Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase

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The enzyme cyclo-oxygenase catalyses the oxygenation of arachidonic acid, leading to the formation of prostaglandins. Recently two forms of cyclo-oxygenase have been described: a constitutive (COX-1) enzyme present in most cells and tissues, and an inducible (COX-2) isoenzyme observed in many cells in response to pro-inflammatory cytokines. Constitutive and inducible forms of human cyclo-oxygenase (hCOX-1 and hCOX-2) were cloned and expressed in insect cells, utilizing a baculovirus expression system. hCOX-1 had a specific activity of 18.8 μmol of O_2/mg with a K_m of 13.8 μM for arachidonate and $V_{max.}$ of 1500 nmol of O_2/nmol of enzyme, whereas hCOX-2 had a specific activity of 12.2 μmol of O_2/mg with a K_m of 8.7 μM for arachidonate and a $V_{max.}$ of 1090 nmol of O_2/nmol of enzyme. Indomethacin inhibited both hCOX-1 and hCOX-2, whereas NS-398 and Dup-697 selectively inhibited hCOX-2. Both NS-398 and Dup-697 exhibited time-dependent inactivation of hCOX-2, as did indomethacin on both enzymes. The competitive inhibitor of hCOX-1, mefenamic acid, also displayed competitive inhibition of hCOX-2. These results demonstrate the ability to generate selective non-steroidal anti-inflammatory drugs (NSAIDs), which could provide useful improvement therapeutically in the treatment of chronic inflammatory disease.

INTRODUCTION

The formation of prostaglandins (PGs) is initiated by the action of PG synthase, which catalyses two separate reactions, the first being the oxygenation of arachidonic acid to the unstable PGG_2 by a cyclo-oxygenase function and the second the further conversion of PGG_2 into the more stable PGH_2 by a peroxidase function. Hence, this 'cyclo-oxygenase' (COX) enzyme performs the critical initial reaction in the arachidonic metabolic cascade leading to the formation of the prostaglandins, thromboxane and prostacyclin (Needleman et al., 1986). Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit PG formation by inactivation of the COX enzyme (Smith and Willis, 1971; Vane, 1971; Vane and Bottin, 1987). Originally thought to be a single enzyme, COX was known to produce both pro-inflammatory PGs at the site of inflammation and homoeostatic PGs in tissues such as the stomach and kidney. These homoeostatic PGs are linked to normal gastric and renal function (Haynes, 1990).

Recently, a second form of the COX enzyme was isolated whose expression is inducible by cytokines and growth factors (COX-2) (Hla and Nielson, 1992; Jones et al., 1993; Kennedy et al., 1993; Kujubu et al., 1991; Xie et al., 1991). This inducible COX-2 is linked to inflammatory cell types and tissues, and is believed to be the target enzyme for the anti-inflammatory activity of the NSAIDs (Fu et al., 1990; Masferrer et al., 1992, 1994; Raz et al., 1988; Sano et al., 1992; Seboldt et al., 1990). NSAIDs currently available for clinical use inhibit both COX-1 and COX-2 (Meade et al., 1933; O'Neill et al., 1994). This suggests that clinically useful NSAIDs inhibit pro-inflammatory PGs derived from the activity of COX-2, as well as PGs in tissues such as the stomach and kidney (via COX-1). It is possible that a selective COX-2 inhibitor may eliminate the side effects associated with COX-1 inhibition while providing anti-inflammatory COX-2 inhibition.

Ram seminal vesicle has provided a rich source of COX-1 that allows the ready purification of the enzyme from the native sources (Hemler et al., 1976; Miyamoto et al., 1976; Van Der Ouderaa et al., 1977). Sufficient amounts of COX-1 have been purified from ram seminal vesicles for crystallography (Picot and Garavito, 1989) and other experiments utilizing large quantities of enzyme. On the other hand, a rich source of the native COX-2 enzyme has not been identified, although small amounts of the rat enzyme have been purified (Sirois and Richards, 1992).

The baculovirus expression system has been used to express a wide variety of proteins that are often enzymically, immunologically and functionally similar to their authentic counterparts (Luckow, 1991). The insect cell expression of sheep COX-1 has been reported (Shimokawa and Smith, 1992). We report the expression and purification of both human (h) COX-1 and COX-2 from insect cells, using recombinant baculoviruses. The availability of these purified enzymes has permitted us to evaluate selective inhibitors of COX-1 and COX-2.

MATERIALS AND METHODS

Materials

Arachidonic acid was purchased from Nu-Chek-Prep Inc. (Elysian, MN, U.S.A.); CHAPS, haemin chloride, *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) and Tris were pur-

Abbreviations used: COX, cyclo-oxygenase; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.

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chased from Sigma (St. Louis, MO, U.S.A.); all other reagents were purchased from Fisher Scientific. NS-398 [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide] was provided by Dr. John Talley, and Dup697 [5-bromo-2-(4-fluorophenyl)-3-(4-methylsulphonylphenyl)-thiophen] was provided by Dr. Len Lee, both of Monsanto Corporate Research, St. Louis.

cDNA cloning and expression

cDNA libraries were constructed from interleukin-1β+cycloheximide-treated human dermal fibroblast cells (HDF) for COX-2, and from retinoic acid-differentiated HL-60 cells for COX-1 (Honda et al., 1990; Raz et al., 1988). Briefly, HDF cells were stimulated for 2 h with interleukin 1β (1 ng/ml) and cycloheximide (10 μM) to induce the expression of hCOX-2 mRNA. Human HL-60 cells (A.T.C.C.) were cultured in the presence of 1 μM retinoic acid + 5 % (v/v) fetal-calf serum in RPMI medium for 3 days to induce hCOX-1 mRNA. First-strand cDNA for either hCOX-1 or hCOX-2 was synthesized from 5 μg of poly(A)$^+$ RNA by using Superscript II, followed by second-strand synthesis using DNA polymerase I and RNAase H. The ends of the double-stranded cDNA were flushed with T-4 DNA polymerase, and excess nucleotides were separated by using a Promega Magic DNA prep. *Eco*RI adaptors were ligated to the cDNA and treated with T-4 kinase before electrophoresis through a low-melting 0.8 % (w/v)-agarose gel. cDNA greater than 2 kb in length was eluted from the gel with Gene Clean (Bio 101) and ligated into Lambda-ZAP. The ligation reaction was packaged *in vitro* and used to infect XL-1 Blue cells.

Approx. 150000 recombinant plaques were screened by using hCOX-1 or -2 cDNA probes (kindly provided by Dr. T. Hla, American Red Cross, Bethesda, MD, U.S.A.). Positive clones were rescued into pBluescript vector by using X Assist helper phage and SOLR cells. cDNA clones encoding hCOX-1 and -2 were characterized by restriction analysis, and completely sequenced by dideoxy chain-termination using Sequenase. Sequencing reactions were analysed by using a Dupont Genesis 2000 DNA analysis system. COX cDNA fragments that contained only the coding sequence of each gene, with all 5′ and 3′ untranslated sequences being deleted, were cloned into baculovirus transfer vector pVL1393 for expression in insect cells.

Large-scale propagation of insect cells

A 10-litre Biostat ES fermenter (B. Braun Biotech; Allentown, PA, U.S.A.) was used for the large-scale propagation of cultured Sf21 insect cells. These cells were derived from the ovarian tissue of the fall armyworm *Spodoptera frugiperda* (Vaughn et al., 1977). The fermenter was configured for insect-cell culture by installation of a marine impeller and oxygen enrichment. Two 1.0-litre spinner cultures of Sf21 cells growing in ITF medium (IPL-41 supplemented with 10 % fetal-bovine serum, tryptose phosphate broth and 0.3 % Pluronic F-68) were used to inoculate the fermenter at an initial cell density of 2.5×10^5 cells/ml. Before infection, the cells were maintained at 27 °C, agitated at 200 rev/min, sparged with 30 %-O_2-enriched air at a flow rate of 100 ml/min with 2 lb/in^2 (13.8 kPa) fermenter back-pressure. When the cell density reached 5×10^5 cells/ml, the 10-litre culture was infected with virus stock at a multiplicity of infection = 0.1. Dissolved O_2 was maintained above 50 % of atmospheric O_2 by increasing O_2 enrichment and by increasing sparger airflow rates. Cells were harvested 72 h after infection at a final cell density of $(0.8–1.2) \times 10^6$ cells/ml. The cells were isolated by centrifugation at 4000 *g* for 15 minutes. Final cell paste (40–60 g wet cell wt./10 litres) was stored at −80 °C before purification.

COX purification from infected insect cells

Cells (40–60 g wet wt.) were thawed and suspended in 10 vol./wet wt. 25 mM Tris/HCl (pH 8.1)/0.25 M sucrose. The pellet was collected by centrifugation at 10000 *g* for 20 min and resuspended in the same buffer. CHAPS was added to 1 % (w/v), from a 10 % stock solution, and the mixture was stirred for 2 h at 4 °C. The supernatant was removed after centrifugation at 50000 *g* for 20 min and passed through a 3 μm-pore filter. The supernatant was loaded directly on to a 50 ml anion-exchange column (Macro-Prep High Q; Bio-Rad, Richmond, CA, U.S.A.), equilibrated with 25 mM Tris/HCl (pH 8.1)/0.4 % CHAPS. COX was eluted with a linear gradient of increasing salt concentration to 0.3 M. Fractions were assayed for peroxidase activity and pooled. The pool was concentrated 10-fold with a stirred cell (Amicon) YM-30 membrane. A 10 ml portion of the concentrate was loaded on a 500 ml (2.6 cm × 94 cm) Sephacryl S-200 (Pharmacia LKB) size-exclusion column, equilibrated with 25 mM Tris/HCl (pH 8.1)/ 150 mM NaCl/0.4 % CHAPS. Fractions were assayed for peroxidase activity and pooled. Molecular masses were determined by measuring elution volume versus the log of the molecular mass of the following standards: aprotinin (6.5 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), BSA (67 kDa), α-amylase (200 kDa) and apoferritin (443 kDa). For COX-1, the size-exclusion-column pool was diluted 4-fold with 25 mM Tris/HCl/0.4 % CHAPS, and the anion-exchange-column chromatography was repeated on an 8 ml Mono-Q column (Pharmacia) with the same conditions described above. Pools were divided into portions and stored at −80 °C for further use.

Characterization methods

Separation of the samples by SDS/PAGE was performed by the method of Laemmli (1970), using an 8 cm × 10 cm 10–20 % gradient gel and stained with Pro-Blue (ISS, Hyde Park, MA, U.S.A.). A 2 μg portion of each sample was loaded after boiling in reducing sample buffer. Molecular-mass standards were from Sigma and used at 4 μg per band. Total protein concentration was determined by the method of Bradford (1976) with BSA as a standard. N-terminal sequencing was performed on an Applied Biosystems (Foster City, CA, U.S.A.) model 470 A gas-phase sequencer, with respective amino acid phenylthiohydantoins derivatives being identified by reversed-phase h.p.l.c. Amino acids composition was determined with a Beckman model 6300 ninhydrin-based system, after 6 M HCl vapour-phase hydrolysis.

Enzyme assays

COX enzyme assay *in vitro*

Expression of COX protein in insect cells was determined by assessing PG-synthetic capability in homogenates from cells incubated for 3 days with COX-1 or COX-2 baculovirus. Cells expressing COX-1 or COX-2 were homogenized as previously described (Needleman et al., 1977) and incubated with arachidonic acid (10 μM). COX activity was determined by monitoring PG production. No COX activity was detected in mock-infected Sf9 cells. Commercial NSAIDs, NS-398 or Dup697 (0.001–100 μM), were preincubated with crude 1 % CHAPS homogenates (2–10 μg of protein) for 10 min before addition of arachidonic acid. PGE_2 formed was detected by e.l.i.s.a. (Caymen), after a 10 min incubation.

Peroxidase assay

Purification was monitored by using a peroxidase assay adapted to a 96-well plate format, using conditions previously described (Kulmacz and Lands, 1987), which can be summarized as follows: 0.1 M Tris/HCl pH 8.1, 100 μM arachidonic acid, 170 μM TMPD, 1 μM haem (bovine haemin chloride in dimethyl sulphoxide), 0–2 μg of purified enzyme. The A_{590} was measured after 5 min at room temperature. One unit is defined as the amount of enzyme necessary to generate a change of 1 A_{590} unit during a 5 min incubation.

O_2-uptake assay

The COX activity of PGH_2 synthase was assayed by the direct measurement of O_2 consumed. The instrument used was an Instech Laboratories (Horsham, PA, U.S.A.) model 203 oxygen sensor, which features a Clark-style polarographic electrode in a 600 μl reaction vessel with constant stirring. Assay conditions were as follows: 0.1 M Tris/HCl, pH 8.0, 500 μM phenol, 1 μM haem, 100 μM arachidonic acid, 37 °C, 0–20 μg of protein. The assay was initiated by addition of arachidonate; rate measurements were taken in the linear portion of the assay (the first 10–15 s). For IC_{50} determinations, inhibitors to be tested were incubated with 200 nM enzyme for 1 min before addition of arachidonate.

RESULTS

Cloning and expression of hCOX-1 and hCOX-2

Poly(A)$^+$ RNA was isolated from HDF cells grown for 2 h in the presence of interleukin-1β and cycloheximide for construction of a cDNA library as a source of a hCOX-2 cDNA. A second cDNA library was synthesized from poly(A)$^+$ RNA from retinoic acid-differentiated HL-60 cells to obtain a cDNA for hCOX-1. Both libraries were constructed from 5 μg of poly(A)$^+$ RNA, and contained approx. 300000 recombinant plaques: 150000 plaques were screened from the HL-60 and HDF libraries for hCOX-1 and hCOX-2 respectively.

Screening of the HL-60 library resulted in the identification of six plaques that hybridized positively to the hCOX-1 cDNA probe. Two of the six plaques remained positive through subsequent rounds of hybridization. One of the positive clones (pMON 23950) contained a cDNA insert of 2600 bp, and sequence analysis confirmed the identity as the homologue of the published human COX-1 (Yokoyama and Tanabe, 1989). The cDNA includes 31 bp of 5′ untranslated sequence, an open reading frame coding for 599 amino acids, and 750 bp of 3′ untranslated sequence, including the polyadenylation signal AATAAA. The peptide sequence differs from the published sequence at only one position, a proline at residue 17 instead of leucine. This substitution occurs within the putative signal peptide of hCOX-1.

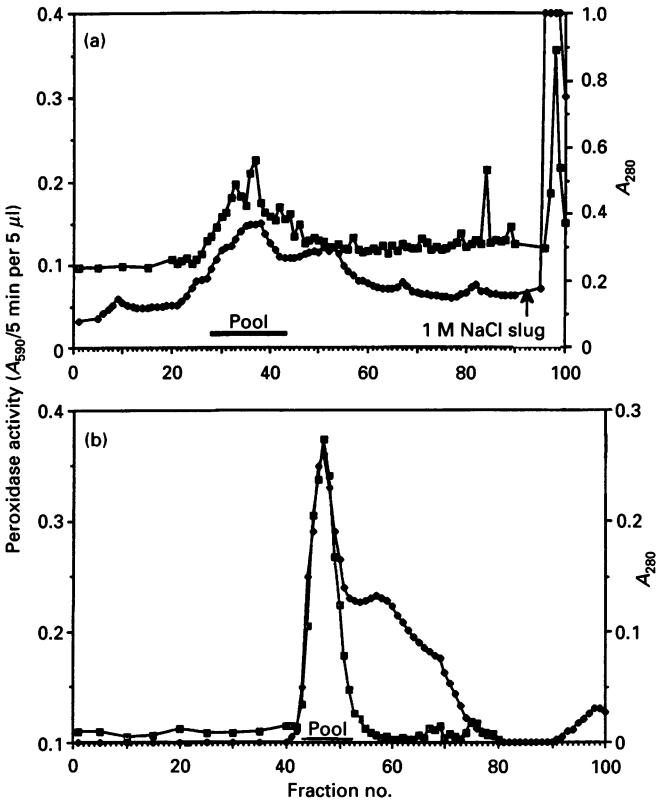

Figure 1 Purification of human COX-2

COX enzyme was purified to homogeneity by extraction with 1% CHAPS, followed by anion-exchange (**a**) and size-exclusion chromatography (**b**). The A_{280} and peroxidase activity with arachidonate as substrate are plotted: fractions pooled are indicated.

Screening of the HDF cDNA library with a hCOX-2 cDNA probe (150000 plaques) resulted in 330 positive clones. Of these, 12 clones were chosen at random and carried through two additional rounds of screening; 11 cDNA clones remained positive by virtue of hybridization. Two clones with insert sizes of 1.9 kb and 2.1 kb were mapped by restriction analysis and shown to be overlapping hCOX-2 cDNAs. The longer clone contained 97 bp of 5′ untranslated sequence, as well as the entire coding sequence followed by several hundred bases of 3′ untranslated sequence. The shorter clone isolated was a truncated cDNA that contains the 3′ end of the coding sequence, and the entire 3′ untranslated sequence, including a poly(A) tail. The complete nucleotide sequence contains 3371 bp. The deduced

Table 1 Human COX-2 purification table

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Activity purification (fold)	Yield (%)
CHAPS extract	1001	13968	14		
Anion-exchange chromatography	58	8990	155	11	64
Size-exclusion chromatography	29	6840	226	16	46

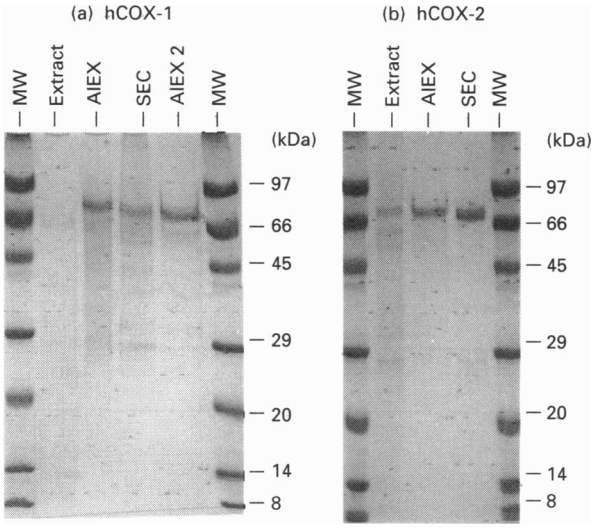

Figure 2 Purification of human COX-1 (a) and COX-2 (b)

Portions (2 μg) of total protein from each step were loaded on to a polyacrylamide gel and, after electrophoresis, stained with colloidal Coomassie Blue. Steps indicated are as follows: CHAPS detergent extract, anion-exchange pool (AIEX), size-exclusion pool (SEC) and anion-exchange step 2 (MW, molecular-mass markers).

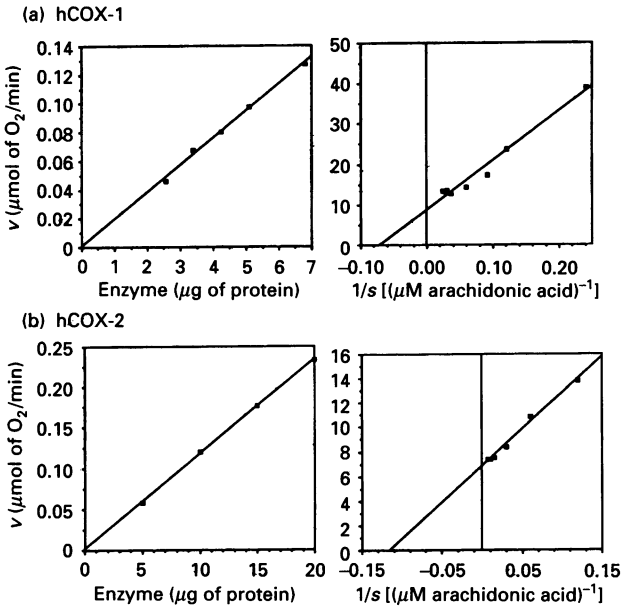

Figure 3 O_2-uptake assay of hCOX-1 (a) and hCOX-2 (b)

Dose/response curves, with rate of O_2 consumed measured for varying amounts of enzyme (2–20 μg) at 100 μM arachidonate, and Lineweaver–Burk plots, constructed with 200 nM enzyme and 4–100 μM arachidonate.

sequence of hCOX-2 differs from the published hCOX-2 sequences at either two positions (Hla and Nielson, 1992), a glutamine to lysine at residue 58, and glycine to glutamine at 165 or at only amino acid 58 (Jones et al., 1993). The cDNAs for hCOX-1 and -2 were then engineered for expression in insect cells.

Purification and characterization of hCOX-1 and hCOX-2

The purification of human COX-2 is summarized in Table 1. Cells from 10 litres of fermentation yielded 46 g wet wt. of cells, which after extraction with CHAPS yielded 1001 mg of total protein. Anion-exchange chromatography produced an 11-fold purification and 64% yield. The chromatogram indicates multiple forms of the enzyme, with three or more major peaks being eluted between 100 and 150 mM NaCl (Figure 1a); the major peak of peroxidase activity was pooled and concentrated. Size-exclusion chromatography provided an additional 1.45-fold purification with a 72% step yield. The elution volume from the size-exclusion column indicates a molecular mass of approx. 150 kDa, which is the molecular mass of dimer COX (Figure 1b). This process yielded 29 mg of purified protein with a 16-fold purification and total yield of 46%. The protein was shown to be > 90% pure by reducing SDS/PAGE, with an apparent molecular mass of 72 kDa (Figure 2a). N-terminal sequencing produced a major signal of ANPCCSHPCQ....., which is the correctly processed form beginning at residue 18 of the coding sequence. Quantification of total protein by the Bradford (1976) method was within 1% of that obtained from the amino acid composition. Purified COX-2 has a specific activity in the O_2 assay of 12.2 μmol of O_2/min per mg of protein (Figure 3b), with a K_m for arachidonate of 8.7 μM and a $V_{max.}$ of 1088 nmol of O_2/nmol of protein.

The purification of COX-1 is summarized in Table 2. A 10-l fermentation yielded 66 g wet wt. of cells, from which 2.3 mg of purified COX was produced with an overall yield of 75% and a 246-fold purification. The protein was shown to be > 80% pure by SDS/PAGE, with an apparent molecular mass of 72 kDa (Figure 2b). N-terminal sequencing revealed a major signal of AXPGAPTPVN....., which is the correctly processed form of the enzyme beginning at residue 25 of the coding sequence. The aspartic acid at the second position in the coding sequence could not be seen, due to a buffer artifact. Quantification of total protein by the Bradford (1976) method was within 1% of that obtained by amino acid composition. Purified COX-1 has a specific activity in the O_2 assay of 18.8 μmol of O_2/min per mg of protein, with a K_m for arachidonate of 13.8 μM and a $V_{max.}$ of 1519 nmol of O_2/nmol of protein (Figure 3a).

Pharmacological manipulation of hCOX-1 and hCOX-2

When either hCOX-1 or hCOX-2 was incubated with arachidonic acid and then assayed for PGE_2 production, it was found that indomethacin inhibited both forms of the enzyme, with an IC_{50} of approx. 100 nM for hCOX-1 and 900 nM for hCOX-2 (Table 3). Likewise, several commercially available NSAIDs were examined, and inhibited both hCOX-1 and hCOX-2 *in vitro*. These results differed significantly from the inhibitory profile of either NS-398 or Dup-697, which both demonstrated selectivity for the hCOX-2 enzyme (IC_{50} 0.1 and 0.01 μM respectively) versus the hCOX-1 enzyme (> 100 and 0.8 μM respectively) (Table 3). The selectivity of NS-398 and Dup-697 for the COX-2 enzyme was confirmed when COX activity was measured directly by O_2 consumption (Figure 4). With 200 nM of the purified enzyme preparation in the presence of 100 μM arachidonic acid, both NS-398 and Dup-697 inhibited hCOX-2 (IC_{50} 1.6 and 0.2 μM respectively) with greater potency compared with hCOX-1 (IC_{50} > 250 and 4.5 μM respectively), whereas indomethacin inhibited both forms of the enzyme (IC_{50}: COX-1, 1 μM; COX-2, 10 μM). As previously reported, indomethacin showed some selectivity for inhibition of hCOX-1 versus hCOX-2 (Meade et al., 1993; O'Neill et al., 1994).

Table 2 Human COX-1 purification table

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Activity purification (fold)	Yield (%)
CHAPS extract	792	858	1.1		
Anion-exchange chromatography (AIEX)	133	982	7.4	6.7	114
Size-exclusion chromatography	35.5	768	21.6	20	90
Second AIEX	2.3	645	280	246	75

Table 3 Selective pharmacology of NSAIDs

NSAIDs (0.001–100 μM) were preincubated with membranes containing COX-1 or COX-2 before addition of arachidonic acid (10 μM) for 10 min. COX activity was measured as PGE_2 formed/min per mg.

IC_{50} (μM)	hCOX-1	hCOX-2
Indomethacin	0.1	0.9
Naproxen	1.1	36
Fluribiprofen	0.1	0.4
Flufenamic acid	2	29.5
Mefenamic acid	0.04	3
Ketoprofen	7.5	7.6
Ibuprofen	3.3	37.5
Piroxicam	13	> 100
Diclofenac	0.04	0.1
NS-398	> 100	0.1
Dup-697	0.8	0.01

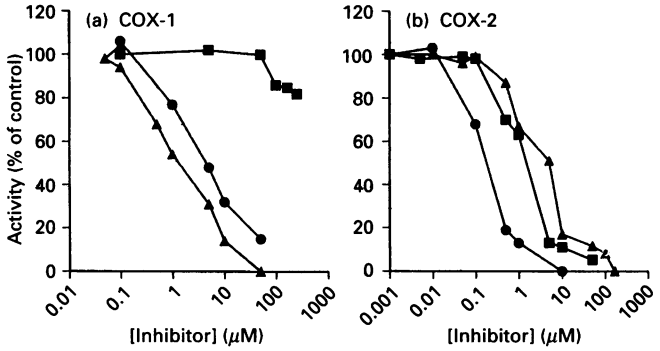

Figure 4 Dose/response curves for inhibition by indomethacin and selective COX-2 inhibitors of COX-1 (a) and hCOX-2 (b)

Indomethacin (▲), NS-398 (■) and Dup-697 (●) (1.0–250 μM) were incubated with 200 nM enzyme for 1 min before addition of 100 μM arachidonate. O_2 uptake activity was then measured.

Inhibition of hCOX-2 by indomethacin, NS-398 or Dup-697 increased with time after preincubation of the enzyme with inhibitor before addition of arachidonic acid, as measured by the O_2-consumption assay (Figure 5). We found that 166 μM indomethacin, 1 μM NS-398 and 0.1 μM Dup-697 inhibited hCOX-2 activity by 20–30% at 5 s and by 70–80% after 1 min. Mefenamic acid at 5 μM showed no such time-dependence, with

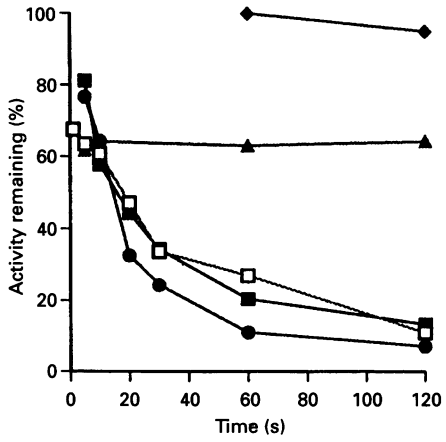

Figure 5 Time course for inactivation of human COX-2 by NSAIDs

Indomethacin (■; 166 μM), NS-398 (●; 1 μM), Dup697 (□; 0.1 μM) and mefenamic acid (▲; 5 μM) were incubated with 200 nM COX-2 for 5–120 s, followed by the addition of arachidonic acid (◆, control). Initial rates were plotted against time of incubation.

35% inhibition of hCOX-2 either at 5 s or after 2 min of preincubation with inhibitor before addition of arachidonic acid.

DISCUSSION

The critical step in the formation of pro-inflammatory PGs is the oxygenation of arachidonic acid by COX enzyme. Recently two forms of COX enzyme have been identified: COX-1, which is constitutively expressed in many tissues, and COX-2, which is associated with pro-inflammatory PG production in several tissues and cell types. Analysis of mRNA from normal tissues in the rodent indicate that COX-1 mRNA is found in nearly every tissue examined, including stomach, kidney, heart, brain, liver and spleen, whereas COX-2 mRNA was nearly undetectable in these normal tissues (Seibert et al., 1994). Conversely, in models of inflammation (e.g. carrageenan-induced rat paw oedema and granuloma pouch) oedema is produced that correlates with a significant induction of COX-2 mRNA and protein (Masferrer et al., 1992; Seibert et al., 1994). The identification of an inducible form of the COX enzyme led us to the hypothesis that selective inhibition of COX-2 would be anti-inflammatory without the gastric toxicity associated with NSAIDs that are at present available clinically. Indeed, we have recently reported that NS-398 selectively inhibits murine COX-2 enzyme activity, and that

it is anti-inflammatory *in vivo* without gastric toxicity (Masferrer et al., 1994).

Insect-cell expression of the human COX-1 and COX-2 clones provided a rich source of active enzyme for purification and for pharmacological evaluation of potentially selective compounds. Initial experiments to determine extraction conditions indicated that CHAPS was at least as good as Tween-20 and Nonidet P-40 and superior to octyl glucoside. CHAPS was chosen because it is easily dialysable and exchanged with other detergents. Western-blot analysis of COX-1 and COX-2 extracts indicates a significant portion of immunoreactive protein not extracted (results not shown). COX-2 was extracted approx. 10-fold more efficiently than COX-1, as borne out by assays of cell extracts, Western-blot analysis and purification yields. The reason for this difference is unclear, although one explanation lies in the difference in the signal sequence of COX-2 from that of COX-1, which might affect insertion into the membrane. Purified recombinant COX-1 has approximately the same specific activity as reported for ram seminal vesicle COX-1 (Smith and Marnett, 1991). As with the purified sheep seminal-vesicle COX-1 enzyme, addition of haem was necessary for activity, with O_2 uptake increased 20-fold by addition of 1 μM haem.

None of the commercially available NSAIDs tested demonstrated any significant selectivity for COX-2; indomethacin actually appeared somewhat more potent in its ability to inhibit human COX-1 that human COX-2, in agreement with similar observations using murine recombinant enzyme (Meade et al. 1993). We report that both NS-398 and Dup-697 selectively inhibit human COX-2, with significantly less ability to inhibit COX-1. This is consistent with their reported anti-inflammatory activity in rats without gastric toxicity (Futaki et al., 1993a,b; Gans et al., 1989). Mechanistically, the commercially available NSAIDs fall into two categories: those that inhibit COX-1 reversibly (e.g. mefenamic acid) and those that demonstrate an irreversible time-dependent inhibition of activity *in vitro* (e.g. indomethacin) (Kulmacz and Lands, 1985; Rome and Lands, 1975). This study demonstrates that indomethacin, in addition to the two COX-2-selective compounds (NS-398 and Dup-697), also inhibit COX-2 in an irreversible time-dependent manner, whereas the reversible inhibitor mefenamic acid does not. Recently the three-dimensional structure for COX-1 was solved (Picot et al., 1994); availability of the crystal structure for COX-2 along with selective inhibitors should allow for greater understanding of the interaction of inhibitors with the active site and their mechanism of inhibition.

The potential clinical advantage of a selective COX-2 inhibitor is unquestioned. Current NSAID therapy, albeit anti-inflammatory, is often limited in its usefulness due to mechanism-based toxicities of these drugs, especially gastrointestinal lesions resulting from inhibition of stomach and intestinal PG formation (Allison et al., 1992). Development of a selective COX-2 inhibitor may represent a major advance in the treatment of patients with acute and chronic inflammatory disorders. Availability of purified COX-1 and COX-2 enzyme will allow further mechanistic and pharmacological evaluation of these closely related enzymes, positively influencing the development of additional safe and effective COX-2 inhibitors.

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