ARSENIC AND ADAPTATION TO SEAWATER IN KILLIFISH (FUNDULUS HETEROCLITUS)

Caitlin R. Stanton[1], Druanne Prescott[2], Alexander Lankowski[3], Katherine Karlson[3], John E. Mickle[4], Joseph Shaw[5], Joshua Hamilton[5] and Bruce A. Stanton[3]
[1]Brown University, Providence, RI 02912, [2]Boston University, Boston, MA 02215, [3]Department of Physiology, Dartmouth Medical School, Hanover, NH 03755, [4]Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21287, and [5]Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755

The killifish *Fundulus heteroclitus* is an excellent model to study the effects of environmental toxins, including arsenic, on physiological systems. In addition, the killifish is an excellent model to study the physiology of CFTR Cl channels. In seawater (SW) adapted fish, CFTR is located in the apical plasma membrane of chloride cells found in the branchial epithelium (i.e., gill, opercula) and is responsible for Cl secretion. When fish migrate from freshwater (FW) to SW the opercular membrane secretes more Cl by increasing the number of CFTR Cl channels in the plasma membrane. This increase in Cl secretion is a vital adaptive response that allows the fish to maintain NaCl balance. Studies have suggested that the increase in plasma cortisol that occurs when fish migrate from FW to SW is required for the adaptive increase in CFTR Cl secretion (Marshall et al., *J.Exp.Biol.* 202:1535-1544, 1999). Cortisol is thought to bind to the glucocorticoid receptor and active CFTR gene transcription, resulting in an increase in CFTR protein expression.

Arsenic is a metalloid that occurs naturally in soil, food, water and air (Abernathy et al. *Environ Health Perspect* 107:593-597, 1999). Arsenic is present in high concentrations in many toxic waste sites and can accumulate in groundwater and well water (National Research Council, Arsenic in Drinking Water, National Academy Press, Washingotn, DC, 1999). In several countries, including the US, arsenic levels greater than 100-800 ppb have been measured in well water, values that are far in excess of the current EPA drinking water standard (10 ppb; U.S. EPA, 66 CFR 6976, 2001). Epidemiological studies have suggested that exposure to high levels of arsenic may increase the incidence of atherosclerotic diseases and diabetes mellitus, as well as lung, skin, liver, bladder and other cancers (National Research Council, Arsenic in Drinking Water, National Academy Press, Washingotn, DC, 1999 and Karagas, et al., *Environ Health Perspect* 106 Suppl 4:1047-1050, 1998). Thus, arsenic contamination of the water supply poses a serious threat to public health in the US. Many of the actions of arsenic are mediated by alterations in signal transduction pathways and by activation or inactivation of transcription factors that may alter gene expression. For example, arsenic blocks glucocorticoid receptor mediated transcriptional activation (Kaltreider et al., *Environ.Health Perspect.* 109:245-251, 2001). Accordingly, the goal of our research was to test the hypothesis that arsenic blocks the cortisol-induced, adaptive increase in CFTR gene and protein expression and thereby blocks the ability of killifish to adapt to an increase in salinity.

Killifish were collected from Northeast Creek (Bar Harbor, ME) and held in aquaria containing running seawater at the MDIBL (June-July, 2002) or static artificial seawater (Instant Ocean) at the Dartmouth Medical School (August-December, 2002). Fish were kept in seawater for at least one week to acclimate. Adaptation to fresh water was achieved by gradually (~1 hr)

reducing the aquaria salinity to 10% and maintaining the fish in 10% seawater for two weeks. Subsequently, the water was changed gradually (~1 hr) to dechlorinated tap water. Fish were maintained in fresh water for at least two weeks prior to experimentation. Thereafter, vehicle or sodium arsenite were injected into the abdominal cavity in a 100 ul volume using a Hamilton syringe fitted with a 30 guage needle. FW adapted fish were either maintained in FW or returned to 100% SW. Two days after vehicle or arsenic treatment, the opercular membranes were isolated and mounted in Ussing chambers for measurements of CFTR-mediated Cl secretion (Isc) as described (Karnaky et al., *Science* 195:203-205, 1977). Similar results were obtained at the MDIBL and Dartmouth.

Initial studies were conducted to establish an appropriate dose of arsenic (50 to 1.25 μmol/kg, which produce environmentally relevant levels of arsenic in blood and tissue). The higher doses of arsenic examined were either lethal (50 μmol/kg caused 100% mortality within 5 minutes) or caused significant toxicity (20 to 10 μmol/kg) after 96 hrs (assessed by blood volume and measurements of transepithelial resistance across opercular membranes). A lower dose of arsenic (5 μmol/kg) did not produce any overt cellular toxicity, however, this dose significantly reduced CFTR mediated Cl secretion (Isc) across opercular membranes. In FW fish arsenic reduced the Isc from 109 ± 10 to 54 ± 13 μA/cm^2 ($P<0.05$) and in SW fish arsenic reduced the Isc from 333 ± 73 to 141 ± 60 μA/cm^2 ($P<0.05$). Although all vehicle-treated fish survived the transfer from FW to SW, none of the arsenic (5 μmol/kg) injected fish survived the transfer from FW to SW. Thus, experiments involving fish transferred from FW to SW were repeated with lower doses of arsenic (2.5 and 1.25 μmol/kg). Both groups of fish injected with the lower doses of arsenic survived the transfer from FW to SW for 48 hrs. However, the opercular membranes in the arsenic injected fish transferred from FW to SW were very fragile (probably because of the dual stress of the switch from FW to SW and arsenic) and Isc could not be measured. Thus, additional studies are required to determine an appropriate dose of arsenic for our studies. These data are consistent with our hypothesis that arsenic blocks the cortisol-induced, adaptive response in CFTR mediated Cl secretion.

In addition, studies were initiated to examine the effect of arsenic on killifish CFTR (kfCFTR) gene expression by real time PCR. Total RNA was isolated from killifish operculum, and heart (control tissue since heart does not express CFTR) using the Purescript RNA Isolation Kit (Gentra, Minneapolis,MN) and DNase treated (DNA-Free, Ambion, Austin,TX) to remove contaminating DNA. Two-Step RT-PCR was performed utilizing Multiscribe Reverse Transcriptase with random hexamers and Taqman Universal PCR Master mix. Primers and probes for Real Time PCR were synthesized using the Assays-by-Design service (Applied Biosystems). Sequences for kfCFTR and kfβ-actin were submitted to Applied Biosystems for primer-probe design and probe target was set to predicted exon-exon junctions. Probes were 6-FAM dye-labeled with an MGB (minor groove binding) modification and non-fluorescent quencher on the 3' end of the probe. The Assay-by-Design probes and primers, premixed to a concentration of 18μM for each primer and 5μM for each probe, were combined with Taqman Universal Master Mix and cDNA and were placed in a 96-well format spectrofluorometric thermal cycler (ABI Prism 7700 Sequence Detection System). Real time PCR was performed with serial dilutions of the templates to construct standard curves for kfCFTR and kfβactin. PCR products were run on an agarose gel to confirm product size. Sequence anaylsis confirmed the identity of the amplicons. Preliminary results indicate consistent and reliable transcription for

kfCFTR and kfβactin with high precision and reliability. Standard curves indicate a broad linear dynamic range of over three orders of magnitude. These preliminary studies demonstrate that we have the ability to accurately and reproducibly examine the effects of arsenic on kfCFTR gene expression by real time PCR. (Supported by a MDIBL New Investigator Award to J. E. M, a Cystic Fibrosis Foundation award to C.R.S., a Cystic Fibrosis Foundation Research Development Program Award to B.A.S., AHA New England Summer Fellowship to D.P., and an NIEHS award to J.H.).