The Journal of Experimental Biology 209, 1696-1708
Published by The Company of Biologists 2006
doi:10.1242/jeb.02198

COX2 in a euryhaline teleost, *Fundulus heteroclitus*: primary sequence, distribution, localization, and potential function in gills during salinity acclimation

Keith P. Choe[1,2,*], Justin Havird[1,2], Rachel Rose[1,2], Kelly Hyndman[1,2], Peter Piermarini[3] and David H. Evans[1,2]

[1]*Department of Zoology, University of Florida, Gainesville, FL 32611, USA,* [2]*Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672, USA and* [3]*Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520, USA*

*Author for correspondence at present address: T-4202 Medical Center North, 1161 21st Ave South, Anesthesiology Research, Vanderbilt Medical Center, Nashville, TN 37232-2520, USA (e-mail: keith.p.choe@vanderbilt.edu)

Accepted 7 March 2006

Summary

In the kidneys of mammals, cyclooxygenase type 2 (COX2) is expressed in medullary interstitial cells, the macula densa and epithelial cells of the cortical thick ascending limb where it generates prostaglandins that regulate hormone secretion, inhibit ion transport, and support cell survival during salt loading and dehydration. In teleosts, the gills are in direct contact with an aquatic environment and are the dominant site of osmoregulation. During transfers between salinities, specialized cells in the gills (chloride cells) rapidly regulate NaCl secretion for systemic osmoregulation while they simultaneously are exposed to acute osmotic shock. This study was conducted to determine if COX2 is expressed in the gills, and if so, to evaluate its function in cellular and systemic osmoregulation. Degenerate primers, reverse transcription–PCR and rapid amplification of cDNA ends were used to deduce the complete cDNA sequence of a putative COX2 enzyme from the gills of the euryhaline killifish (*Fundulus heteroclitus*). The 2738 base pair cDNA includes a coding region for a 610 amino acid protein that is over 70% identical to mammalian COX2. A purified antibody generated against a conserved region of mouse COX2 labeled chloride cells, suggesting that the enzyme may control NaCl secretion as an autocrine agent. Real-time PCR was then used to demonstrate that mRNA expression of the COX2 homologue was threefold greater in gills from chronic seawater killifish than in gills from chronic freshwater killifish. Expression of $Na^+/K^+/2Cl^-$ cotransporter and the cystic fibrosis transmembrane conductance regulator were also greater in seawater, suggesting that chronic COX2 expression in the gills is regulated in parallel to the key ion transporters that mediate NaCl secretion. Real-time PCR was also used to demonstrate that acute transfer from seawater to freshwater and from freshwater to seawater led to rapid, transient inductions of COX2 expression. Together with previous physiological evidence, the present molecular and immunological data suggest that constitutive branchial COX2 expression is enhanced in seawater, where prostaglandins can regulate NaCl secretion in chloride cells. Our data also suggest that branchial COX2 expression may play a role in cell survival during acute osmotic shock.

Key words: osmoregulation, prostaglandin, chloride cell, mitochondrion-rich cell, killifish, *Fundulus heteroclitus*.

Introduction

In vertebrates, prostaglandins have roles in diverse processes including blood clotting, ovulation, initiation of labor, bone metabolism, wound healing, kidney function, blood vessel tone and inflammation (Dubois et al., 1998). In the kidneys of mammals, prostaglandins regulate vascular tone, salt and water homeostasis, hormonal action and cellular osmotic stress responses (Harris and Breyer, 2001). Systemic osmotic imbalances such as chronic salt loading and hydration stimulate renal prostaglandin production that facilitates salt excretion *via* complex pathways. One effect of renal prostaglandins is to inhibit salt transport by cells in the cortical thick ascending limb and collecting ducts (Stokes and Kokko, 1977; Stokes, 1979; Guan et al., 1998), another is to facilitate cell survival during acute osmotic stress in medullary interstitial cells (Yang, 2003).

Cyclooxygenase (COX; prostaglandin synthase; G_2/H_2) is the enzyme responsible for the initial rate-limiting conversion of arachidonic acid to prostaglandin G_2 and then to prostaglandin H_2. A COX enzyme was first purified from sheep

seminal vesicles and was first cloned from the same tissue by DeWitt and Smith (DeWitt and Smith, 1988). This first isoform was found to be constitutively expressed in many tissues and was later named COX1; a second isoform, named COX2, was subsequently cloned from mouse and chicken fibroblast cell cultures (Kujubu et al., 1991; Xie et al., 1991; O'Banion et al., 1992). Early reports on COX expression and function suggested that constitutive expression of COX1 predominated over COX2 expression, because basal expression of COX2 was found to be low in some cell types (Funk, 2001; Simmons et al., 2004). COX2, and not COX1, expression was shown to be greatly increased by stimulation with mitogens and cytokines, and COX2 was considered as the inducible isoform that mediates inflammatory responses (Kujubu et al., 1991; Xie et al., 1991; O'Banion et al., 1992). However, COX2 was later found to be constitutively expressed in the kidneys of mammals (Harris et al., 1994; Guan et al., 1997; Khan et al., 1998) where it regulates blood flow and ion transport (Harris and Breyer, 2001). Specifically, COX2 is expressed constitutively in the macula densa and the adjacent cortical thick ascending limb of the loop of Henle where it appears to be an important intermediary step in the control of renin secretion by juxtaglomerular cells (Harris and Breyer, 2001). COX2 is also expressed constitutively in medullary interstitial cells where it is important for cell survival during dehydration and for mediating NaCl excretion during salt loading and dehydration (Yang et al., 2002; Yang, 2003).

Unfortunately, very little is known about the functions of COX enzymes in non-mammalian vertebrates, including their roles in osmotic and ionic regulation in fishes. A COX homologue was recently cloned from shark (*Squalus acanthias*) rectal glands, where NaCl secretion was reduced by a COX inhibitor, but the authors were unable to determine if the cDNA was an orthologue of COX1 or COX2 (Yang et al., 2002). COX1 and COX2 cDNAs have been cloned from rainbow trout (*Onchorynchus mykiss*) (Zou et al., 1999), brook trout (*Salvelinus fontinalis*) (Roberts et al., 2000) and zebrafish (*Danio rerio*) (Grosser et al., 2002), and genome sequences predict the presence of both isoforms in puffer fish (*Fugu rubripes*), demonstrating that teleosts have both isoforms (Jarving et al., 2004). A probable candidate tissue for COX-mediated regulation of ion transport is the gill epithelium of teleosts. This tissue is the primary site of osmoregulation in fishes and contains specialized cells that secrete NaCl (chloride cells) *via* a mechanism that is similar to the NaCl transport mechanism of shark rectal glands and the mammalian cortical thick ascending limb of the kidney (Evans et al., 2005). Using isolated opercular epithelia of killifish (*Fundulus heteroclitus*), a well-established model for the gill epithelium that can be mounted in modified Ussing chambers (Karnaky, Jr et al., 1977; Eriksson et al., 1985), we and others have demonstrated that prostaglandins inhibit short circuit currents (I_{sc}) that are a result of active Cl^- secretion (Van Praag et al., 1987; Evans et al., 2005). Using specific pharmacological inhibitors, we also determined that basal I_{sc} may be moderately inhibited by COX2-mediated prostaglandin synthesis and that COX2 mediated a large fraction of endothelin-induced inhibition of I_{sc} (Evans et al., 2005).

Killifish are euryhaline and can osmoregulate in environmental salinities ranging from extremely hypoionic (freshwater, 0.1 mmol l^{-1} NaCl) to extremely hyperionic (4× seawater, ~2000 mmol l^{-1} NaCl) by absorbing or secreting NaCl from their gills (Griffith, 1974). Remarkably, killifish can tolerate acute transfer from freshwater to seawater and from seawater to freshwater with only minor, transient alterations in blood plasma osmolarity and Na^+ concentration by rapidly controlling NaCl transport (Jacob and Taylor, 1983; Wood and Marshall, 1994; Marshall et al., 1999; Wood and Laurent, 2003). The key ion transporters and cells (chloride cells) that mediate NaCl secretion from teleost gills are well described, and have been reviewed extensively (Perry, 1997; Karnaky, 1998; Marshall, 2002; Hirose et al., 2003; Evans et al., 2005). The model of NaCl secretion from teleost gills is similar to other secretory epithelia, where basolateral Na^+/K^+-ATPase (NKA) is the primary active transporter that creates electrochemical gradients that favor Cl^- entry *via* basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and Cl^- exit *via* apical cystic fibrosis transmembrane conductance regulator (CFTR). Na^+ then leaves paracellularly through leaky junctions. All three key ion transporters have been cloned from killifish and the isoforms expressed in the gills have been identified; NKA α1 (Semple et al., 2002; Scott et al., 2004), NKCC1 (Scott et al., 2004) and CFTR (Singer et al., 1998). Although the mechanism of NaCl secretion from teleost gills is well described, little is known about the role of transcriptional regulation of transporters in relation to paracrine signaling agents (such as COX2) that may control ion transport.

The first goal of this study was to clone a COX2 orthologue from the euryhaline killifish so that its distribution and primary sequence could be characterized. The second goal of this study was to localize COX2 in the gills relative to chloride cells to determine if it could regulate ion transport as an autocrine signaling component. The last goal of this study was to measure the effect of salinity on the branchial expression of COX2 and several ion transporters, to gain insights into how the enzyme might contribute to salinity acclimation.

Materials and methods

Animals and standard holding conditions

The University of Florida, Institutional Animal Care & Use Committee approved all procedures. Killifish (*Fundulus heteroclitus* L., approximately 5–10 g) were captured from tidal creeks near Salisbury Cove, ME, USA. They were transported to the University of Florida in Gainesville, FL, USA where they were held in a 380 l Rubbermaid tank containing 100% seawater from the Atlantic Ocean. The water pH was maintained between 7.8 and 8.2 with a commercial aquarium carbonate buffer (Seachem, Stone Mountain, GA, USA), NH_3 and NO_3^- were maintained below 1 ppm with a biological filter, and temperature was maintained at between 22 and 26°C. The room that housed the killifish was on a

12 h:12 h light:dark cycle, and killifish were fed commercial pellets to satiation 3–4 times a week.

Reverse transcriptase-polymerase chain reaction (RT–PCR), cloning and sequencing

Killifish were pithed, and gill filaments were removed with sterile, RNAse-free tools, and frozen in liquid nitrogen. Total RNA was then isolated with TRI reagent (Sigma, St Louis, MO, USA), and first-strand cDNA was synthesized from 2 μg of total RNA with a Superscript™ II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) using oligo(dT) as a primer. A degenerate primer pair taken from Yang et al. (Yang et al., 2002) that was designed to amplify conserved regions of vertebrate COX proteins (Table 1), was used for initial cloning and sequencing of killifish COX2. Each PCR was performed on 1/20th of a reverse transcriptase reaction with a FastStart Taq DNA Polymerase kit (Roche Applied Science, Indianapolis, IN, USA) in a PCR Express thermocycler (ThermoHybaid, Franklin, MA, USA) with standard cycling parameters. PCR products were visualized by ethidium bromide staining in 1.0–1.5% agarose gels, ligated into pCR®4-TOPO vectors, and transformed into TOP10 chemically competent cells using a TOPO TA Cloning® Kit for sequencing (Invitrogen). Plasmid DNA was then sequenced in both directions at the Marine DNA Sequencing Facility at the Mount Desert Island Biological Laboratory (Salisbury Cove, MA, USA).

After the sequencing of initial fragments with vector-specific primers, more of the cDNA for COX2 was cloned and sequenced by 5′ and 3′ rapid amplification of cDNA ends (5′ and 3′ RACE). Briefly, 5′ and 3′ RACE cDNA was prepared with a GeneRacer™ Kit (Invitrogen) according to the manufacturer's protocols. PCRs for 5′ RACE were completed with antisense primers 5′ R1199 and 5′ R1090 and sense primers that were included in the kit, and PCRs for 3′ RACE were completed with sense primers 3′ F1495 and 3′ F1631 and antisense primers that were included in the kit (Table 1). PCR, cloning, and sequencing were performed as above, except for the use of touchdown cycle parameters and nested PCR to increase specificity.

Sequence analysis

Sequence results for each initial degenerate primer pair were assembled and the resulting amino acid translations were analyzed with the basic local alignment search tool (Blast) on the National Center for Biotechnology Information website. COX2 fragment sequences were assembled with GeneTools software (BioTools Inc., Edmonton, Alberta, Canada) and the assembled nucleotide sequence was searched for open reading frames. The predicted amino acid sequence was aligned with other full-length vertebrate COX proteins using PepTools software (BioTools Inc.). The expected locations of enzymatic activity and regions important for regulation of the enzyme were taken from previously published reports (Simmons et al., 2004). MEGA software (Kumar et al., 2001) was used to make an unrooted phylogenetic tree of chordate cyclooxygenases with the neighbor-joining method and Poisson-corrected evolutionary distances (Nei and Kumar, 2000). Branches were then tested for statistical significance by bootstrapping with 1000 replicates. COX sequences from *Ciona*, *Fugu* and *Danio*, were derived from genome databases.

Multiple tissue semi-quantitative PCR

To determine the distribution of COX2 among tissues, semi-quantitative RT–PCR was performed on total RNA from gill, opercular membrane, brain, heart, stomach, intestine and kidney tissue as described previously (Choe et al., 2004b). Briefly, cDNA was produced from the tissues of a seawater killifish as described above, but random primers were used so that ribosomal and messenger RNA would be reverse transcribed. Non-degenerate primer pairs (Table 2) were designed to amplify a product with high efficiency (e.g. high melting temperature). To minimize the chance of amplifying contaminating genomic DNA, the primer pair was designed to include at least one intron–exon boundary that is conserved between vertebrate COX homologues (Hla and Neilson, 1992; Kosaka et al., 1994; Scott et al., 2002). A QuantumRNA™ 18S internal standard primer kit (Ambion, Woodward Austin, TX, USA) was used to control for variability in RNA quality and quantity between the different tissues tested. Multiplex PCR with primers for 18S and COX2 were then optimized to ensure that the reactions were terminated during the exponential phase and that the kinetics of 18S amplification approximated those of COX2. Lastly, the products were visualized by ethidium bromide staining in 1.5% agarose gels and photographed with Polaroid 667 film.

Table 1. *Primers used for cloning*

Name	Orientation	Nucleotide sequence (5′ to 3′)
L8 F1*	Sense	GGA TAC ATC AAG GGA ATC GTG AAR GAY ATH AT
L8 R1*	Antisense	CCG AAA GGG TGC TCC CAN GGR TTC AT
F4 COX 912*	Sense	ATG TAT GCT ACC ATT TGG CTC CGT GAG CAC AAC CGT GTC TGT G
R4 COX 1571*	Antisense	CCT TTG AGA GAG TAG GGA GCT CCC ATT TCC ACC ATA GTC TCC
3′ F1495	Sense	TCC TCC ATG AAG CCC TAC ACC TC
3′ F1631	Sense	ACG CCA TCT TTG GGG AGA CTA TG
5′ R1199	Antisense	AGC GAT GCG GTT TTG GTA CTG GA
5′ R1090	Antisense	ATC TTG ATA GTC TCG CCA ATA AGG AT

*Degenerate primer.

Table 2. *Primers used for real-time PCR and distribution analysis*

Name	Orientation	Nucleotide sequence (5′ to 3′)
L8 F2	Sense	CGT TTC AAG AAA AGG ACG GAG C
L8 R2	Antisense	GGA GAT GAC GGT GGC GTA G
COX2 F2	Sense	ATC CTT ATT GGC GAG ACT ATC A
COX2 R2	Antisense	ATT GAG GGA TTG GTA ACG CAT TT
nNOS F3	Sense	ACC GTG TTT TGT CCG AAG TG
nNOS R3	Antisense	AAG GTG CTT GGT TGT CTT TG
NKA F2	Sense	AGC CCT GGT GGT ACG TGA TG
NKA R2	Antisense	GGA AGC TAA GGT GGC AAT ACG AC
NKCC F1	Sense	CCC GCA GCC ACT GGT ATT
NKCC R1	Antisense	GCC ATC TGT GGG TCA GCA A
CFTR F2	Sense	GGC GTC ATA CTG CGT AGT GTT AC
CFTR R2	Antisense	TCA TGC CTT CCT CTA AGC GTA G
COX2 DF1	Sense	TCA CTG AAG CCT ACG CCT AAC AC
COX2 DR1	Antisense	CTC CAG GTT CTC GCC ATA AA

Immunohistochemistry

Immunohistochemistry was completed on paraffin-wax-embedded sections as described previously (Choe et al., 2004a; Choe et al., 2004b), with minor modifications. Seawater killifish were pithed and gill arches were removed and immersion fixed (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. Fixed tissues were dehydrated in an ethanol series and embedded in paraffin wax. Sections were cut at 6 μm and dried onto poly-L-lysine-coated slides. Tissue sections were deparaffinized in Citrisolv (Fisher Scientific, Pittsburgh, PA, USA), and rehydrated in an ethanol series followed by phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited by incubating with 3% H_2O_2 for 25 min at 24°C. Non-specific binding sites on the tissues were blocked by incubating with a protein blocker (Biogenex, San Ramon, CA, USA) (BPB: normal goat serum with 1% bovine serum albumin, 0.09% NaN_3 and 0.1% Tween 20) for 20 min.

Sections were incubated with a commercial affinity purified antibody (#160126, Cayman Chemical, Ann Arbor, MI, USA) generated against mouse COX2 amino acids 584–598 (diluted 1:1000 to 1:2000 in BPB) overnight at 4°C, in a humidified chamber. Negative control sections were incubated with BPB lacking antibodies or antibody 160126 that was pre-absorbed with 5 mg ml^{-1} antigen (Caymen Chemical #360106). Unbound primary antibodies were removed with a 5 min rinse in PBS. Sections were then incubated with BPB multilink solution (biotinylated goat anti-mouse, rabbit, guinea pig and rat antibodies diluted in BPB), followed with horseradish-peroxidase–streptavidin solution (Biogenex) for 20 min at 24°C each. After another wash in PBS for 5 min, antibody binding was visualized by incubating with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 5 min at 24°C. Sections were then rinsed with running tapwater for 5 min, dehydrated in an ethanol-Citrosolv series, and mounted with a coverslip using Permount (Fisher Scientific).

To determine if COX2 protein was expressed in chloride cells, some sections were stained with an antibody for NKA (α5) diluted 1:500. Bound antibody was detected as described above, except Vector SG, which produced a blue reaction product, was used for NKA. Antibody α5 was developed by Douglas Fambrough and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. It was made against the avian Na^+/K^+-ATPase α subunit and binds to all isoforms. This antibody recognizes fish NKA, and is now used commonly for studies on fish branchial cells (e.g. Piermarini and Evans, 2000; Wilson et al., 2000; Wilson et al., 2002; Choe et al., 2004a; Choe et al., 2004b).

Salinity transfers and quantitative real-time PCR (qRT–PCR)

A total of 56 killifish were captured from creeks near Salisbury Cove, ME, USA, transported to the University of Florida, and held in 100% seawater as above. After at least 1 month of acclimation to captive conditions, the killifish were separated into two groups; one group remained in 100% seawater [approximate concentrations (Choe and Evans, 2003) in mmol l^{-1}: Na^+ 517, Ca^{2+} 9, K^+ 12, Cl^- 486] and the other was transferred to a separate 380 l tank that contained freshwater [buffered Gainesville tapwater, approximate concentrations, taken from Choe and Evans (Choe and Evans, 2003) in mmol l^{-1}: Na^+ 4, Ca^{2+} 1, K^+ 0.03, Cl^- 0.40]. Killifish then remained in either seawater or freshwater for at least 37 days before initiating the salinity transfer series. For all experimental series, total RNA was prepared from killifish gills as described above. The first series included six killifish from freshwater and six from seawater, without any further salinity transfers. For the second series, 15 killifish were transferred directly from freshwater to seawater and were sampled (five per time point) after 3, 8 and 24 h; five killifish that remained in freshwater were sampled as pre-treatment controls

(transferred from freshwater to freshwater as a sham control). For the third series, 18 killifish were transferred directly from seawater to freshwater and were sampled (six per time point) after 3, 8, and 24 h; six killifish that remained in seawater (transferred from seawater to seawater as a sham control) were sampled as pre-treatment controls.

To measure relative expression levels, poly(A)$^+$ RNA was reverse transcribed as described above and the resulting cDNA was subjected to PCR in the presence of SYBR® Green (Molecular Probes, Inc., Eugene OR, USA) binding dye in a real-time thermal cycler (Bustin, 2002). Primers (Table 2) were designed from killifish-specific cDNA sequences that we either cloned using degenerate primers (COX2:AY532639) or derived from GenBank (L8:DQ066926, CFTR:AF000271, NKCC:AY533706, NKA1:AY057072). All qRT–PCR reactions were run in triplicate and included 0.2 μl of cDNA (2.0 μl of a 1/10 dilution of original cDNA), 7.4 pmoles of each primer and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μl. All qRT–PCR reactions were run in an ABI 5700 sequence detection thermal cycler (Applied Biosystems) or a Stratagene MX4000 qRT–PCR system (Stratagene, La Jolla, CA, USA) with the following cycling parameters: initial denaturing for 10 min at 95°C, followed by 40 cycles of 35 s at 95°C, 30 s at 58 or 60°C, and 30 s at 72°C. The final cycle was followed by a melting curve analysis to verify the amplification of a single product in each well. Quantitative RT–PCR reactions with gill RNA samples that were not reverse transcribed verified that either no products were amplified from contaminating genomic DNA, or that the genomic contamination was inconsequential background compared to cDNA amplification (genomic template was less than 1:1000 cDNA template for all primers).

Relative gene expression was calculated from relative standard curves that used a pooled gill cDNA sample as the template, and all results were normalized to ribosomal protein L8 gene expression, a highly conserved gene for which expression in gills remains constant during salinity and acid-base changes (Choe et al., 2004b; Choe et al., 2005). Unpaired Student's *t*-tests (two-tailed) were used to compare relative expression levels between seawater and freshwater killifish in the first series. ANOVAs with Dunnett's *post-hoc* tests were used to compare the relative expression levels of acutely transferred killifish to pre-treatment control killifish in the second and third series. Significance P=0.05 for all statistical tests.

Results

Molecular identification of COX2

In initial PCR reactions, oligonucleotide primers F4 COX 912^d and R4 COX 1571^d supported the amplification of a 700 bp product from killifish gill cDNA that matched the size expected from vertebrate COX nucleotide alignments. After cloning and sequencing, the killifish nucleotide sequence fragment was found to be 73.3% identical to mouse COX2 and 68.6% identical to mouse COX1. Blast e-values for the putative killifish COX2 sequence fragment were less than 10^{-25} for mammalian COX2s.

The complete putative killifish COX2 cDNA (accession number AY532639) contains 2738 nucleotides with a 1830 nucleotide open reading frame that codes for a 610 amino acid protein (Fig. 1). The probable start codon (ATG) is 102 nucleotides downstream from the 5′ end of the cDNA obtained by RACE, and a probable in-frame stop codon (TAA) is 807 nucleotides upstream from the 3′ end of the cDNA. A transcript cleavage sequence (AATAAA) is 22 nucleotides upstream from a poly(A)$^+$ tail. Killifish COX2 is well conserved with other vertebrate COX sequences, including the dimerization domains, the membrane-binding domain and cyclooxygenase and peroxidase active sites (Fig. 1). Importantly, the region of mouse COX2 that was used as an antigen to generate antibody 160126, is well conserved with killifish COX2 (13/15 similar amino acids). The complete killifish protein grouped with other vertebrate COX2 homologues in phylogenetic analysis (Fig. 2).

Distribution of putative transporters

Multiplex RT–PCR with cDNA from gill, opercular membrane, brain, heart, stomach, intestine and kidney was conducted to determine the distribution of COX2. After 30 cycles of PCR with killifish-specific COX2 primers (COX2 DF1 and COX2 DR1), the expected 479 bp product was most abundant in gill, followed by opercular membrane, kidney, heart, stomach, intestine, and brain (Fig. 3). The 315 bp product expected from the 18S internal control primers was observed for all tissues, at roughly equivalent levels (Fig. 3).

Fig. 1. Amino acid alignment of chordate cyclooxygenases (labeled by genus). Gaps (dashes) were introduced to maintain alignment. All amino acids that are identical or similar to those of the mouse COX2 are shaded (Blosum 62 scoring matrix with the following amino acid groups considered similar: DN, EQ, ST, KR, FYW and LIVM). The dimerization domains were well conserved in all the vertebrates, but not in the urochordate (*Ciona*). The membrane-binding domain was well conserved in all species. The catalytic domain begins just after the second dimerization domain and constitutes about 80% of the protein. When folded properly, the catalytic domain is further divided into cyclo-oxygenase and peroxidase active sites. Within the cyclo-oxygenase site, active site tyrosine and serine (ASA-acetylated) residues are conserved in all species (Simmons et al., 2004). Importantly, the substitution of an isoleucine for a valine within the cyclooxygenase active site of COX1 only occurs in mammalian COX1. This substitution allows some drugs to be COX2 specific (Gierse et al., 1996a), and therefore these drugs would not be expected to discriminate between COX1 and COX2 of non-mammalian vertebrates. Within the peroxidase active site, the proximal histidine that binds heme is conserved in all species. 13–15 amino acids of mouse COX2 that were used as an antigen to generate antibody 126 are conserved in killifish COX2. GenBank accession numbers are, from top to bottom: COX2 – *Mus* (Q05769), *Gallus* (P27607), *Fundulus* (AAS21313), and *Onchorynchus* (CAB46017); COX1 – *Mus* (NP_032995), *Gallus* (XP_425326), *Onchorynchus* (CAC10360) and *Squalus* (AAL37727); COXa – *Ciona* (scaffold 118); COXb – *Ciona* (scaffold 207).

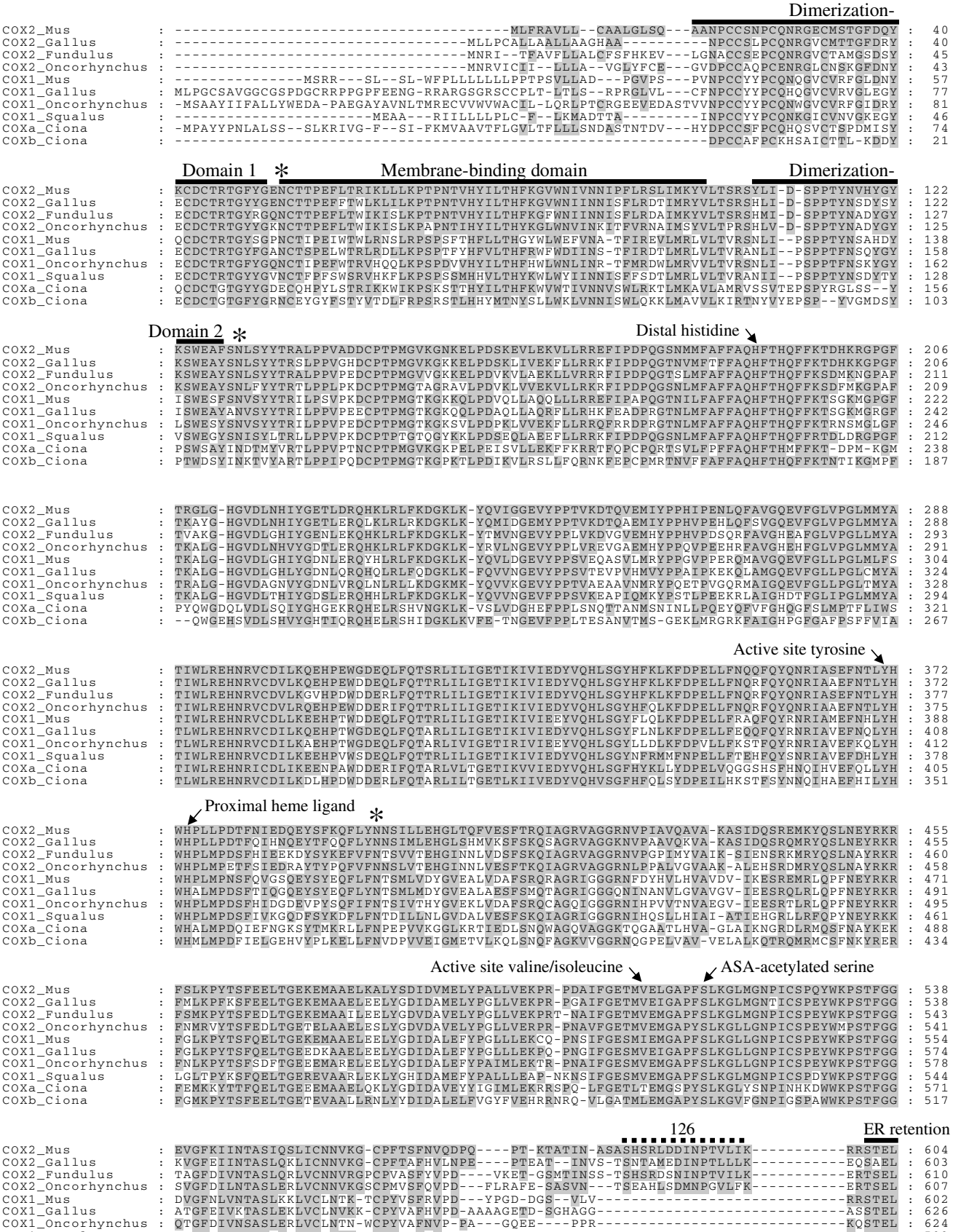
Dimerization-
Domain 1
Membrane-binding domain
Dimerization-
Domain 2
Distal histidine
Active site tyrosine
Proximal heme ligand
Active site valine/isoleucine
ASA-acetylated serine
126
ER retention

Fig. 1.

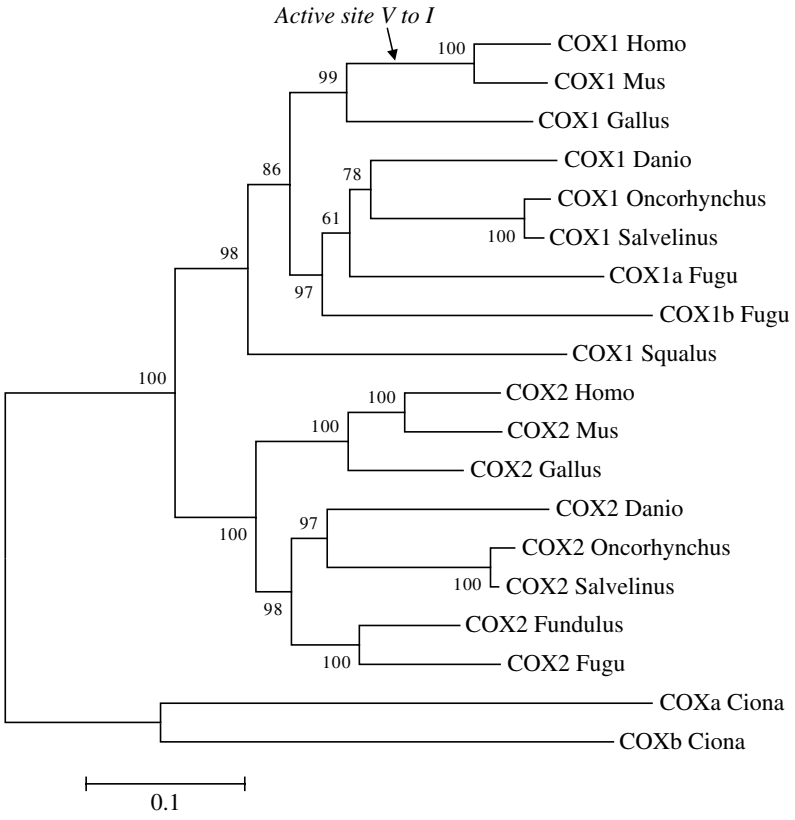

Fig. 2. Phylogenetic tree of chordate COX homologues. Database searches were used to find COX homologues in *Fugu* and *Ciona*. The tree was constructed using the neighbor-joining method with Poisson-correction, and numbers indicate bootstrap values for 1000 replicates. All homologues are labeled by genus. GenBank accession numbers are, from top to bottom: COX1 – *Homo* (AAL33601), *Mus* (NP_032995), *Gallus* (XP_425326), *Danio* (NP_705942), *Onchorhynchus* (CAC10360), *Salvelinus* (AAF14529), *Fugu* 1a (FuguGenscan_21183), *Fugu* 1b (FuguGenscan_4741); COX2 – *Homo* (BAA05698), *Mus* (Q05769), *Gallus* (P27607), *Danio* (NP_705943), *Onchorhynchus* (CAB46017), *Salvelinus* (AAD45896), *Fundulus* (AAS21313), *Fugu* (FuguGenscan_3088); COXa – *Ciona* (scaffold 118), COXb – *Ciona* (scaffold 207). Scale bar represents 20% amino acid replacement per site.

Immunohistochemistry

The anti-mouse COX2 antibody, 160126, reacted strongly with a subpopulation of epithelial cells in the filamental epithelium of seawater killifish (Fig. 4A). No staining was observed when sections were incubated with bpB, pre-immune serum, or when antibody 160126 was pre-incubated with antigen (Fig. 4B). Staining of serial sections demonstrated that antibody 160126 labeled cells that were immunoreactive for NKA (Fig. 4C,D). We also performed immunohistochemistry on gills from freshwater killifish and killifish following salinity transfers (not shown). In all cases, the location of COX2 immunoreactivity was the same (i.e. in NKA-rich cells) and qualitative changes in protein labeling were not apparent.

Quantitative real-time PCR (qRT–PCR)

The mRNA levels of gill COX2, CFTR and NKCC1 were 3.1, 2.0 and 1.6-fold greater in chronic seawater killifish than in chronic freshwater killifish, respectively (Fig 5A–C). By contrast, the expression of gill NKA1 mRNA was not different between the fish from the two salinities (Fig. 5D).

Transcript levels were also measured during the first 24 h following acute transfers between the two salinities to evaluate the kinetics of mRNA expression. Acute transfer from freshwater to seawater resulted in a rapid, large (3.4-fold), but transient, increase of COX2 mRNA levels (Fig. 6A). By 8 h following transfer to seawater, COX2 mRNA levels decreased back to near pre-transfer levels and remained there for up to 24 h (Fig. 6A). Acute transfer from seawater to freshwater resulted in a similar rapid increase (2.6-fold) in COX2 mRNA levels that decreased back to near pre-transfer levels by 8 h. Unlike in seawater, COX2 mRNA levels continued to decrease to 25% of pre-transfer levels by 24 h in freshwater. Sham-treatment control series were also performed for COX2 in freshwater and seawater killifish to determine if handling stress contributed to the acute increase in COX2 mRNA levels. There was no effect of handling on acute COX2 mRNA levels in freshwater (1.00±0.04, 0.80±0.21, 1.01±0.53 and 0.56±0.17; mean ± s.e.m., N=3–5) or seawater (1.00±0.37, 0.95±0.15, 1.48±0.34 and 1.59±0.65; mean ± s.e.m., N=4–5) fish.

Acute transfer from freshwater to seawater resulted in a rapid, large (2.8-fold) and stable increase of CFTR mRNA levels that peaked at 8 h (Fig. 6B). Conversely, acute transfer

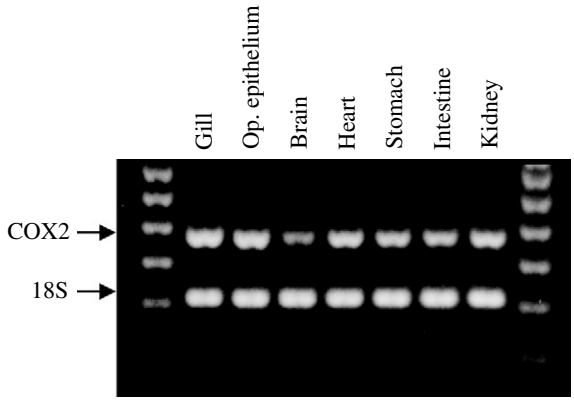

Fig. 3. Multiple tissue semi-quantitative PCR. Multiplex PCRs with primer pairs for COX2 and a 315 bp fragment of the 18S ribosome were conducted with randomly primed killifish cDNA as a template. The 479 base pair COX2 fragment was amplified, in decreasing order, from gill, opercular epithelium, kidney, heart, stomach, intestine and brain. 18S was amplified approximately equally from the cDNA of all tissues. 100 bp ladder molecular mass markers were run in the lanes to the left and right of samples.

from seawater to freshwater resulted in a rapid, large and stable decrease in CFTR mRNA levels that reached 14.2% of pre-transfer levels by 8 h (Fig. 6B). Acute transfer from freshwater to seawater resulted in a slow, moderate (1.9-fold) increase of NKCC1 mRNA levels (Fig. 6C). Conversely, acute transfer from seawater to freshwater resulted in a rapid, large decrease in NKCC1 mRNA levels that reached less than 33% of pre-transfer levels by 24 h (Fig. 6B). NKA1 mRNA levels increased only slightly (1.4-fold) 24 h after transfer from freshwater to seawater, and did not change following transfer from seawater to freshwater (Fig. 6D).

Discussion

Our study is the first to use molecular and immunological techniques to demonstrate that an orthologue of mammalian COX2 is present in branchial chloride cells of killifish where it may function in systemic and cellular osmoregulation. These conclusions are based on our molecular identification of a COX2 orthologue in killifish gills, localization of COX2 protein in NKA-rich cells, demonstration of over threefold greater expression of COX2 mRNA in seawater fish than freshwater fish, and demonstration of transient induction of COX2 expression during acute osmotic shock.

Molecular identification of killifish COX2

The phylogenetic analysis of our full-length cDNA sequence with available COX sequences, demonstrates that it is probably a teleost orthologue of mammalian COX2. Our killifish sequence groups well with other COX2 sequences of teleosts, and it contains an elongated carboxyl tail that distinguishes COX2 from COX1 (Fig. 1). The high homology of the killifish and mammalian COX2s (>70% identical amino acids) suggests that COX2 has changed very little in the teleost and tetrapod lineages (including mammals) since these two groups separated over 400 million years ago (Nelson, 1994). As demonstrated by a previous study (Jarving et al., 2004), our tree shows that teleosts contain orthologues of mammalian COX1 and COX2 that have no direct equivalents in urochordates (e.g. *Ciona*), suggesting that COX1 and COX2 originated following a gene duplication event during early vertebrate evolution (Fig. 2). Included in our tree is an elasmobranch COX from *Squalus acanthias* that appears to be an orthologue of COX1 (Yang et al., 2002). This would suggest that elasmobranchs, like teleosts, also contain an orthologue of COX2 and that the duplication occurred before cartilaginous fishes separated from the rest of the vertebrate lineage over 450 million years ago (Coates and Sequeira, 2001).

The alignment of COXs has important

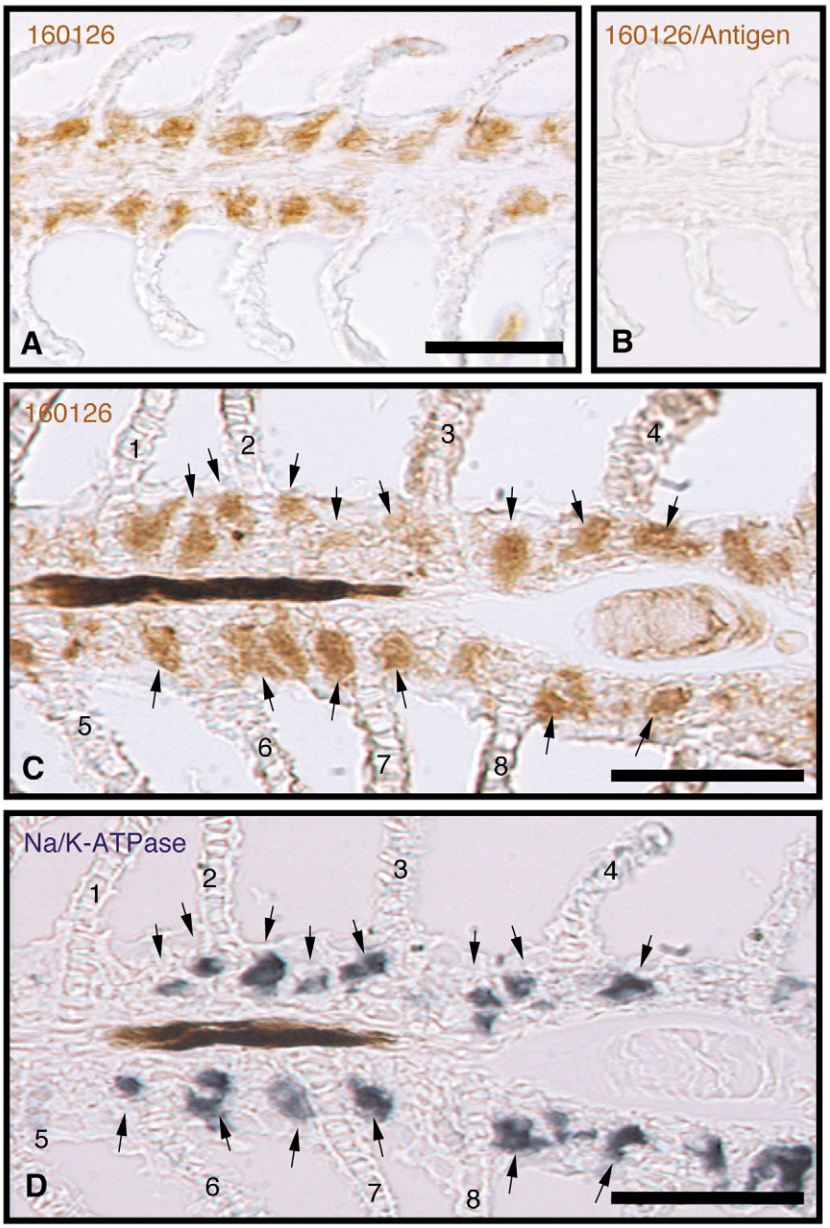

Fig. 4. Representative light micrographs of gill sections from killifish that demonstrate the localization of COX2 protein. Gill sections were incubated with antibody 160126 (A,C), antibody 160126 and antigen (B), or antibody α5 (D). Peroxidase substrates used to label antibodies were brown (160126=COX2) and blue (Na^+/K^+-ATPase). No immunolabeling was observed in negative control sections that were incubated with antibody 160126 and excess antigen followed by multilink (anti-mouse, rabbit and donkey) secondary antibodies (B); however, strong immunolabeling occurred in a population of epithelial cells with antibody 160126 (A). In serial sections, immunolabeling with antibody 160126 was always in cells with Na^+/K^+-ATPase (C,D); arrows mark cells that stained with both antibodies. Lamellae are numbered for clarity. Scale bars, 50 μm.

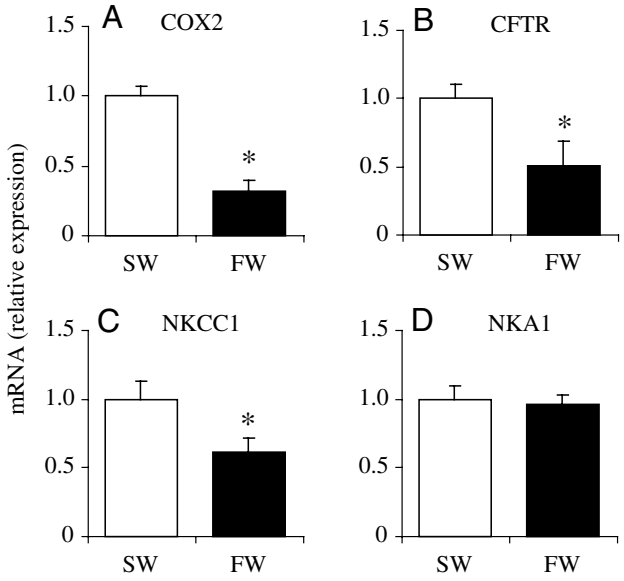

Fig. 5. Relative expression of (A) COX2, (B) CFTR, (C) NKCC1 and (D) NKA1 mRNA was measured by quantitative real-time PCR in gills following chronic acclimation to seawater (SW; white bars) or freshwater (FW; black bars). Values are means ± s.e.m., N=6, *P<0.05.

implications on the specificity of isoform-specific pharmacological inhibitors in non-mammalian vertebrates, including fishes. A single amino acid substitution of isoleucine to valine at position 509 in mammalian COX1 was shown to confer sensitivity to COX2-specific inhibitors such as NS-398 (Gierse et al., 1996b). In mammals, COX1 at this position contains an isoleucine and COX2 contains a valine (Fig. 1). Conversely, all other vertebrate COX proteins contain a valine at this position (including killifish COX1; J. Havird, K. P. Choe and D. H. Evans, unpublished results), regardless of whether they are COX1 or COX2, suggesting that the ancestral residue at this position is valine (Fig. 1). Therefore, NS-398 would be expected to inhibit both COX isoforms in non-mammalian vertebrates and should not be used to discriminate between COX1 and COX2 in any vertebrate other than a mammal.

In light of this finding, we must reconsider the relative roles of COX1 and COX2 in the endothelin signaling cascade that inhibits NaCl secretion (measured as Isc) in killifish opercular epithelia. We previously hypothesized that COX2 was more important than COX1 in regulating NaCl secretion, because NS-398 blocked the inhibitory effect of endothelin by nearly 90% in opercular epithelia, and SC560, a COX1 specific inhibitor (Smith et al., 1998), blocked the endothelin effect by only 47% (Evans et al., 2004). Our sequence analysis of vertebrate COX homologues suggests that NS-398 probably inhibited both isoforms, explaining why the effects of NS-398 and SC560 were not additive. The mechanism of discrimination for the COX1-specific inhibitor SC560 is not known, and therefore it is unclear if SC560 is COX1-specific in non-mammalian vertebrates. If SC560 is COX1-specific in killifish, then COX1 and COX2 may mediate the inhibitory effects of endothelin roughly equally in isolated opercular epithelia, because the effects of SC560 on I_{sc} in killifish opercular epithelia was about half that of the general COX inhibitor indomethacin (Evans et al., 2004). Presumably, COX2 was responsible for the other approximate half of the total COX activity.

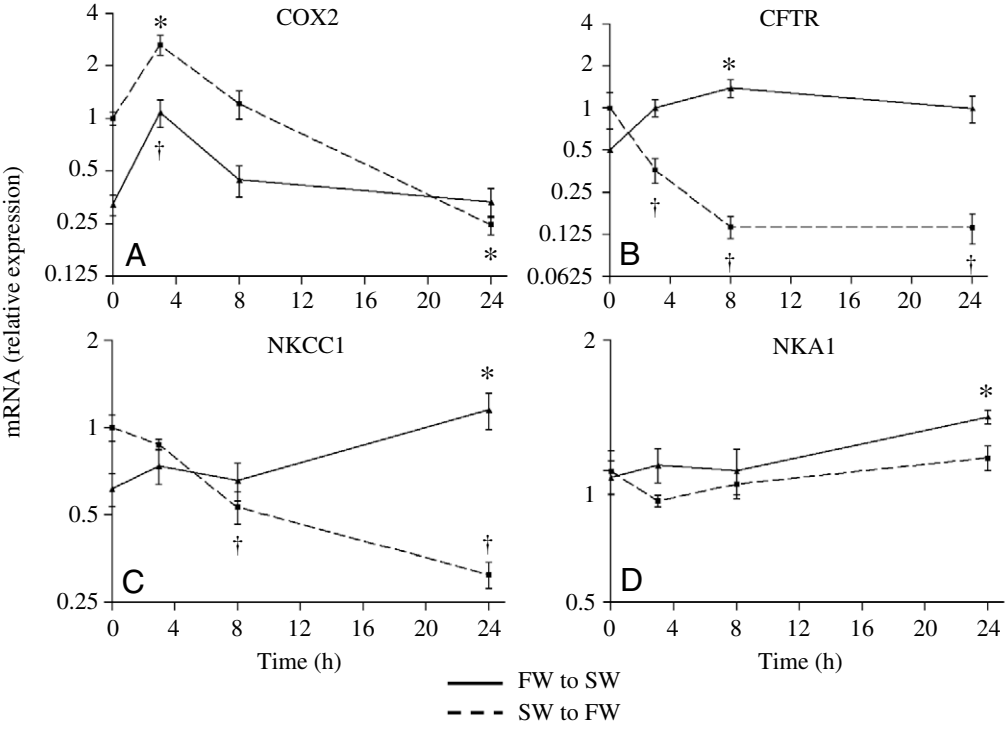

Fig. 6. Relative expression of (A) COX2, (B) CFTR, (C) NKCC1 and (D) NKA1 mRNA was measured by quantitative real-time PCR in gills following acute transfer from freshwater (FW) to seawater (SW; solid lines) or from seawater to freshwater (broken lines). To adjust for different starting expression levels between the two salinities, the relative expression values of the freshwater to seawater series was standardized to the relative chronic salinity means of Fig. 5. Values are means ± s.e.m., N=5 or 6, *P<0.05 for FW to SW, †P<0.05 for SW to FW.

COX2 distribution and localization in gills

The distribution of COX2 in killifish tissues is similar to the distribution of COX2 in zebrafish (Grosser et al., 2002). Specifically, the high constitutive expression of COX2 in killifish gills, opercular epithelia and kidney suggest a role in regulating ion transport as has been shown in mammalian kidneys (Harris and Breyer, 2001; López et al., 2003) and shark rectal glands (Yang et al., 2002). Expression of COX2 was also found to be highest in the gills of zebrafish (Grosser et al., 2002), suggesting that COX2 is constitutively expressed at high levels in the gills of all teleosts.

High expression of Na^+/K^+-ATPase is a well-established characteristic of teleost chloride cells (Marshall, 2002; Evans et al., 2005). Therefore, the clear colocalization of COX2 and Na^+/K^+-ATPase immunoreactivity in killifish demonstrates that, in the gills, COX2 is predominantly expressed in chloride cells where prostaglandins can regulate ion transport rates as autocrines (Fig. 4). Similarly, COX2 is constitutively expressed in epithelial cells of the mammalian renal cortical thick ascending limb (Harris et al., 1994; Guan et al., 1997), and prostaglandins were shown to inhibit NaCl transport by this segment (Stokes, 1979). Taken together, these results suggest that COX2 is expressed in diverse vertebrate epithelial tissues, where it can have direct effects on ion transport.

Chronic salinity acclimation

The greater expression of NKCC1 and CFTR mRNA in gills that we observed for in chronic seawater killifish *versus* chronic freshwater killifish are generally consistent with previous studies and with the well-established roles of these transporters in basolateral (NKCC1) and apical (CFTR) Cl^- transport (Singer et al., 1998; Pelis et al., 2001; Singer et al., 2002; Tipsmark et al., 2002; Scott et al., 2004; Tipsmark et al., 2004; Evans et al., 2005).

We previously demonstrated that COX2 regulates NaCl secretion in isolated opercular epithelia (Evans et al., 2004). The immunolocalization and real-time PCR results of the current study demonstrate that COX2 is expressed in seawater-type chloride cells and that COX2 expression levels are regulated by environmental salinity. Chronic acclimation to seawater results in a suite of morphological and biochemical alterations (including increased NKCC1 and CFTR expression) that assemble the NaCl secretory mechanism (Perry, 1997; Marshall et al., 1999; Katoh and Kaneko, 2003), and we hypothesize that COX2 expression also increases as a fine-tuning negative regulatory mechanism. COX2 expression is also dramatically increased in the mammalian renal medulla by chronic dehydration and salt loading (Yang, 2003). The resulting increased prostaglandin synthesis then inhibits transport in surrounding collecting ducts and promotes salt excretion in urine (Brater, 1999). Interestingly, although chronic COX2 expression appears to be stimulated by similar osmotic and ion conditions in killifish gills and mammalian renal medulla, and prostaglandins inhibit ion transport in both tissues, the resulting systemic effects are opposite (inhibition of salt excretion by killifish gills and promotion of salt excretion by mammalian kidneys).

Acute transfer from freshwater to seawater

The real-time PCR results following acute transfers between salinities reveal some novel kinetics of NKCC1 and CFTR mRNA expression that were not revealed by previous studies that either focused on longer time intervals (Scott et al., 2004; Tipsmark et al., 2004) or that did not consider transfer from seawater to freshwater (Singer et al., 1998; Singer et al., 2002; Scott et al., 2004). Transfer of killifish from freshwater to seawater causes an increase in plasma osmolarity and $[Na^+]$ in the first 24 h. Between 24 and 48 h following transfer, plasma osmolarity and $[Na^+]$ begin to decrease in concert with an increase in I_{sc} measured from isolated opercular epithelia (Marshall et al., 1999; Hoffmann et al., 2002). Our results demonstrate that gill NKCC1 and CFTR mRNA levels are elevated in the first 24 h following transfer to seawater, presumably preceding an increase in transporter protein and activity levels that facilitate NaCl secretion and osmoregulatory compensation between 24 and 48 h.

Interestingly, the kinetics of COX2 mRNA levels following acute transfer from freshwater to seawater were markedly different than the kinetics of NKCC1 and CFTR mRNA (Fig. 6), and suggest a role for branchial COX2 in addition to regulation of ion transport. The rapid, large and transient induction of COX2 may be an important mechanism of cell survival during acute osmotic stress. Several mammalian cell types have been shown to increase COX2 expression in response to hyperosmotic shock with kinetics strikingly similar to those that we observed for killifish gills (Hao et al., 1999; Arbabi et al., 2000; Hao et al., 2000; Arbabi et al., 2001; Rao et al., 2004). Most relevant are studies on medullary interstitial cells, which experience large changes in extracellular osmolarity similar to teleost gills (Hao et al., 1999; Hao et al., 2000). Antisense and pharmacological COX2 inhibition abolishes the ability of medullary interstitial cells to survive hyperosmotic stress (Hao et al., 1999; Hao et al., 2000). The exact mechanism of COX2-mediated cell survival is unclear, but is believed to depend on prostaglandin control of blood flow and ion transport (Hao et al., 2000). We hypothesize that the rapid induction of COX2 that we observed in killifish gills promotes chloride cell survival, as it does in mammalian cells.

It was recently demonstrated (Fiol and Kultz, 2005) that acute transfer from freshwater to seawater stimulated rapid, transient expression (increased protein expression within 2 h) of a candidate immediate early transcription factor (osmotic stress transcription factor 1) in the gills of tilapia (*Oreochromis mossambicus*). Interestingly, the tilapia osmotic stress transcription factor 1 contains a putative phosphorylation site for MAP kinases (p38 and extracellular signal-regulated kinase); and p38 MAP kinase is known to stimulate COX2 expression in cultured cTALH cells of mammals (Arbabi et al., 2000; Cheng et al., 2000). Therefore, it is tempting to speculate that the rapid and transient increase in COX2 expression in killifish gills may be mediated by osmotic stress transcription factor 1, which may be enhanced by MAP kinases.

The reason that COX2 mRNA levels fell back to near pre-transfer levels and did not increase toward chronic seawater

levels (Fig. 6A) 8 h after transfer to seawater may be related to the role of the enzyme in regulating NaCl secretion (Evans et al., 2005). During the first 48 h after transfer to seawater when plasma osmolarity and NaCl secretion are still being corrected (Marshall et al., 1999; Hoffmann et al., 2002), increased COX2 expression, prostaglandin synthesis and inhibition of NaCl secretion would be counterproductive to acclimation. Presumably, in chronic seawater conditions levels of COX2 expression (Fig. 5A) and fine-tune control of NaCl secretion is achieved as a new osmotic and transport steady state is reached.

Acute transfer from seawater to freshwater

Importantly, our results are the first to demonstrate that decreases in NKCC1 and CFTR mRNA levels following transfer to freshwater occur more rapidly, and more extensively, than increases in NKCC1 and CFTR mRNA levels following transfer to seawater. Transfer of killifish from seawater to freshwater causes a transient decrease in plasma [Na^+] that is completely removed by 12 h (Marshall et al., 2000). This decrease in [Na^+] presumably reflects a decrease in osmolarity and hypotonic shock of NaCl secreting MRCs in the gills *via* the basolateral membrane. Hypotonic shock *via* the basolateral membrane is an acute inhibitor of I_{sc} in isolated opercular epithelia (Marshall et al., 2000; Marshall et al., 2005) and therefore NaCl secretion by the gills is probably inhibited directly by lowered plasma osmolarity immediately following transfer to freshwater. In our study, NKCC1 and CFTR mRNA levels had decreased 47 and 86%, respectively by 8 h following transfer to freshwater, coinciding with restoration of plasma osmolarity and removal of hypotonic inhibition of NaCl secretion (Marshall et al., 2000; Marshall et al., 2005). Therefore, our results suggest that rapid decreases in NKCC1 and CFTR mRNA levels, which presumably precede decreased protein and activity levels, may be an important mechanism of decreasing NaCl secretion in killifish gills that immediately follows inhibition of NaCl secretion by hypotonic shock.

The kinetics of COX2 mRNA during the first 8 h after transfer to freshwater were virtually identical to those after transfer to seawater, again suggesting a cell survival role during an acute osmotic shock. Similar results were observed for human amniotic cells exposed to hypotonic shock (Lundgren et al., 1997). In that study, volume expansion was shown to induce a rapid and transient increase in COX2 mRNA (Lundgren et al., 1997). In our study, the apical side of killifish gill cells were transferred from a solution of about 1000 mOsm (seawater) to a solution of less than 50 mOsm (freshwater). This would be expected to cause cell volume expansion and activation of hypotonic stress signal transduction pathways that lead to regulatory volume decrease (RVD). The potential mechanism of increased COX2 expression is unclear, because the signal transduction pathways that are initially activated by cell volume expansion and lead to gene transcription have not been identified for vertebrate cells (Jakab et al., 2002). In yeast, hypotonic stress activates a protein kinase C pathway that is required for survival in hypotonic media (Davenport et al., 1995). Further work will be required to determine if a homologous pathway regulates COX2 expression in killifish gills. The function of the transient induction of COX2 expression following hypotonic shock is unknown, but may be related to regulation of effector ion channels that contribute to RVD. Prostaglandins have been shown to activate K^+ channels that promote RVD in mammalian epithelial cells (Civan et al., 1994). Collectively, the results of our acute salinity transfer on fish gills together with previous results on mammalian cells suggest that, among many other functions, COX2 is an osmotic response gene that may promote cell survival. This work also establishes killifish gills as an in vivo model in which to study volume-sensitive signal transduction pathways that promote COX2 mRNA expression.

Conclusions

In summary, we have cloned and characterized COX2 from the gills of the euryhaline killifish. The killifish COX2 shares a high degree of homology with other vertebrate COX2s and is constitutively expressed at high levels in the gills. Immunohistochemistry demonstrated that COX2 is expressed in chloride cells in the gills. We also measured expression levels of COX2 mRNA and ion transporters that mediate NaCl secretion following chronic and abrupt salinity transfers, to gain insights into the function of COX2 in the gills. Long-term acclimation of fish led to a higher expression of COX2 mRNA in seawater than in freshwater that was consistent with the relative expression of NKCC1 and CFTR mRNA. Collectively, these data suggest that constitutive COX2 expression in killifish gills is regulated by external salinity and that one role of the enzyme may be to regulate NaCl secretion as an autocrine signaling enzyme. Acute transfer from seawater to freshwater and from freshwater to seawater led to rapid, transient inductions of COX2 expression that suggest an addition role in cell survival during osmotic shock.

We thank J. B. Claiborne at Georgia Southern University for assistance with cloning of the initial COX2 sequence fragment. This work was supported by National Science Foundation grants IBN-0089943 and IOB-0519579 to D.H.E.

References

Arbabi, S., Rosengart, M. R., Garcia, I. and Maier, R. V. (2000). Hypertonic saline solution induces prostacyclin production by increasing cyclooxygenase-2 expression*1. *Surgery* **128**, 198-205.

Arbabi, S., Rosengart, M. R., Garcia, I., Jelacic, S. and Maier, R. V. (2001). Epithelial cyclooxygenase-2 expression: a model for pathogenesis of colon cancer. *J. Surg. Res.* **97**, 60-64.

Brater, D. C. (1999). Effects of nonsteroidal anti-inflammatory drugs on renal function: focus on cyclooxygenase-2-selective inhibition. *Am. J. Med.* **107**, 65-70.

Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29**, 23-39.

Cheng, H.-F., Wang, J.-L., Zhang, M.-Z., McKanna, J. A. and Harris, R. C. (2000). Role of p38 in the regulation of renal cortical cyclooxygenase-2 expression by extracellular chloride. *J. Clin. Invest.* **106**, 681-688.

Choe, K. and Evans, D. (2003). Compensation for hypercapnia by a euryhaline elasmobranch: effect of salinity and roles of gills and kidneys in fresh water. *J. Exp. Zool.* **297A**, 52-63.

Choe, K. P., O'Brien, S., Evans, D. H., Toop, T. and Edwards, S. L. (2004a). Immunolocalization of Na^+/K^+-ATPase, carbonic anhydrase II, and vacuolar H^+-ATPase in the gills of freshwater adult lampreys, *Geotria australis*. *J. Exp. Zool.* **301A**, 654-665.

Choe, K. P., Verlander, J. W., Wingo, C. S. and Evans, D. H. (2004b). A putative H^+/K^+-ATPase in the Atlantic stingray, *Dasyatis sabina*: primary sequence and expression in gills. *Am. J. Physiol.* **287**, R981-R991.

Choe, K. P., Kato, A., Hirose, S., Plata, C., Sindic, A., Romero, M. F., Claiborne, J. B. and Evans, D. H. (2005). NHE3 in an ancestral vertebrate: primary sequence, distribution, localization, and function in gills. *Am. J. Physiol.* **289**, R1520-R1534.

Civan, M. M., Coca-Prados, M. and Peterson-Yantorno, K. (1994). Pathways signaling the regulatory volume decrease of cultured nonpigmented ciliary epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **35**, 2876-2886.

Coates, M. I. and Sequeira, S. E. K. (2001). Early sharks and primitive gnathostome interrelationships. In *Major Events in Early Vertebrate Evolution* (ed. P. E. Ahlberg), pp. 241-262. London: Taylor and Francis.

Davenport, K. R., Sohaskey, M., Kamada, Y., Levin, D. E. and Gustin, M. C. (1995). A second osmosensing signal transduction pathway in yeast. *J. Biol. Chem.* **270**, 30157-30161.

DeWitt, D. L. and Smith, W. L. (1988). Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. USA* **85**, 1412-1416.

Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S. A., Van De Putte, L. B. and Lipsky, P. E. (1998). Cyclooxygenase in biology and disease. *FASEB J.* **12**, 1063-1073.

Eriksson, O., Mayer-Gostan, N. and Wistrand, P. J. (1985). The use of isolated fish opercular epithelium as a model tissue for studying intrinsic activities of loop diuretics. *Acta Physiol. Scand.* **125**, 55-66.

Evans, D. H., Rose, R. E., Roeser, J. M. and Stidham, J. D. (2004). NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. *Am. J. Physiol.* **286**, R560-R568.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Fiol, D. F. and Kultz, D. (2005). Rapid hyperosmotic coinduction of two tilapia (*Oreochromis mossambicus*) transcription factors in gill cells. *Proc. Natl. Acad. Sci. USA* **102**, 927-932.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871-1875.

Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M. and Seibert, K. (1996). A single amino acid difference between cyclooxygenase-1 (COX-1) and -2 (COX-2) reverses the selectivity of COX-2 specific inhibitors. *J. Biol. Chem.* **271**, 15810-15814.

Griffith, R. W. (1974). Environment and salinity tolerance in the genus *Fundulus*. *Copeia* **2**, 319-331.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M. A. and FitzGerald, G. A. (2002). Developmental expression of functional cyclooxygenases in zebrafish. *Proc. Natl. Acad. Sci. USA* **99**, 8418-8423.

Guan, Y., Chang, M., Cho, W., Zhang, Y., Redha, R., Davis, L., Chang, S., DuBois, R. N., Hao, C. M. and Breyer, M. (1997). Cloning, expression, and regulation of rabbit cyclooxygenase-2 in renal medullary interstitial cells. *Am. J. Physiol.* **273**, F18-F26.

Guan, Y., Zhang, Y., Breyer, R. M., Fowler, B., Davis, L., Hebert, R. L. and Breyer, M. D. (1998). Prostaglandin E2 inhibits renal collecting duct Na^+ absorption by activating the EP1 receptor. *J. Clin. Invest.* **102**, 194-201.

Hao, C.-M., Komhoff, M., Guan, Y., Redha, R. and Breyer, M. D. (1999). Selective targeting of cyclooxygenase-2 reveals its role in renal medullary interstitial cell survival. *Am. J. Physiol.* **277**, F352-F359.

Hao, C.-M., Yull, F., Blackwell, T., Komhoff, M., Davis, L. S. and Breyer, M. D. (2000). Dehydration activates an NF-κB-driven, COX2-dependent survival mechanism in renal medullary interstitial cells. *J. Clin. Invest.* **106**, 973-982.

Harris, R. C. and Breyer, M. D. (2001). Physiological regulation of cyclooxygenase-2 in the kidney. *Am. J. Physiol.* **281**, F1-F11.

Harris, R. C., McKanna, J. A., Akai, Y., Jacobson, H. R., Dubois, R. N. and Breyer, M. D. (1994). Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *J. Clin. Invest.* **94**, 2504-2510.

Hirose, S., Kaneko, T., Naito, N. and Takei, Y. (2003). Molecular biology of major components of chloride cells. *Comp. Biochem. Physiol.* **136B**, 593-620.

Hla, T. and Neilson, K. (1992). Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA* **89**, 7384-7388.

Hoffmann, E. K., Hoffmann, E., Lang, F. and Zadunaisky, J. A. (2002). Control of Cl^- transport in the operculum epithelium of *Fundulus heteroclitus*: long- and short-term salinity adaptation. *Biochim. Biophys. Acta* **1566**, 129-139.

Jacob, W. F. and Taylor, M. H. (1983). The time course of seawater acclimation in *Fundulus heteroclitus* L. *J. Exp. Zool.* **228**, 33-39.

Jakab, M., Fuerst, J., Gschwentner, M., Bottà, G., Garavaglia, M. L., Bazzini, C., Rodighiero, S., Meyer, G., Eichmueller, S., Woell, E. et al. (2002). Mechanisms sensing and modulating signals arising from cell swelling. *Cell. Physiol. Biochem.* **12**, 235-258.

Jarving, R., Jarving, I., Kurg, R., Brash, A. R. and Samel, N. (2004). On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages. *J. Biol. Chem.* **279**, 13624-13633.

Karnaky, K. J. (1998). Osmotic and ionic regulation. In *The Physiology of Fishes*, Vol. 2 (ed. D. H. Evans), pp. 159-178. Boca Raton, FL: CRC Press.

Karnaky, K. J., Jr, Degnan, K. J. and Zadunaisky, J. A. (1977). Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. *Science* **195**, 203-205.

Katoh, F. and Kaneko, T. (2003). Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique. *J. Exp. Biol.* **206**, 4113-4123.

Khan, K. N., Venturini, C. M., Bunch, R. T., Brassard, J. A., Koki, A. T., Morris, D. L., Trump, B. F., Maziasz, T. J. and Alden, C. L. (1998). Interspecies differences in renal localization of cyclooxygenase isoforms: implications in nonsteroidal antiinflammatory drug-related nephrotoxicity. *Toxicol. Pathol.* **26**, 612-620.

Kosaka, T., Miyata, A., Ihara, H., Hara, S., Sugimoto, T., Takeda, O., Takahashi, E. and Tanabe, T. (1994). Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2. *Eur. J. Biochem.* **221**, 889-897.

Kujubu, D., Fletcher, B., Varnum, B., Lim, R. and Herschman, H. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.* **266**, 12866-12872.

Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244-1245.

López, R., Roig, F., Llinás, M. T. and Salazar, F. J. (2003). Role of cyclooxygenase-2 in the control of renal haemodynamics and excretory function. *Acta Physiol. Scand.* **177**, 429-435.

Lundgren, D. W., Moore, R. M., Collins, P. L. and Moore, J. J. (1997). Hypotonic stress increases cyclooxygenase-2 expression and prostaglandin release from amnion-derived WISH cells. *J. Biol. Chem.* **272**, 20118-20124.

Marshall, W. S. (2002). Na^+, Cl^-, Ca^{2+}, Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *J. Exp. Zool.* **293**, 264-283.

Marshall, W. S., Emberley, T. R., Singer, T. D., Bryson, S. E. and McCormick, S. D. (1999). Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. *J. Exp. Biol.* **202**, 1535-1544.

Marshall, W., Bryson, S. and Luby, T. (2000). Control of epithelial Cl(–) secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. *J. Exp. Biol.* **203**, 1897-1905.

Marshall, W. S., Ossum, C. G. and Hoffmann, E. K. (2005). Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. *J. Exp. Biol.* **208**, 1063-1077.

Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Nelson, J. S. (1994). *Fishes of the World*. New York: John Wiley and Sons.

O'Banion, M., Winn, V. and Young, D. (1992). cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA* **89**, 4888-4892.

Pelis, R. M., Zydlewski, J. and McCormick, S. D. (2001). Gill Na^+-K^+-$2Cl^-$ cotransporter abundance and location in Atlantic salmon: effects of seawater and smolting. *Am. J. Physiol.* **280**, R1844-R1852.

Perry, S. F. (1997). The chloride cell: structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol.* **59**, 325-347.

Piermarini, P. M. and Evans, D. H. (2000). Effects of environmental salinity

on Na^+/K^+-ATPase in the gills and rectal gland of a euryhaline elasmobranch (*Dasyatis sabina*). *J. Exp. Biol.* **203**, 2957-2966.

Rao, R., Hao, C.-M. and Breyer, M. D. (2004). Hypertonic stress activates glycogen synthase kinase 3β-mediated apoptosis of renal medullary interstitial cells, suppressing an NFκB-driven cyclooxygenase-2-dependent survival pathway. *J. Biol. Chem.* **279**, 3949-3955.

Roberts, S. B., Langenau, D. M. and Goetz, F. W. (2000). Cloning and characterization of prostaglandin endoperoxide synthase-1 and -2 from the brook trout ovary. *Mol. Cell. Endocrinol.* **160**, 89-97.

Scott, B. T., Hasstedt, S. J., Bovill, E. G., Callas, P. W., Valliere, J. E., Wang, L., Wu, K. K. and Long, G. L. (2002). Characterization of the human prostaglandin H synthase 1 gene (PTGS1): exclusion by genetic linkage analysis as a second modifier gene in familial thrombosis. *Blood Coagul. Fibrinolysis* **13**, 519-531.

Scott, G. R., Richards, J. G., Forbush, B., Isenring, P. and Schulte, P. M. (2004). Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. *Am. J. Physiol.* **287**, C300-C309.

Semple, J. W., Green, H. J. and Schulte, P. M. (2002). Molecular cloning and characterization of two Na/K-ATPase isoforms in *Fundulus heteroclitus*. *Mar. Biotech.* **4**, 512-519.

Simmons, D. L., Botting, R. M. and Hla, T. (2004). Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol. Rev.* **56**, 387-437.

Singer, T. D., Stephen, J. T., Marshall, W. S. and Higgins, C. F. (1998). A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. *Am. J. Physiol.* **274**, C715-C723.

Singer, T. D., Clements, K. M., Semple, J. W., Schulte, P. M., Bystriansky, J. S., Finstad, B., Fleming, I. A. and McKinley, R. S. (2002). Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. *Can. J. Fish. Aquat. Sci.* **59**, 125-135.

Smith, C. J., Zhang, Y., Koboldt, C. M., Muhammad, J., Zweifel, B. S., Shaffer, A., Talley, J. J., Masferrer, J. L., Seibert, K. and Isakson, P. C. (1998). Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc. Natl. Acad. Sci. USA* **95**, 13313-13318.

Stokes, J. B. (1979). Effect of prostaglandin E2 on chloride transport across the rabbit thick ascending limb of Henle. Selective inhibitions of the medullary portion. *J. Clin. Invest.* **64**, 495-502.

Stokes, J. B. and Kokko, J. P. (1977). Inhibition of sodium transport by prostaglandin E2 across the isolated, perfused rabbit collecting tubule. *J. Clin. Invest.* **59**, 1099-1104.

Tipsmark, C. K., Madsen, S. S., Seidelin, M., Christensen, A. S., Cutler, C. P. and Cramb, G. (2002). Dynamics of Na^+,K^+,$2Cl^-$ cotransporter and Na^+,K^+-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and atlantic salmon (*Salmo salar*). *J. Exp. Zool.* **293**, 106-118.

Tipsmark, C. K., Madsen, S. S. and Borski, R. J. (2004). Effect of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). *J. Exp. Zool.* **301A**, 979-991.

Van Praag, D., Farber, S. J., Minkin, E. and Primor, N. (1987). Production of eicosanoids by the killifish gills and opercular epithelia and their effect on active transport of ions. *Gen. Comp. Endocrinol.* **67**, 50-57.

Wilson, J. M., Laurent, P., Tufts, B. L., Benos, D. J., Donowitz, M., Vogl, A. W. and Randall, D. J. (2000). NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J. Exp. Biol.* **203**, 2279-2296.

Wilson, J., Morgan, J., Vogl, W. and Randall, D. (2002). Branchial mitochondria-rich cells in the dogfish *Squalus acanthias*. *Comp. Biochem. Physiol.* **132A**, 365-374.

Wood, C. M. and Laurent, P. (2003). Na^+ versus Cl^- transport in the intact killifish after rapid salinity transfer. *Biochim. Biophys. Acta* **1618**, 106-119.

Wood, C. M. and Marshall, W. S. (1994). Ion balance, acid-base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus*-a euryhaline estuarine teleost. *Estuaries* **17**, 34-52.

Xie, W., Chipman, J., Robertson, D., Erikson, R. and Simmons, D. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. USA* **88**, 2692-2696.

Yang, T. (2003). Regulation of cyclooxygenase-2 in renal medulla. *Acta Physiol. Scand.* **177**, 417-421.

Yang, T., Forrest, S. J., Stine, N., Endo, Y., Pasumarthy, A., Castrop, H., Aller, S., Forrest, J. N., Jr, Schnermann, J. and Briggs, J. (2002). Cyclooxygenase cloning in dogfish shark, *Squalus acanthias*, and its role in rectal gland Cl secretion. *Am. J. Physiol.* **283**, R631-R637.

Zou, J., Neumann, N. F., Holland, J. W., Belosevic, M., Cunningham, C., Secombes, C. J. and Rowley, A. F. (1999). Fish macrophages express a cyclo-oxygenase-2 homologue after activation. *Biochem. J.* **340**, 153-159.