EVIDENCE FOR REGULATION OF NHE-1-LIKE PROTEIN EXPRESSION IN THE GILLS OF THE LONG-HORNED SCULPIN *(MYOXOCEPHALUS OCTODECIMSPINOSUS)* DURING ACIDOSIS

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pH homeostasis in fishes is challenged by intrinsic H^+ production (from by-products of metabolism) and a relatively high sensitivity to environmental stress (i.e. hypercapnia). Despite this, fish are able to maintain their pH between 7.7 and 8.0 primarily by vectorial H^+ transport across the branchial epithelia (blood to water). Although the molecular identity of H^+ transporters in marine fish gills is still underway (Blackston et al., this vol. and MacKenzie et al., this vol.), some findings implicate a Na^+/H^+ exchanger (NHE) in the apical membrane. Experiments with sculpin adapted to low Na^+ water showed that net acid efflux is dependent on an external solute concentration that favors Na^+ entry across the apical membrane (Claiborne, et al., *J. Exp. Zool.* 279:509-520, 1997). In addition, amiloride and a more specific NHE inhibiting analog (5-N,N-hexamethylene-amiloride), independently caused more than a 60% decrease in net H^+ efflux from acidotic sculpin suggesting that apical Na^+/H^+ exchange may be responsible for the majority of net H^+ efflux during recovery from acidosis (Claiborne, et al., *Bull. MDIBL* 35:48-49, 1996).

In contrast, basolateral Na^+/H^+ activity would decrease the efficiency of whole animal H^+ efflux by lowering the Na^+ and H^+ gradients required for apical exchange on the other side of the cell (Kurtz, *J. Clin. Invest.* 83:616-622, 1989). Five mammalian Na^+/H^+ exchanger isoforms have been cloned and characterized, each different with respect to location, regulation, and membrane targeting in polarized cells. NHE-1 is the "ubiquitous" isoform normally targeted to the basolateral membrane of epithelial cells (Tse et al., *J. Mem. Biol.* 135:93-108, 1993). Its expression was detected in sculpin gills by Northern blot analysis with a 1.9 kb human NHE-1 probe (Harris et al., *Bull. MDIBL* 32:128-130, 1993). If present, degradation of this transporter would reduce basolateral Na^+/H^+ activity and increase net H^+ efflux across the apical membrane. Such an adaptive response would help explain the rapid increase in acid excretion and eventual alkalosis observed after sculpin are given an acid load (Claiborne et al., 1997). Thus, in the present study, an antibody raised against amino acids 514-818 of porcine NHE-1 (Cox et al., *Cell* 91:139-148, 1997) was used to: 1) determine if NHE-1-like proteins are present in sculpin gills and 2) investigate alterations in their expression during recovery from acidosis.

After a pre-adaptation to dilute seawater (20%, ~100 mM) for 9-10 days (as in Claiborne et al., 1997), sculpin were given an intraperitoneal injection of either 0.1 M HCl (2 mM/kg) or an equivalent volume of deionized water (control fish). After 5 hours of recovery, fish were pithed and a membrane enrichment was prepared from dissected gill filaments. First, cells were disrupted with a polytron homogenizer (on ice for 20 s, medium setting) in ice cold buffer (250 mM sucrose, 1 mM EDTA, 2 ug/ml aprotinin, 2 ug/ml leupeptin, 100 ug/ml PMSF, and 30 mM Tris-Cl at pH 7.4). Cell debris was then removed in a low speed spin (3000g for 5 min) and membranes were pelleted in a final high speed spin (50,000g for 30 min). Pellets were then resuspended in a minimal volume of homogenization buffer and proteins in the suspension were solubilized by adding a modified Laemmli sample buffer (*Nature* 227:680-685, 1970; no bromophenol blue or β-mercaptoethanol). After determining the total protein concentration (Biorad DC, detergent compatible assay), 2% β-mercaptoethanol and 0.01% bromophenol blue were added to each sample. A 100 ug aliquot of total protein was separated in a 7% polyacrylamide gel (3 h at 200 V) then transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore) according to the manufacturer's protocol. Immunoblotting procedures were as follows: Blocking, Blotto (5% nonfat dry milk in TBS, pH 7.4) overnight at 4°C; Primary antibody incubation, monoclonal antibody 4E9 (culture supernatant diluted 1/5 in

Blotto) for 2 h at ~25°C; Primary antibody wash, 3 X TBST (0.1% Tween-20 in TBS pH 7.4); secondary antibody incubation, HRP-conjugated goat anti-mouse IgG (1:5000 diluted in Blotto; Pierce, Rockford, IL) 1.5 h at ~25°C. Antibody binding was detected by exposing Kodak X-OMAT-AR scientific imaging film to a Chemiluminescent signal (SuperSignal System; Pierce, Rockford, IL) according to the manufacturer's protocol. A digitized image was produced from the resulting negative (Hewlett Packard ScanJet IIcx flatbed scanner) followed by analysis with NIH Image software version 1.61 (National Institute of Health, USA) on a Power Macintosh 6500/225. Mouse monoclonal antibody 4E9 was graciously provided by Drs. Bliss Forbush and Daniel Biemesderfer at Yale University School of Medicine.

As shown in figure 1, a diffuse band centered at approximately 90 kDa was detected in both deionized water (control) and acid loaded sculpin gills indicating the presence of NHE-1-like antigens in these tissues. The optical density of the 90 kDa band in acid loaded sculpin was fourty fold lower than in control fish indicating a reduction in NHE-1-like protein abundance during acidosis. Two additional bands of lower molecular weight were also detected.

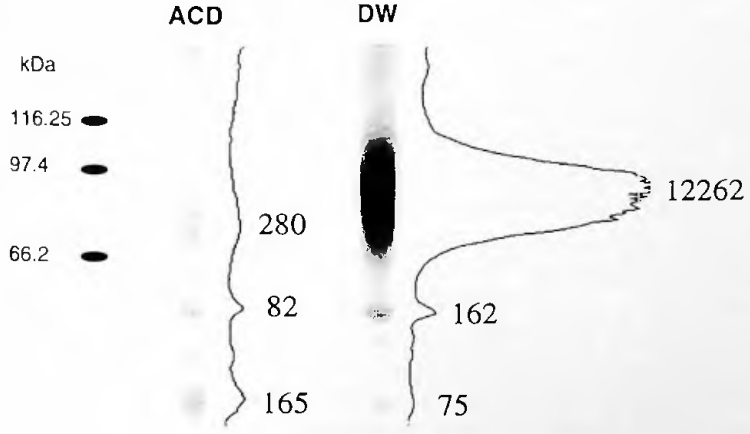

Figure 1: Representative immunoblot of gill tissue from acid (ACD) and deionized water loaded (DW) sculpin using monoclonal antibody 4E9. 100 ug of total protein was loaded in each lane. The uncalibrated optical density of each lane was graphed and the relative area (arbitrary units) under each peak is to the right of the respective lane.

These results suggest that NHE-1-like proteins are expressed in sculpin gills and support preliminary Northern blot studies which detected mRNA transcripts with a human cDNA probe. Although the apparent molecular mass of 90 kDa is smaller than mammalian NHE-1 (97 kDa, kidney tissue blotted under the same conditions; data not shown), the signal profile is similarly diffuse, indicating that it may also be a heavily glycosylated protein (Sardet et al., *Science* 247:723-725 & 747, 1989). The identity of the two small molecular weight bands is unknown but may represent degradative fragments of the functional protein.

NHE-1 has recently been localized to the basolateral membrane of turtle (Harris et al., *Am. J. Physiol.* 272:G1594-G1606, 1997) and amphibian epithelial tissue (Coupaye-Gerard et al., *Am. J. Physiol.* 271:C1639-C1645, 1996). If basolateral targeting is a conserved feature of NHE-1 in fish gills, a reduction in serosal Na^+/H^+ exchange by NHE-1 degradation would increase the efficiency of whole animal net acid excretion. Thus, the dramatic reduction in NHE-1-like protein observed 5 h after inducing metabolic acidosis may represent an adaptive response to a reduction of systemic pH. Future immunohistochemistry studies will investigate the membrane localization and relative quantity of NHE-1 homologues under basal and acidotic conditions.

This research was funded by NSF grant RUI-94-19849 to J. B. C.