Cyclooxygenase cloning in dogfish shark, *Squalus acanthias*, and its role in rectal gland Cl secretion


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Yang, T., S. J. Forrest, N. Stine, Y. Endo, A. Pasumarty, H. Castrop, S. Aller, J. N. Forrest, Jr., J. Schnermann, and J. Briggs. Cyclooxygenase cloning in dogfish shark, *Squalus acanthias*, and its role in rectal gland Cl secretion. Am J Physiol Regul Integr Comp Physiol 283: R631–R637, 2002. First published May 23, 2002; 10.1152/ajpregu.00743.2001.—The present studies were carried out in nonmammalian vertebrates. Recently, a COX-2 homologue has been cloned from rainbow trout macrophages, and COX-1 and COX-2 have been cloned in a wide variety of mammalian species (4, 7, 10, 11, 13, 16, 18, 20, 24, 39) as well as in some avian vertebrates (38). Large numbers of studies carried out in mammals have extensively characterized the two COX isoforms. In general, COX-1 is constitutively expressed in a wide variety of tissues and thought to have “housekeeping” functions, whereas COX-2 is selectively expressed in certain cell types and is highly inducible by cytokines, growth factors, and osmotic stress (6).

In contrast to the large body of information on biosynthesis and function of PGs in mammals, few studies have been carried out in nonmammalian vertebrates. Recently, a COX-2 homologue has been cloned from rainbow trout macrophages, and COX-1 and COX-2 homologues from brook trout ovary, but little func-

A large body of evidence in mammals suggests that renal PGs contribute to the adaptation to high salt stress by facilitating the excretion of sodium chloride and water. For example, systemic infusion of PGE1 produces natriuresis and diuresis in humans (27), dogs (25), and rodents (2). Production of endogenous PGs in the kidney, especially in the renal medulla, is stimulated by chronic salt loading (22, 23, 37). Furthermore, pharmacological blockade of PG synthesis in humans reduces urinary salt and water excretion (5, 26). PGs regulate salt excretion in mammals through a complex mechanism. In vitro studies have demonstrated that PGs such as PGE2 can directly inhibit NaCl transport in isolated thick ascending limbs and collecting ducts (14, 33, 34). In addition, it has been shown that PGE2 augments renal medullary blood flow and attenuates the hydroosmotic effect of antidiuretic hormone (3), effects that alone or in combination promote urinary sodium and water excretion.

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tional evidence of the cloned COX isoforms was provided in these studies (30, 40). One may expect that elasmobranch species living in seawater may require a more activated PG system compared with freshwater teleosts. An early study more than 20 years ago isolated and identified PGE2 from shark gastrointestinal tracts (28). Recently, it has been shown that whole blood cells from the gray shark possess the highest capacity for PG synthesis among 11 fish species assayed (35). Furthermore, PGs, not nitric oxide, have been shown to mediate endothelium-dependent dilation in ventral aorta of sharks as well as carps (9, 29). However, the presence of COX in shark has not been reported.

In their existence in a high-salt environment, sharks use the rectal gland as a vital organ in regulation of NaCl excretion. Shark rectal glands provide a unique opportunity to study hormonal regulation of the salt excretory process. Over the past decades, remarkable progress has been made in cloning various ion transporters and hormone receptors and in studying intracellular signaling mechanism for regulation of the salt excretory process by using the shark rectal gland (1, 8, 15, 31). In the present study, we describe the cloning of COX cDNA from shark rectal glands, the first COX so far identified in an elasmobranch species. We further provide evidence for the role of COX product in regulation of chloride secretion in the perfused shark rectal gland.

MATERIALS AND METHODS

Animals and tissue collection. Male dogfish sharks, weighing 2–4 kg, were captured in Frenchman’s Bay, ME, and kept in a saltwater tank at Mt. Desert Island Biological Laboratory, Salisbury Cove, ME. After a shark was killed by spinal cord pithing, various tissues including brain, heart, kidney, rectal gland, spleen, liver, gill, and fin were removed for isolation of RNA, frozen on dry ice, and kept at −80°C until use.

RNA isolation, cDNA synthesis, PCR, and cloning of PCR fragment. Total RNA from 1 g of shark rectal gland was isolated using TRI-reagent. Single-strand cDNA was synthesized using Superscript II (Life Technologies) and oligo(dT) (Amersham Pharmacia). Two sets of long primers were chosen at the 5'-end of the original 600-bp PCR fragment sequence, antisense 1, 5'-GCAGTCGGATCC-AGCTTCTCA-GTGGGACTTTAAAAGGGGACACGCCTTACACAAAGCC-3', and antisense 1, 5'-GCTCAACAGCAACCCGGTTCCAGAGCTTGTTTGCTGAAGG-3'; sense 2, 5'-ATGATATGATCCATATTTGCTTGAGACACACCCTTGCTG-3', and antisense 2, 5'-CCCTTTGAGAGGACTGAGCCCTTTCATTCACCATACTCC-3'. PCR amplification was performed under low-stringency conditions using Expand High Fidelity PCR kit (Roche). The PCR reaction was started with denaturation for 3 min at 94°C, followed by 35 cycles at 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min. Out of four different combinations of primers, only one pair of primers, sense 2 and antisense 2, gave a 600-bp PCR product of the predicted size. PCR products were separated on a 2% agarose gel, purified, subcloned to TA cloning vector, and sequenced.

5'-RACE. Poly(A) RNA from shark rectal glands was isolated using Oligotex mRNA kit (Qiagen, Valencia, CA). One microgram of poly(A) RNA was used to synthesize adapter-ligated cDNA according to manufacturer's instruction (Marathon cDNA amplification kit, Clontech). Two antisense primers were chosen at the 5'-end of the original 600-bp PCR fragment sequence, antisense 1, 5'-GCAGTCGGATCC-AGCTTCTCA-GTGGGACTTTAAAAGGGGACACGCCTTACACAAAGCC-3', and antisense 2, 5'-CGAGGACTTCGTGGCAGACACCCGGTTCCAGAGCTTGTTTGCTGAAGG-3'. A first round of PCR was performed using shark-specific antisense 1 and a sense primer that anneals to the adapter sequence attached to the 5'-ends of shark cDNA. PCR reactions containing high-fidelity polymerase were denatured for 3 min at 94°C, followed by 35 cycles at 94°C for 40 s, 60°C for 1 min, and 68°C for 2.5 min. A second round of PCR was performed using shark-specific antisense 2, and the adapter primer was prepared under the same cycling conditions except at an annealing temperature at 63°C. PCR products were gel purified and sequenced as described above.

RT-PCR detection of shark COX mRNA. Total RNA from different tissues was isolated by TRI-reagent. One microgram of total RNA from each tissue was reverse transcribed by Superscript II (Life Technologies). Primers were chosen from the cloned 600-bp PCR fragment sequence, sense 5'-ATATCCTGAAAGAGAGCATCC-3', and antisense 5'-ACACATTGAGTCATGGA-3'. PCR reactions were denatured for 3 min at 94°C, followed by 33 cycles at 94°C for 40 s, 58°C for 40 s, and 72°C for 40 s. PCR products were separated on 2% agarose gels.

In vitro perfusion of shark rectal glands. For perfusion studies, shark rectal glands with artery and vein were cannulated and perfused as previously described (21). Rectal glands were placed in a glass perfusion chamber, maintained at 15°C with running seawater, and perfused with elasmobranch Ringer solution containing (in mM) 270 NaCl, 4 KCl, 3 MgCl2, 2.5 CaCl2, 1 KH2PO4, 8 NaHCO3, 350 urea, 5 glucose, and 0.5 Na2SO4 and adjusted to pH 7.5 by bubbling with 99% O2 and 1% CO2. Results are expressed as micro-equivalents of Cl− secreted per hour per gram wet weight.

Extraction of total lipids from shark tissues and PGE2 enzyme immunoassay. Shark tissues were homogenized in PBS and precipitated at 80% ethanol at 4°C. The supernatant was 1.8 diluted in 0.1 M phosphate buffer and was then subjected to Sep-Pak C18 cartridge (Waters) purification. The cartridge purification procedure was performed according the instruction from Cayman. Total lipids dissolved in appropriate amounts of enzyme immunoassay buffer were assayed for PGE2 concentration using an enzyme immunoassay kit (Cayman). In performance of the assay, the tissue lipid samples, along with a serial dilution of PGE2 standard samples, were mixed with appropriate amounts of acetylcholinesterase-labeled tracer and PGE2 antiserum and incubated at room temperature for 18 h. After the wells were emptied and rinsed with wash buffer, 200 μl of Elman’s reagent containing substrate for acetylcholinesterase was added. The enzyme reaction was carried out on a slow shaker at room temperature for 1 h. The plates were read at 415 nm, and the results were analyzed by KC4 software (Bio-Tek Instrument).

RESULTS

Cloning of shark COX. With the use of long primers (43 bp) derived from an EST database-derived zebrafish COX-2 sequence, a 600-bp product was amplified from shark rectal gland cDNA under low-stringency amplification conditions and was found to be
almost equally homologous to mammalian COX-1 and COX-2 (~65%). The remaining 5’-sequence was obtained by analyzing two overlapping 5’-RACE products of 1.3 and 1 kb. The 3’-sequence was obtained by sequencing an EST clone generated by the EST Project of the MDIBL Marine DNA Sequencing Center. The procedure for cloning the full-length cDNA is illustrated in Fig. 1. The gene, designated as shark COX (sCOX), contains a 216-bp 5’-untranslated region (UTR), a 1,779-bp reading frame, and a 233-bp 3’-UTR. The transcript encodes a 593-amino acid protein that is 68 and 64% identical to mammalian COX-1 and COX-2, respectively. sCOX contains virtually all functional sites of known COX, including active site (tyrosine385, histidine 388, and serine 530), substrate binding site (arginine120), N-glycosylation sites (asparagines68, 144, and 410), and sites crucial for peroxidase activity (glutamine203 and histidine207) (Fig. 2). sCOX contains valine523 that has been shown to be a key residue determining the sensitivity to COX-2-specific inhibitors including NS-398 (12).

To confirm that the different fragments of cDNA obtained by different approaches were originated from a single transcript, full-length cDNA was amplified using primers chosen from 5’-UTR and 3’-UTR, subsequently yielding 2-kb PCR products. Sequence analysis revealed that the 2-kb products contained two clones that are identical, except a size difference in 117 bp with the short clone lacking 39 amino acids without shifting reading frame (Fig. 3). Out of 10 clones derived from the 2-kb products, 7 were the long clone identical to sCOX and 3 were the short clone.

Distribution of sCOX mRNA and production of PGE2 in shark tissues. RT-PCR was performed to detect sCOX mRNA in different tissues using sCOX-specific primers. A 567-bp product of predicted size was observed from cDNA derived from shark rectal gland but not in RT control sample (not shown). Identity of the product was confirmed by sequencing. sCOX mRNA was found in all tissues including rectal gland, kidney, spleen, gill, liver, brain, and heart, except fin. The message was most abundantly expressed in rectal gland (Fig. 4). PGE2 production, determined by enzyme immunoassay, was higher in rectal gland and kidney than that in liver (Fig. 5).

Effect of COX inhibition on chloride secretion in the isolated perfused shark rectal gland. To assess a potential functional role of sCOX, we determined the effect of NS-398 on VIP-stimulated chloride secretion in rectal glands from Squalus acanthias. Rectal glands were perfused in vitro with shark Ringer solution for 30 min to reach basal levels of chloride secretion (<250 μeq-h⁻¹-g⁻¹). VIP at a concentration of 5 nM was then added in the presence or absence of the COX-2 inhibitor NS-398 (50 μM) and chloride secretion was measured at 1-min intervals for additional 60 min. NS-398 was removed from the perfusate at 60 min to determine reversibility. The diluent (DMSO) was present in all experiments. As can be seen in Fig. 6, NS-398 inhibited both the peak response (3,108 ± 479 vs. 2,131 ± 307 μeq-h⁻¹-g⁻¹, n = 4, P < 0.05) and the sustained response to VIP (50–60 min). When NS-398 was removed at 60 min, there was a prompt recovery of chloride secretion to control value.

DISCUSSION

Using PCR- and EST-based strategies, we obtained a full-length shark cDNA sequence that is highly homologous to mammalian COX over the entire coding region. A 593-amino acid protein encoded by this cDNA is 68% identical to mammalian COX-1 and 64% identical to mammalian COX-2. This protein contains key residues (tyrosine385, histidine388, and serine530) in the active site of COX and “EL” as ending sequence at the COOH terminal, both of which are highly conserved in all known COXs. It also contains other consensus sequences of COX, including arachidonic acid binding site (arginine120), three N-glycosylation sites (asparagines68, 144, 410), and sites crucial for peroxidase activity (histidine207, glutamine203) (ovine COX-1 numbering). Thus we conclude it is a shark COX and designate it as sCOX. To our knowledge, this is the first COX so far identified from elasmobranch species. The identification of a COX enzyme complements previous observations that certain cells from shark possess a high capability of PG production (35) and that PG plays a role in regulation of vascular tone in shark carps (9, 29). sCOX mRNA was widely distributed in different tissues, suggesting that the enzyme may play a role in multiple organ systems in sharks.

Whether sCOX should be viewed as the elasmobranch homologue of COX-1 or of COX-2 is not clear. The modest difference in homology (68% to COX-1 vs. 64% to COX-2) would appear to be insufficient to discriminate the two. Certain features of sCOX favor considering it to be closer to COX-1: similar to mammalian COX-1, it lacks 18 amino acids at the COOH-terminal end found in mammalian COX-2, a highly consistent difference between the two COX isoforms; sCOX mRNA was found widely in all tissues tested, except fin, suggesting a constitutive expression pattern. However, sCOX also contains a number of key residues specific to mammalian COX-2, most notably...
valine\textsuperscript{523} (valine\textsuperscript{509} in COX-2 numbering) that determines the sensitivity to COX-2-specific inhibitors including NS-398 \textsuperscript{(12)}. COX-1 and COX-2 differ strikingly in membrane binding domain (MBD) regions near Arg\textsuperscript{277} (COX-1 numbering), but sCOX does not exhibit significant homology to either one of the COX isoforms in the regions (51\% to COX-1 and 39\% to COX-2 in MBD regions). Recently, Valmsen et. al. \textsuperscript{(36)} and Koljak et al. \textsuperscript{(19)} identified a form of COX found in coral, COX0, that also could not be clearly classified as either COX-1 or COX-2 (about 50\% to both). The sequence we have identified shows <50\% homology to this sequence.

In the process of analyzing the full-length cDNA of sCOX, we identified two different forms of sCOX. The two clones are identical except for a size difference in 117 bp with the short clone showing an in-frame 39-amino acid deletion. This finding suggests the possibility that sCOX exists in two different isoforms generated by alternative splicing. Out of 10 clones derived from PCR amplification of full-length sCOX cDNA, 7 were the long form and 3 were the short clone, indicating that the short clone may be expressed at relatively lower abundance. Identity of the short form of sCOX needs to be clarified in future studies.

sCOX appears to participate in the control of the salt excretory function of the shark rectal gland. Among shark tissues analyzed, sCOX expression levels were highest in the SRG, consistent with the high capacity of PGE2 production in this organ. These findings suggest the possibility that some sCOX product may be involved in the regulation of chloride secretion. Indeed, we observed that NS-398 significantly inhibited VIP-stimulated Cl\textsuperscript{-} secretion in the in vitro perfused shark rectal gland. Because sCOX contains valine\textsuperscript{523} (valine\textsuperscript{509} in COX-2 numbering), the single residue that has been shown to confer sensitivity to NS-398, it is likely that inhibition of sCOX accounts for the effect of NS-398. This finding suggests that an endogenous COX product stimulates salt secretion in the rectal gland. This notion, however, is not consistent with a previous study by J. S. Stoff et al. (unpublished observations) who show that indomethacin has no obvious effect on VIP-stimulated Cl\textsuperscript{-} secretion in perfused shark rectal gland. The reason behind the inconsistent results between this study and ours is not clear. This could be related to the differences in sensitivities of sCOX to inhibition by the two classes of compounds. It is also possible that similar to mammals, sharks may

***Fig. 2. Amino acid alignment of sCOX with known COX-1 and COX-2 from different species. Identical (*) and similar (.) residues identified by the CLUSTAL program are indicated. Amino acid numbering is based on ovine COX-1. Consensus sequences of COX are active site of COX (tyrosine\textsuperscript{385}, histidine\textsuperscript{388}, and serine\textsuperscript{530}), substrate binding site (arginine\textsuperscript{120}), N-glycosylation site (asparagines\textsuperscript{68, 144, and 410}), and sites crucial for peroxidase activity (glutamine\textsuperscript{203} and histidine\textsuperscript{207}). Residue in bold is valine\textsuperscript{523} (valine\textsuperscript{509} in COX-2 numbering) that determines sensitivity to NS-398. Trout COX-1 and COX-2 are from brook trout. Coral COX0 represents 15R-specific COX. Nucleotide sequence of sCOX has been submitted to the GenBank with accession number AF420317.***
have another COX isoform in addition to sCOX, two isoforms of which may have different roles in regulation of salt excretion. In mammal studies, it is not uncommon to see different effects of selective and non-selective COX blockers due to different functions of the two COX isoforms. It has been suggested that the two COX isoforms in the mammalian kidney may exert opposite actions in regulation of salt excretion, with COX-2 promoting salt excretion and COX-1 inhibiting it.

Regulated Cl secretion by the rectal gland is a critical survival mechanism of elasmobranchs in the high salt marine environment. Over the past few decades, the mechanisms for regulation of the chloride secretory process have been extensively investigated. It is well established that chloride secretion is under the control of various vasoactive hormones. In response to volume expansion, C-type natriuretic peptide (CNP) is released from the heart and acts in the rectal gland. CNP causes release of VIP from nerves within the gland and together with VIP initiates chloride secretion. CNP and VIP, via intracellular cGMP and cAMP, respectively, both activate cystic fibrosis transmembrane conductance regulator and stimulate chloride secretion (32). Inhibition of VIP-stimulated Cl secretion by NS-398 in the present study suggests that a COX product in part mediates the action of VIP. Interactions of PG and VIP have been observed previously in vascular
tissue. Kagstrom et al. (17) showed that PG synthesis mediates VIP-induced relaxation in small arteries of rainbow trout. The demonstration of a role of PG synthesis in the regulation of chloride secretion in the present study contributes to our understanding of the complex mechanism for hormone regulation of the excretory function of the rectal gland.

The mechanism involving PG regulation of chloride secretion in shark rectal gland may be applicable to mammalian kidney. A large body of evidence in mammals has demonstrated the natriuretic and diuretic nature of PGs. Exogenous PGs such as PGE2 inhibit NaCl transport in both thick ascending limbs and collecting ducts (14, 33, 34). In whole animal experiments, chronic salt loading increases PG synthesis in the renal medulla (22, 23, 37). Systemic COX inhibition in humans results in reduction of urinary salt excretion (5, 26). These findings strongly suggest that PGs, produced in the renal medulla in response to salt loading, promote salt excretion. However, assessment of the role of endogenous PGs in salt excretion in mammalian kidney has been difficult. The present study using the perfused shark rectal gland provides evidence supporting a role of endogenous PG in regulation of NaCl excretion, thus complementing the observations in mammals. Taken together, these observations suggest a well-conserved function of the PG system in salt balance across different species.

In summary, using low-stringency PCR and EST approaches, we cloned a shark COX (sCOX) from the rectal gland, the first COX so far identified in an elasmobranch species. sCOX mRNA was widely distributed in different tissues with relatively more abundant expression in shark rectal gland. Furthermore, we demonstrated that COX inhibition significantly reduces VIP-stimulated chloride secretion in the in vitro perfused shark rectal gland, suggesting an important role of PG in regulation of the NaCl transport process in the gland.

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