Fish macrophages express a cyclo-oxygenase-2 homologue after activation

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In mammals, the increased generation of prostaglandins (PG) during the onset of inflammatory responses and activation of immune cell types has been attributed to the induction of a novel cyclo-oxygenase (COX) isoform, termed COX-2, which is distinct from the well-characterized constitutive activity (COX-1). Goldfish (*Carassius auratus*) macrophages exposed to bacterial lipopolysaccharide and leucocyte-derived macrophage-activating factor(s) showed a significant increase in the generation of the major COX product, PGE₂, within the first 6 h of stimulation. The selective COX-2 inhibitor, NS398, inhibited this elevated generation of PGE₂, whereas the basal level of this product synthesized by unstimulated macrophages was unaffected by such exposure. PGE₂ generation by goldfish macrophages was similarly inhibited by the glucocorticoid, dexamethasone, and an inhibitor of protein synthesis, cycloheximide, suggesting that this stimulation may be due to an inducible enzyme equivalent to

mammalian COX-2. The complete coding sequence of rainbow trout (*Oncorhynchus mykiss*) COX-2 was obtained by PCR. The gene contains a 61 bp 5'-untranslated region (UTR), a 1821 bp open reading frame and a 771 bp 3'UTR containing multiple copies of an mRNA instability motif (ATTTA). The predicted translation product had high homology to known mammalian and chicken COX-2 (83–84%) and COX-1 (77%) sequences. Reverse-transcriptase PCR with cDNA from control and bacterially challenged fish revealed that trout COX-2 expression was not constitutive but could be induced. Overall, these studies show for the first time that the inducible isoform of COX has a long evolutionary history, probably dating back to the evolution of fish over 500 million years ago.

Key words: goldfish, leukotriene, *Oncorhynchus mykiss*, prostaglandins, trout.

INTRODUCTION

The synthesis of prostaglandins (PG) by cyclo-oxygenase (COX), also termed prostaglandin H synthase, and their physiological functions in various mammalian cells and tissues, have been well characterized [1]. Aspirin and other non-steroidal anti-inflammatory drugs have been shown to inhibit prostanoid formation by inactivation of COX, thereby implicating a role for PGs in inflammatory processes [1]. It has been discovered recently that the generation of such biologically active compounds in mammals can be attributed to the existence of two distinct COX isoenzymes [2–5], the structures of which have now been fully elucidated (see e.g. [6,7]). The first form (COX-1) is generally regarded as the 'housekeeping' enzyme and is constitutively expressed in most cells and tissues. The second form (COX-2) is inducible and is associated with several cell types, including inflammatory cells such as fibroblasts, mast cells, monocytes and macrophages. In the latter cells COX-2 is expressed in response to various inflammatory signals, including growth factors, cytokines and lipopolysaccharide (LPS) [5,8,9]. In addition, the stimulated synthesis of PG observed in most cell types has been attributed to the selective expression of COX-2, with no effect on the contribution by COX-1 [9].

PGs have been shown to be formed in a diverse range of invertebrates and vertebrates. However, in 'lower' invertebrates, such as corals, their synthesis is independent of COX and appears to involve allene synthase activity [10]. In most 'higher' invertebrates, such as arthropods and molluscs, COX-like activity

is apparently responsible for PG synthesis [11,12], although no such enzyme has been purified and sequenced. With the evolution of the first vertebrates, including fish, over 600 million years ago, significant changes occurred in the level of organ complexity. In particular, in modern day fish the immune system is well-developed, with the first evolutionary appearance of lymphocytes and specific inducible immunoglobulin, as well as 'true' macrophages capable of stimulation by cytokines and bacterial products such as LPS [13]. Many studies have demonstrated the involvement of tissue macrophages in piscine inflammatory responses [14] and PG synthesis has been shown to accompany such events [15]. Furthermore, as in mammals, the release of PGE₂ by fish macrophages has been reported to modulate both immune reactivity and inflammatory responses [16,17]. Recent studies have demonstrated the individual and synergistic activities of LPS and fish-derived cytokines [macrophage-activating factors (MAF)] in stimulating or inducing respiratory burst [18], as well as nitric oxide [19] and PGE₂ [17] generation in piscine macrophages.

The aim of this study was to determine whether an inducible form of COX is present in fish macrophages. Previous preliminary studies have shown elevated levels of PG generation in rainbow trout (*Oncorhynchus mykiss*) macrophages [17], although this was found to be highly variable in terms of magnitude and dynamics from individual to individual. Therefore, in this present study we have used a novel macrophage cell line derived from goldfish (*Carassius auratus*) to overcome such problems.

Abbreviations used: COX, cyclo-oxygenase; DEPC, diethyl pyrocarbonate; EIA, enzyme immunoassay; HBSS, Hanks balanced salt solution; MAF, macrophage-activating factor(s); GMCL, goldfish macrophage cell line; LPS, lipopolysaccharide; LT, leukotriene; PG, prostaglandin; RT, reverse transcriptase; UTR, untranslated region.

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MATERIALS AND METHODS

Goldfish (*C. auratus*) macrophages

The goldfish head kidney macrophage cell line, designated GMCL, was used in most experiments. The characteristics and growth requirements of this cell line have been described in detail previously [20]. GMCL were routinely maintained in goldfish leucocyte medium consisting of Leibovitz L-15 and Dulbecco's modified Eagle's medium (Gibco) containing 5% goldfish serum, 10% calf serum (Hyclone), 50 $\mu\text{g}\cdot\text{ml}^{-1}$ gentamicin and 0.4% Hepes. All cultures were maintained at 20 °C for 7–10 days before experimentation. In a few experiments, primary cultures of goldfish macrophages were used. These cells were isolated from head kidney and maintained as described elsewhere [21].

Production of MAF by goldfish leucocytes

Supernatants enriched with MAF were obtained through the mitogenic stimulation of mixed goldfish head kidney leucocyte culture as described by Neumann and Belosevic [22]. Briefly, fish were anaesthetized with MS-222 (Sigma), killed by cervical dislocation, and head kidneys were removed and pushed through stainless steel meshing while rinsing with serum-free medium supplemented with 50 units $\cdot\text{ml}^{-1}$ heparin (Sigma), 50 $\mu\text{g}\cdot\text{ml}^{-1}$ gentamicin (Gibco), 100 $\mu\text{g}\cdot\text{ml}^{-1}$ streptomycin (Sigma) and 100 units $\cdot\text{ml}^{-1}$ penicillin (Sigma). Released cells were subsequently layered over 51% Percoll gradients (Pharmacia) and centrifuged at 400 g for 25 min. Cells fractionating at the medium/Percoll interface were removed and washed twice in serum-free medium. Leucocytes isolated from a number of fish were pooled and seeded into 75 cm^2 tissue culture flasks containing medium supplemented with 5% goldfish serum, 10% calf serum and antibiotics (as above) to a density of 4×10^6 cells $\cdot\text{ml}^{-1}$. After an initial incubation of 12 h at 20 °C, leucocytes were stimulated with concanavalin A (10 $\mu\text{g}\cdot\text{ml}^{-1}$; Boehringer Mannheim), PMA (10 $\text{ng}\cdot\text{ml}^{-1}$; Sigma) and calcium ionophore A23187 (100 $\text{ng}\cdot\text{ml}^{-1}$; Sigma) for 6 h. Subsequently, cell monolayers were washed three times with Hanks balanced salt solution (HBSS) and cultured in complete medium containing 5% goldfish serum, 10% calf serum and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ gentamicin. Cell supernatants were harvested after 72 h of stimulation, filter-sterilized and stored at -20 °C.

Kinetics of induction of PGE generation in GMCL and primary cultures of macrophages

GMCL were seeded at a density of 5×10^5 cells in 500 μl of goldfish leucocyte medium and stimulated with a 1:5 dilution of MAF and/or 5 $\mu\text{g}\cdot\text{ml}^{-1}$ *Salmonella typhimurium* LPS (Difco) for 6, 12 or 24 h at 20 °C. Unstimulated cells were incubated with medium alone. After these incubation periods, cells were challenged with 5 μM calcium ionophore A23187 for 20 min at 20 °C, followed by centrifugation of cell supernatants at 400 g for 10 min at 4 °C to remove any debris. The resultant supernatants were stored at -80 °C before PGE₂ or leukotriene B₄ (LTB₄) enzyme immunoassay (EIA). Experiments using primary cultures of goldfish macrophages were performed similarly to those using GMCL, with the exception that cell stimulation was with 2 $\mu\text{g}\cdot\text{ml}^{-1}$ *S. typhimurium* LPS alone.

Effect of dexamethasone, cycloheximide and COX-1 and COX-2 inhibitors on PGE₂ generation by goldfish macrophages

GMCL were seeded to a density of 5×10^5 cells/500 μl and stimulated for 12 h with or without LPS (5 $\mu\text{g}\cdot\text{ml}^{-1}$) in the presence or absence of either 2 μM dexamethasone (Sigma) or

Figure 1 Primers and PCR products for the COX-2 gene in trout

The position of primers (F1–F6; R1–R4; T3; T7) used to probe for the trout COX-2 gene sequence and PCR products obtained (P1–P3).

2 μM cycloheximide (Sigma). After the incubation period, cells were challenged with 5 μM calcium ionophore A23187 for 20 min at 20 °C and supernatants were centrifuged at 400 g for 10 min at 4 °C before storage at -80 °C. To determine the effect of COX-1 and COX-2 inhibitors on PGE₂ generation, GMCL or goldfish primary macrophage cultures were incubated with or without 5 $\mu\text{g}\cdot\text{ml}^{-1}$ *S. typhimurium* LPS for 12 h and after this time they were either pre-incubated for 30 min with or without 0.1–10 μM of the selective COX-2 inhibitor, NS-398 ([23]; Cayman Chemicals), 20 or 500 μM of a selective COX-1 inhibitor, valerylalicylate ([24]; Cayman) or 50 μM of the non-selective COX inhibitor, indomethacin (Sigma) for 30 min. Cells were subsequently challenged with calcium ionophore A23187 for 20 min at 20 °C in the presence or absence of these inhibitors. Cell supernatants were harvested and stored at -80 °C before PGE analysis.

LTB₄ and PGE₂ EIA

The amounts of LTB₄ and PGE₂ immunoreactive material generated by GMCL and primary cultures of macrophages were determined without extraction using commercially available EIA kits (Amersham Life Sciences), the sensitivities of which were 40 and 6 $\text{pg}\cdot\text{ml}^{-1}$ respectively. The antibody to PGE₂ had cross-reactivity with PGE₁ (7%), PGF_{2 α} (4.3%), PGD₂ and PGB₂ (1%), whereas the antibody to LTB₄ had cross-reactivity with 20-OH-LTB₄ (2.5%) and 6-*trans*-LTB₄ (16.6%).

Cloning of COX-2 cDNA

Two pairs of degenerate primers (F1 = 5'-AARGARGTNYT-NGARAARGT-3', F2 = 5'-RCATTCTTTGCCAGCACTT-CAC-3', R1 = 5'-CATYTCNACYTGNGTRTCYTT-3' and R2 = 5'-AGCCAKATBGTDGCATACATCAT-3' [R = A/G, K = G/T, Y = C/T, B = C/G/T, D = G/A/T, N = A/G/C/T]) were designed against conserved regions of the known mammalian COX-2 genes in the GenBank Database (Figure 1). These primers were used for PCR with cDNA from a previously prepared trout head kidney leucocyte cDNA library [25]. This library was derived from macrophage-enriched head kidney leucocyte RNA, collected 2 days after challenge of trout with 2×10^8 attenuated (*aroA*⁻) *Aeromonas salmonicida* bacteria. Further primers were designed against the initial trout COX-2 sequence obtained to allow cloning of the 5' and 3' ends of the molecule (F3 = 5'-AGGTTGGGGCCGAGATGCAT-3', F4 = 5'-AAGCTGAGGCTGTTCAAGGATG-3' and R3 = 5'-

GCTGGTCCTTTCATGAAGTCTG-3') using anchored PCR [25] with either T3 (5'-AATTAACCTCACTAAAGGG-3') or T7 (5'-GTAATACGACTCACTATAGGGC-3'). In addition, two primers were designed for sequencing purposes (F5 = 5'-TGAAACCTTCAGCATTGAGG-3' and R4 = 5'-TGGTAAACACCTGAGGGATG-3'). PCR amplification was performed in 50 μ l reaction mixtures containing the following components: 2 μ l of forward (F) and reverse (R) primers (each 10 μ M), 1 μ l of dNTP mixture (2.5 mM each), 0.5 μ l of *Taq* polymerase (5 units/ μ l; Promega), 5 μ l PCR buffer (10 $\times$; Promega), 4 μ l of $MgCl_2$ buffer (25 mM; Promega), 10 μ l of Q buffer (Qiagen), 23.5 μ l sterile distilled water and 2 μ l of cDNA template. Control reactions were set up in the absence of cDNA template to ensure that products did not arise due to contamination or from primer-dimer effects. All reaction mixtures were overlaid with mineral oil. The cycling protocol was 1 cycle of 94 $^{\circ}$ C for 5 min, 35 cycles of 94 $^{\circ}$ C for 1 min, 52 $^{\circ}$ C for 1 min and 72 $^{\circ}$ C for 2 min, followed by 1 cycle of 72 $^{\circ}$ C for 10 min. PCR products were visualized on 0.8–1.5% agarose gels containing ethidium bromide (100 ng·ml $^{-1}$) using a 100 bp DNA ladder as a size marker (Gibco). The products obtained were cloned using a TOPO TA cloning kit (Invitrogen). Plasmid DNA was prepared from at least three clones using a QIAGEN plasmid miniprep kit and sequenced on an ABI automated 373A sequencer. The nucleotide and amino acid sequence data were then compared with entries in the GenBank Database [26], using the basic local alignment search tool (BLAST) [27]. Direct comparisons between two full-length sequences were performed using the GAP alignment program [28] within the Wisconsin Genetics Computer Group (GCG) sequence analysis software package, version 8.1, and results are presented as percentage identity or similarity. Multiple sequence alignments were generated using the CLUSTAL program [29]. Finally, an unrooted phylogenetic tree was constructed based on peptide translations of the precursor molecule using the PHYLIP package [30] and alignments generated with CLUSTAL.

Reverse transcriptase (RT) PCR

To determine whether COX-2 gene expression *in vivo* could be upregulated, rainbow trout were exposed to an attenuated (*aroA* $^{-}$) strain of the bacterial pathogen *A. salmonicida* [31]. Animals were injected intraperitoneally with 2×10^8 bacteria per fish, and 2 days later the head kidneys were collected and macrophage-enriched leucocyte suspensions were prepared as described previously [18]. Total RNA was isolated from the cell pellets using RNazol B (Biogenesis) and stored overnight at -70° C. For reverse transcription, 5 μ g of RNA was recovered from stocks and resuspended in 11 μ l of diethyl pyrocarbonate (DEPC)-treated water. Oligo-dT18 primer (0.5 μ g; Stratagene) was added and the reaction mixture was incubated at 70 $^{\circ}$ C for 10 min. After cooling on ice, 4 μ l ($\times 5$) of reaction buffer, 2 μ l of dNTP mix (10 mM; Boehringer) and 2 μ l of dithiothreitol (0.1 M; Stratagene) were added for 2 min at 42 $^{\circ}$ C, followed by addition of 1 μ l of Superscript MuMLV RNase H $^{-}$ reverse transcriptase (50 units; Gibco) and incubation at 42 $^{\circ}$ C for 50 min. The reaction was terminated by heating to 70 $^{\circ}$ C for 15 min. DEPC-treated water was added to make the reaction volume up to 100 μ l and the cDNA was stored at -20° C. cDNA was also made from unstimulated kidney leucocyte suspensions from control fish. The cDNA was then used for PCR with primers F6 (5'-ATCCTTACTCACTACAAAGG-3'), designed to be COX-2 specific (i.e. not to anneal to COX-1), and R3, using the conditions described above for cloning the trout COX-2 cDNA. Products were again visualized on an agarose gel.

Primers for trout β -actin (5'-ATCGTGGGGCGCCCCAGGC-ACC-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3') were used as a positive control for RT-PCR, since the gene is expressed constitutively in the tissues examined. Primers were designed against consensus β -actin sequences to amplify a product of 541 bp that spanned introns 2 and 3 of the mammalian gene [32]. Reactions with no added cDNA were used as a negative control.

Statistical analyses

Statistical analyses were performed using Student's *t*-test (INSTAT, Graph Pad, San Diego, CA, U.S.A.) where significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Kinetics of eicosanoid generation by LPS- and/or MAF-stimulated GMCL and primary goldfish macrophage cultures

Figure 2(A) demonstrates that the effectivity of LPS and/or MAF in augmenting the generation of immunoreactive PGE by the goldfish macrophage cell-line (GMCL) was dependent upon the length of the stimulatory period. Maximal immunoreactive PGE levels were observed after only 6 h in the presence of LPS

Figure 2 Kinetics of PGE₂ (A) and leukotriene B₄ (B) generation by stimulated goldfish macrophages

Goldfish macrophages were stimulated with *S. typhimurium* LPS and/or MAF for 6–24 h before challenge with the calcium ionophore A23187 (5 μ M). Mean values $\pm$ S.D., $n = 3$. * $P < 0.02$ and ** $P < 0.04$ compared with control or columns marked with arrows.

Figure 3 Inhibition of the *S. typhimurium* LPS-stimulated PGE generation by goldfish macrophages

(A) Stimulated macrophages were incubated in the presence of the selective COX-2 inhibitor NS398 (10 μM), the selective COX-1 inhibitor, valeryl salicylate (200, 500 μM) and the non-selective COX inhibitor, indomethacin (50 μM; the absence of a bar represents this value since 100% inhibition was observed in all samples analysed). Mean values $\pm$ S.D., $n = 4$. * $P < 0.001$ and ** $P < 0.0005$ compared with control. **(B)** Inhibition of the *S. typhimurium* LPS-stimulated PGE generation by GMCL in the presence of the corticosteroid dexamethasone or the protein biosynthesis inhibitor cycloheximide. Mean values $\pm$ S.D., $n = 3$. * $P < 0.0005$ compared with control.

with an approx. 18-fold increase in the level of product. The inclusion of MAF led to a more modest induction of product generation, exhibiting its maximal affect after 12 h of stimulation. Interestingly, when cells were co-stimulated with LPS+MAF only after 24 h did there appear to be a synergistically enhanced generation of PGE, which was found to be significantly greater ($P < 0.004$) than the generation occurring in the presence of LPS alone. The dynamics of changes in PGE₂ generation following LPS challenge of GMCL are similar to those reported for mammalian macrophages such as the murine RAW 264.7 cell line [33].

To determine whether upregulation of lipoxygenase products, such as LTB₄, also occurred under these conditions, the same supernatants were tested for the presence of this product by EIA. Only at 6 h was there any significant increase in the amount of product generated after stimulation with MAF+LPS (Figure 2B). Furthermore, statistically significant decreases in the levels of immunoreactive LTB₄ were observed between MAF and LPS and/or MAF+LPS-stimulated cells after 12 and 24 h of incubation (Figure 2B). Such reductions in LTB₄ generation may have been due to a number of factors, including suppression of lipoxygenase activity by the increased levels of PGE₂, or cytokine/LPS suppression of 5-lipoxygenase expression. Interestingly, interleukin 3 has been shown to upregulate the expression of 5-lipoxygenase in murine bone marrow-derived

mast cells after several weeks exposure [34], suggesting that cytokines can modulate the expression of this enzyme.

Owing to difficulty in obtaining sufficient GMCL for such studies, further experiments were carried out using primary cultures of goldfish macrophages. As with the macrophage cell line, the induction of immunoreactive PGE in these goldfish primary macrophage cultures occurred maximally after a 6 h period in the presence of LPS (results not shown). Furthermore, similar trends were observed for longer incubation periods in the presence of either LPS, MAF or LPS+MAF, as observed with GMCL (results not shown).

Inhibition studies

The incubation of LPS-stimulated GMCL or primary cultures of macrophages with the COX-2-specific inhibitor NS-398 (0.1–10 μM) resulted in a significant decrease in the generation of immunoreactive PGE (Figure 3A). Under the same conditions, indomethacin (50 μM) ablated all generation of PGE₂ as measured by EIA, whereas valeryl salicylate (reported to be a COX-1 selective inhibitor) had less effect than the other two inhibitors tested (Figure 3A). In addition, NS-398 (10 μM) had no inhibitory effect on the amount of immunoreactive PGE generated by unstimulated GMCL (0.054 ± 0.02 ng/10⁶ cells compared with 0.050 ± 0.06 ng/10⁶ cells in the presence of 10 μM NS-398; mean values $\pm$ S.D., $n = 3$), suggesting that the constitutively expressed form of the enzyme (a COX-1 homologue?) was insensitive to this inhibitor. Interpretation of these studies requires some caution as the specificity of the inhibitors used has not been established in any non-mammalian systems. Furthermore, whereas NS-398 is a highly specific COX-2 inhibitor (e.g. purified COX-1 has been reported to be unaffected by NS-398 at $> 10^{-4}$ M; [23]), valeryl salicylate is apparently less specific. For example, 500 μM valeryl salicylate caused the loss of 85% of COX-1 activity from *cos-1* transfected cells, whereas 15% of COX-2 activity was removed at this concentration [24]. Although we have no direct evidence for the existence of COX-1 in these leucocytes, their ability to generate PGE immunoreactive material in the absence of induced COX-2 implies the presence of a constitutive form of the enzyme (a COX-1 homologue?). Therefore it is impossible at this time to deduce whether the inhibition of PGE biosynthesis in stimulated leucocytes (Figure 3A) is due to inhibition of COX-2 alone or to a combination of COX-1 and COX-2.

Significant reductions in the amounts of PGE₂ generated by LPS-stimulated GMCL were observed after the inclusion of dexamethasone (2 μM) or cycloheximide (2 μM), with no effect on the levels of immunoreactive PGE produced by unstimulated cells (Figure 3B). Although the precise mechanism of glucocorticoid inhibition of COX-2 expression in mammals is unresolved, it may act, at least partially, by post-transcriptional mRNA destabilization [35]. In the present study it was surprising that cycloheximide did not lead to the total inhibition of PGE generation, particularly if this is all the product of the induced COX-2. However, such a situation is not ruled out by our observations, as the remaining PGE₂ generation after cycloheximide treatment, may have been due to accumulation of this compound in the medium.

Cloning

PCR with primers F2 and R2 (see Figure 1) gave a product (P1) of the expected size (323 bp), that when cloned and sequenced was found to have high homology to other known COX-2 genes. PCR with primers F1/R1 or F2/R1 gave no products. Furthermore, anchored PCR using primer R3 (based on the initial

AGGAGTCGACTGGAATTTGACATTTTATACCGGATTGCGATTCAATAGAATCTTTCCGGG 1
61
ATGAATAGAGTAATCTGTATAATTTTCTCTTGGCTTGGGCTCTACTTCTGCGAAGGA 121
M N R V I C I I L L L A V G L Y F C E G 20
GTTTACTCTTGGTGTGACAGCCTTGTGAACATAGGGGTTATGTAATTCAGAGGCTTT 181
V D P C C A Q P C E N R G L C N S K G F 40
GACAACATGAGTGCAGCTGCACAAGGCTGATATATGGAAGAACTGCACAACCTCT 241
D N Y E C D C T R T G Y Y G K N C T T P 60
GAATTCCTAACATGGAATAAATCATTTGAAACCGAGCTCCGAACACTATTCACATC 301
E F L T W I K I S L K P A P N T I H Y I 80
CTTACTCACTACAAAGGTTGTGGAACGTCATCAACAGATCACCTTTGTGAGAAACGCT 361
L T H Y K G L W N V I N K I T F V R N A 100
ATAATGAGCTATGTCCTGACACCCCGCTTACATTTGGTGGACAGCCACCGCTTCAAT 421
I M S Y V L T P R S H L V D S P P T Y N 121
GCTGACTATGGCTACAAGAGCTGGGAGGCTCTCAATCTGTTTACTACACCGGAGC 481
A D Y G Y K S W E A Y S N L F Y Y T R T 141
CTCCCCCATTCGCAAGGAGCTCCCTTACAGCTAGGACACGAGGAGGAGGAGCTCT 541
L P P L P K D C C T P M G T A G R A V L 161
CCGAGTCTCAAGCTCGTGGTGGAGAAGTTTGTGTGAGGAAGCGGTTCAATCCGATCT 601
P D V K L V V E K V L L R K R F I P D P 180
CAGGGTTCCAACTCTCATGCTTCTTCCGCCAGGACTTCCACCACGTTCTTCAAA 661
Q G S N L M F A F F A Q H F T H Q T F F K 200
TCAGACTTCATGAAGAGGACGCTTTTACCAAGGCTTGGGCAATGGCGTGGACTTAAAC 721
S D F M K G P A F T K A L G H G V D L N 220
CATGTTTACGGAGACACTCTGAGAGACAGCAGCTAGGCTGTGTTCAAGGATGGCAAA 781
H V Y G D T L E R Q H K L R L F K D G K 240
CTGAAGTATCGGGTTTGAACGGGAGGCTTACCCCGATTTGGTAAGGGAGGTGGGGCC 841
L K Y R V L N G E V Y P P L V R E V G A 260
GAGATGCATTACCCACCCAGGAGCTGCTGAGGAGGAGCTGCTGCTGGGCGCAGAGCAT 901
E M H Y P P Q V P E E H R F A V G H E H 280
TTTGGCTTGGTCCCGGGCTCATGATGATGACCATATGGCTGGCGAGGACAAACGCT 961
F G L V P G L M M Y A T I W L R E H N R 300
GTCTGTGAGCTCTGAGGAGGAGCAGCTCCGAAATGGGAGCAGCAACGATCTTCCAGACC 1021
V C D V L R Q E H P E W D D E R I F Q T 320
ACAGCCCTCATCTTAATTTGGCGAGACCTTAAGATCGTGATTGAGGACTATGTCAGAC 1081
T R L I L I G E T I K I V I E D Y V Q H 340
CTGAGTGGCTACCACTTCCAGCTCAAGTTCGACCCGAGGCTCTCTTCAACCGGCTTTC 1141
L S G Y H F Q L K F D P E L L F N Q R F 360
CAGTACCAAGCCGTATCGCAGCTGAGTTCAATACCTCTACCACTGGCACCCTGATG 1201
Q Y Q N R I A E C F N T L Y H W H P L M 380
CCTGAACCTTCCAGCATTTGAGGACCGGCTACACCTTACCCCGAGTTTGTCTTCAACAC 1261
P E T F S I E D R A Y T Y P Q F V F N N 400
TCCCTGGTACAGAGCAGGCAACCAACCTGGTGGAGTCTCTTCAACAGCAATCGCT 1321
S L V T E H G I N L V E S F T K Q I A 420
GGAAGGGTGGCTGGTGGACGGAACCTGCTCTCTGCACTAGTGGCGTGGCAGCTAAGGCC 1381
G R V A G G R N L P P A L V G V A A K A 440
CTGGAGCAGCAGAGACATGCGTTTACCACTCCCTCAACCGCTACAGGAACCGCTTCAAC 1441
L E H S R D M N R Y Q L N A Y R K R F N 460
ATGAGGGTCTACACCTCCTTTGAGGACCTCAGGAGAGAGAGAGCTGGCTGCGGAACCTA 1501
M R V Y T S F E D L T G E T E L A A E L 480
GAGAGCTATATGGGAGTATGATGCTGTGGAGCTGTACCCAGGCTTCTGTTGGAGCGC 1561
E S L Y G D V D V A E L Y P G L L V E R 500
CCCAGACCTAACGCTGTTTTCGGGGAGACTATGGTGCAGATGGGTGCACCTACTCCCTG 1621
P R P N A V F G E T M V E M G A P Y S L 520
AAAGGCTCTCTGGGAAACCCCATTTGCTCGCGGAATCTGGATGCCAGCAGCTTCGGC 1681
K G L G N P I T C S P E Y W M P S T G 540
GGGAGTGTGGGCTTTGACATCTCAACACTGCTTCAATTTGAGAGGCTGGTGTGCAACAAT 1741
G S V G F D I L N T A S L E R L V C N N 560
GTCAAGGGTTCTTGTCCGATGGTCTCTTTCAAGTGCCTGATTTTGTGAGAGCATTTGAG 1801
V K G S C P M V S F V P D F L R A F E 580
TCGGCGCTCAGTCAACCAAGTGAAGGCCACCTGAGTGACATGAACCCAGGTGTTCTGTTTC 1861
S A S V N T S E A H L S D M N P G V L F 600
AAAGAAAGGACTTCAGAGCTTTAAATAACTTAAACACTAGGTTCTATTCAGTACACAAA 1921
K E R T S E L
TACACCGTTTCTCAGTGTCTTAAAGATGGTCTCGTTTGTAGCTTAAATTTGCTTTATTAGT 1981
TATTATATTTGATTAATTTATGATTTAATTTATTTTGTACTGTGTAATATTGAGTT 2041
GTTATTAAGTTAATAGTCATTTATGTTTAAATATGATACCTATTACAAATGCTCT 2101
TACATGAACAAAGCAAGTTAGTTTACTCTGTATACAAATGGAACCTTGAAGGAGTTGCA 2161
TTTACTTTGAAGAAATAACTATTAGCAATACCTGTATGTTTTCAGCTACTGAATGGATA 2221
CAGCTGGGCTGATTTGTCAATCTTTTGTGTACTTTAAAGACAAAGCCTTTATTTATT 2281
GTAATGGATGAAGTGTATAATGGATGTAGAAATATACCGTTTTCAGAAAGTATGAAG 2341
ACACTGTATTTTTCAGGCCCCATCCCTCAGGTGTTTACAGAGACAGTGGCGGGCTGT 2401
CTGTTTGTGTGTGTTTCACTGCGGATTCGCCCTTAAATGTTATGAAGTCATAAAT 2461
TACATTCCAACTAAAATGGTTAGGCACCTTGAAGAAACATTAAAGACAATCGATCTGTCA 2521
AGATTATCATTTTATCAGTATGATTCAATATATTAATTCATGTCATATGTCAACCCCTT 2581
GACCTTTTCACATTTGTTGTGTACTGCTGCTGATATAAATAAGTATTAATTTGAAAAA 2641
AAAAAATTTTGTGTGTACTGCTGCTGATATAAATAAGTATTAATTTGAAAAA 2653
AAAAAATTTTGTGTGTACTGCTGCTGATATAAATAAGTATTAATTTGAAAAA

trout sequence) with T3 gave a product of 684 bp (P2) that contained the 5'-end of the trout COX-2 gene (Figure 1). However, finding the 3'-end was more problematic, and a product (P3, 1822 bp) was eventually obtained using a nested strategy whereby F4/T7 were used in a first round of PCR, followed by F3/T7 in a second round. Sequencing of this product required two further primers (F5 and R4). Overall, the nucleotide sequence obtained from the three overlapping products translated in one reading frame to give the full-length COX-2 molecule, containing 607 amino acids, with a predicted 20 amino acid signal peptide, a 61 bp 5'-untranslated region (UTR) and a 771 bp 3'-UTR (Figure 4). The 3'-UTR contained multiple copies of an mRNA instability motif (ATTTA) and a polyadenylation signal 15 bp upstream of the poly(A) tail. Multiple alignment of the predicted translation of trout COX-2 with other known COX sequences revealed the high level of conservation within these molecules (Figure 5), including 12 conserved cysteines, two potential glycosylation sites, potential haem-binding domains (KALGH and TIWLREHN RV), an active-site tyrosine (Tyr-374), a distal haem-binding histidine (His-377) and the aspirin acetylation site [Ser-519 (= Ser-530 in the human)] present in both COX-1 and COX-2. The interaction between aspirin and this serine residue in human COX-2 leads to the formation of 15-(R)-hydroxy-eicosatetraenoic acid [36]. This is further converted into novel 15-epi-lipoxins in the presence of 5-lipoxygenase [37]. As fish macrophages are potent in the synthesis of lipoxins [38] it would be of interest to determine if such products are generated in LPS-activated GMCL in the presence of aspirin. Interestingly, the insertion at the 5'-end of the COX-1 molecules, within the signal peptide, is also missing from the trout COX-2 sequence, whereas the insertion at the 3'-end of known COX-2 sequences is present in the trout sequence. Within this extra COX-2 domain are one (trout) or two (chicken and mammals) additional potential glycosylation sites. Many amino acids (69 in total) in the trout sequence can clearly be considered COX-2 specific, including Val-512 (= Val-523 in human COX-2), Arg-502 (= Arg-513 in human COX-2) and Leu-492 (= Leu-503 in human COX-2). Indeed, replacement of Val-523 in human COX-2 by isoleucine significantly alters the behaviour of the enzyme to some inhibitors, including NS-398 [39]. Twelve amino acids are present in trout COX that would previously have been considered COX-1 specific, and several are shared between COX-1 and chicken/trout COX-2 (e.g. Trp-65, Lys-211, Ala-212, Glu-228, Glu-532, Leu-553). The overall homology between the trout COX-2 sequence and other known COX sequences is 83–84% for COX-2 and 77% for COX-1 genes. By comparison, the homology between the human and chicken COX-2 genes is 89–90%, while the homology of human and chicken COX-2 to known COX-1 genes is 77–78%. The predicted translation was also used to construct an unrooted phylogenetic tree (Figure 6). This showed a clear relatedness between the putative trout COX-2 sequence and other known COX-2 sequences.

RT-PCR

RT-PCR analysis revealed that trout COX-2 expression was not constitutive but could be induced after challenge *in vivo* with the bacterial pathogen *A. salmonicida*. Thus, head kidney leucocyte cDNA from control fish gave no products with trout COX-2-

Figure 4 Compiled full-length trout COX-2 sequence

Features in bold include the start and stop codons and the RNA instability motif (attta) in the 3'-UTR. The polyadenylation signal (aataaa) is in bold and underlined and potential glycosylation sites are underlined.

Figure 5 Alignment of the predicted trout COX-2 translation with other known mammalian and avian COXs

Identical (*) and similar [(single dot (.) or double dot (:)] residues identified by the CLUSTAL program are indicated. Features in bold include the conserved cysteines and glycosylation sites, potential haem-binding domains (KALGH and TIWLREHNRV), the active site tyrosine (Tyr-374), a distal haem-binding histidine (His-377) and the aspirin acetylation site (Ser-519). The position of the COX-2 signal peptide is indicated by a double line. Residues shared between trout COX-2 and either COX-1 or COX-2 sequences are boxed.

Figure 6 Unrooted phylogenetic tree showing the relationship between the trout COX-2 amino acid sequence with other known COX sequences

The tree was constructed by the 'neighbour-joining' method using the CLUSTAL W and PHYLIP packages, and was bootstrapped 10 000 times.

Figure 7 Amplification of products from rainbow trout head kidney leucocytes by RT-PCR

Products were amplified from control (lanes 2 and 3) or bacterially challenged (lanes 4 and 5) fish, using COX-2-specific (A) and β -actin-specific (B) primers. In lane 1 no cDNA was added. M represents molecular-mass markers as shown. Note the COX-2 product of 387 bp in (A) lanes 4 and 5 and the β -actin product of 541 bp in (B) lanes 2–5.

specific primers, although products of the correct size were obtained with β -actin primers, confirming that there had been no problem with reverse transcription (Figure 7). Head kidney leucocyte cDNA samples from fish challenged for 2 days with the attenuated strain of *A. salmonicida* gave products of the correct size with both COX-2 and β -actin primers.

Overall, the results of the present study demonstrate the existence of an inducible form of COX in fish that shares key sequence similarities with mammalian and avian [40] COX-2. This is the first study to confirm the presence of an inducible COX isoform in lower vertebrates such as fish. As these animals are the ancestors of some of the first vertebrates it suggests that this isoform arose at least 500 million years ago with the evolution of bony fish. It would be interesting to know if COX-2 is also expressed in different invertebrate phyla with much longer evolutionary histories.

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