IMMUNOLOGICAL DETECTION OF GILL NHE2 IN THE DOGFISH (*SQUALUS ACANTHIAS*)

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During respiratory acidosis, fishes compensate by accumulating plasma bicarbonate (Heisler, N. in *Fish Physiology* ed. W. Hoar and D. Randall, Academic Press Orlando 10:315-392, 1984). Excretion of acid across the gills is enhanced and is thought to be driven by Na^+/H^+ exchangers (NHE). Studies of the mummichog (*Fundulus heteroclitus*) using heterologous antibodies have immunologically demonstrated the presence of NHE2 in gill membrane homogenates (Edwards, S. et al., J. Exp. Biol., submitted; Wall et al., Bull. MDIBL 39:50-51, 2000), but we have been unsuccessful at detecting NHE2 in other fish species using these antibodies designed against mammalian sequence. We have recently cloned a full length cDNA transcript for a NHE2-like isoform from the gills of the dogfish (Morrison-Shetlar et al., Bull. MDIBL, 41:11, 2002). Using this sequence, we have now developed polyclonal antibodies specific for a dogfish NHE2 epitope. The peptide was synthesized against sequence at the end of the carboxyl terminal, believed to be the variable region between isoforms, and was found to be nonhomologous to other isoforms and NHE2 in other fish species. In this study, we have begun the immunological detection of NHE2 in the gills of the dogfish using these specific antibodies.

Following anesthesia in MS-222 in seawater (150 mg l^{-1}) spiny dogfish (*Squalus acanthias*) were perfused through the *conus arteriosus* with elasmobranch Ringer's, pithed, and filaments were removed, weighed and placed in ice cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, 100µg/ml PMSF and 5 mg/ml protease inhibitor cocktail). The solution was centrifuged with the resulting pellet suspended in 50/50 solution of homogenizing buffer and modified Laemmli sample buffer. 30 µg of total protein was resolved on a 7.5% polyacrylamide gel, and transferred to PVDF membranes. Rabbit polyclonal antibodies against AA 636-654 of the dogfish NHE2 sequence (NENQVKEILIRRHESLRES) were created (BioSource International) and used from whole serum (540-4) or affinity purified (541-AP) with the same peptide antigen. The membrane was blocked with 5% Blotto (pH 7.4) for one hour at room temperature (RT), then incubated with NHE2 primary antibody (1:5000 diluted in 5% Blotto) overnight, and washed with primary antibody (TBST: TBS with 0.1% Tween-20 pH 7.4). A secondary 1 h incubation in AP-conjugated goat anti-rabbit IgG (diluted 1:3000 in 5% Blotto) was followed by a secondary antibody wash with 1X TBST with three changes. Bound IgG was detected using an enhanced chemiluminescence system (Biorad 170-5018). Gill filaments were also removed from the arches for immunohistochemistry, and placed in fixative (3% paraformaldehyde, 0.05% glutaraldehyde, 0.05% picric acid in 10 mmol l^{-1} phosphate-buffered saline, pH 7.3) for 24 h at 4°C. Filaments were rinsed in at least three changes of phosphate buffered saline (PBS) at 4°C, cryoprotected in at least two changes of 20% sucrose, 5% polyethylene glycol, and frozen in O.C.T. embedding medium (TissueTek®, Sakura, CA). Cryosections (8-10 mm) were cut with a Reichert-Jung cryostat and dried on positively charged slides (Fisher Scientific). Slides were then thawed at RT, and endogenous peroxidase activity was inhibited by incubating with 0.3% H_2O_2 in block (1.5% normal goat serum, 0.09% NaN_3, and 0.1% Tween-20 in PBS at pH 7.3) for 30 min at RT. Non-specific binding sites on the tissues

were blocked by incubating with block for 30 min. Sections were then incubated with a primary NHE2 antibody (541-AP; 1/750-1/1000) overnight at 4°C, in a humidified chamber and then detected with Vector Laboratories' Vectastain Elite® ABC kit.

As seen in Figure 1, the polyclonal antibody 540-4 (anti-NHE2) was cross-reactive to a protein of ~70 kDa from crude membranes. Incubation of tissue sections with affinity purified antibody 541 produced diffuse cellular staining along the lamellar region (Figure 2). In one of three sharks measured, cells had an apical distribution of NHE2 while in the other two, cytoplasmic and/or basolateral immunoreactivity was detected. NHE2 staining did not appear to be colocalized with Na^+-K^+-ATPase positive cells and no staining was observed in filaments which were incubated in preimmune serum, or in excess antigen controls in which affinity purified antibody 540 was incubated with excess peptide (data not shown).

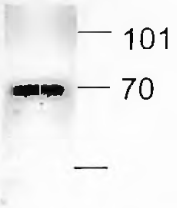

Figure 1: Immunoblot of gill tissue from dogfish. Incubation in 540-4, primary antibody for NHE2, produced bands with a molecular weight of ~70 kDa.

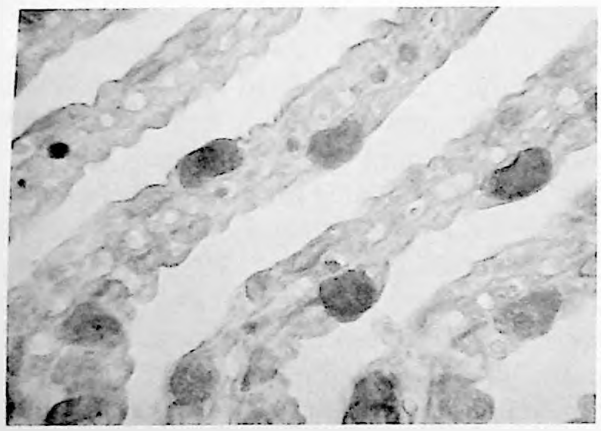

Figure 2: Lamellar staining pattern for NHE2 (541-AP) observed the dogfish gill.

These results indicate that a protein similar to the predicted size of the dogfish NHE2 ORF (84.5 kDa) is found in gill membrane preparations. The 540-4 antibody was designed to be species and isoform specific to an epitope near the 3' end of the dogfish sequence. It recognized specific lamellar cells and was observed both apically and as a diffuse staining throughout the cells. Claiborne et al. (J. Exp. Zool. 261:9-17, 1992) showed that net H^+ excretion in the dogfish increases ~3x fold during a hypercapnia induced respiratory acidosis. Wilson et al. (J. Exp. Zool 278:78-86, 1997) demonstrated the presence of H^+-ATPase in the *S. acanthias* and suggested that this transporter may be involved with acid excretion during acidosis. The present data show for the first time, that NHE2 is also expressed in the dogfish. It remains to be seen what roles these two cellular mechanisms play during acid-base perturbations. This research was funded by NSF grant # 0111073 to JBC.