diffusion (Pane et al., 2004). Whether these two membrane-level phenomena of chronic Ni acclimation are mechanistically related, and the exact manner by which Ni alters the structure and function of cellular membranes during chronic, sublethal exposures, are unknown and worthy of further investigation.

Current methods of water quality criteria determination for certain metals are based on the biotic ligand model (BLM; DiToro et al., 2001), which relies on physiological constants to describe the short-term binding of metals to membrane surfaces within such target organs as the gill of teleost fish. These constants, however, assumed to be stable for the purposes of acute toxicity modeling, are a function of intrinsic membrane properties and consequently, previous metal exposure history (Niyogi and Wood, 2003). The present report describes the subtle in vitro alteration of such a membrane property (i.e. permeability of the renal brush border membrane of the rainbow trout to Ni) induced by chronic, low-level Ni exposure. Accordingly, maturation of the BLM process, including eventual development of chronic biotic ligand models, will be well served by accommodation of variations in physiological parameters over the course of chronic metal exposures.

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