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THE HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF HUMAN PROSTAGLANDIN G/H SYNTHASE-2 (COX-2)

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The open reading frame of human cyclooxygenase-2 was cloned by pcr amplification of IL-1ß stimulated human dermal fibroblast cDNA. The coding region was used to construct a recombinant baculovirus which when used to infect Sf9 cells directed the expression of recombinant human cyclooxygenase-2. The heterologously expressed enzyme was characterized and found to display all salient features of cyclooxygenase. Large-scale microsomal preparations of infected cells yielded more than 20 units of enzyme with a specific activity of 240 nmoles prostaglandin product/mg protein.

Recently, the characterization of an inducible cyclooxygenase (Prostaglandin G/H Synthase-2, COX-2) (1-4) has rekindled interest in the regulation and inhibition of prostaglandin biosynthesis. This discovery clarifies previous observations in the field, notably the fact that dexamethasone can reduce prostaglandin production in cultured cells without affecting COX-1 protein levels (5,6). Most importantly, the existence of a cyclooxygenase induced by proinflammatory factors such as IL-1ß (7) and TNFα (8) suggests that cyclooxygenase activity performs two physiologically distinct functions: first, that the constitutive cyclooxygenase (COX-1) fulfills "housekeeping" functions by forming cyclooxygenase products involved in cellular homeostasis; and second, that an inducible form of the enzyme (COX-2) provides proinflammatory amounts of cyclooxygenase products during an inflammatory response.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin ameliorate many aspects of arthritis and other diseases, but their deleterious side effects (*e.g.*, gastric ulceration and renal in-

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Abbreviations: COX, cyclooxygenase; MMLV, moloney murine leukemia virus; m.o.i., multiplicity of infection; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; TMPD, N,N,N',N'-tetramethylphenylenediamine; 15-HETE, 15-hydroperoxyeicosatetraenoic acid; KLH, Keyhole Limpet Hemocyanin.

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sufficiency) are significant. The model presented above ascribes the beneficial effects of NSAIDs to the inhibition of the inducible form of the enzyme and their untoward effects to inhibition of the constitutive isoenzyme. If this hypothesis is true, selective inhibitors of COX-2 should provide the benefits of NSAIDs with minimized side effects. Following this idea, Meade and coworkers (9) found that, while most NSAIDs show little selectivity for either murine COX-1 or COX-2, some inhibitors show modest selectivity for COX-1 and only one, 6-methylnaphthylacetic acid, preferentially inhibits COX-2 over COX-1.

In order to characterize human COX-2 and its inhibitors more thoroughly, we sought to produce large amounts of the enzyme. Because the enzyme is a membrane glycoprotein, we initially attempted heterologous gene expression in mammalian cells, but found that many commonly used host cells harbor significant endogenous cyclooxygenase activity. The heterologous expression of COX-2 in insect cells was therefore pursued. In this manuscript we describe the preparation of a recombinant baculovirus directing the expression of human COX-2 and demonstrate that the protein expressed displays all key characteristics of COX-2, including localization in the membrane fraction, heme dependence, indomethacin sensitivity, and aspirin-induced shifts in product formation.

MATERIALS AND METHODS

Cloning of COX-2. The open reading frame of COX-2 was cloned by the polymerase chain reaction. We prepared cDNA from IL-1ß stimulated human dermal fibroblasts using MMLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and oligo-dT priming, then used the following primers to isolate the open reading frame of COX-2 as two overlapping fragments: primer 27: 5'-CTTCAGCTCCACAGCCAGA C-3'; primer 1047: 5'-CAAGCAGGCTAATACTGATAGG-3'; primer 1373(-): 5'-CATTCCTACCACCAGCAACC-3'; primer 1914(-): 5'-TTCTACAGTTCAG TCGAACG-3'.

Approximately 1 µg of cDNA was combined with primers 27 and 1373(-) or with primers 1047 and 1914(-) and was subjected to 32 cycles (1 min at 94°C, 1 min at 55°C, 2 min at 78°C) of amplification using Pfu I polymerase (Stratagene, LaJolla,CA). We isolated PUC19 clones of each fragment identical to the sequence published by Jones and coworkers (10). A unique BspHI restriction site at 1247 provided a means to construct the entire coding region.

Recombinant Baculovirus Preparation. The COX-2 coding region was ligated into pVL941. This construct was cotransfected with Baculogold AcMNPV dna (Pharmingen, San Diego, CA) into Sf9 cells. After 4 days, the supernatant was collected as the primary viral stock. From this stock, a high-titer clonal virus (BVCOX-2) was prepared by established procedures (11).

Western Blot of Recombinant Human COX-2. Infected cells from 100 mm dishes were collected and pelleted. The pellet was solubilized in 200 mL lysis buffer (1 % NP-40 and 5 mM EDTA in PBS) on ice. The lysates were run on a 10% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA), then were electrophoretically blotted to nitrocellulose. The nitrocellulose membrane was blocked for 15 min in 5 % powdered milk in PBS and then was manipulated as described in the instructions for the ECL Western Kit (Amersham, Arlington, IL). COX-2 was detected using used a high-titer rabbit antisera raised against the amino acid sequence CINASSSRSGLDDINPTVLLDE (COX-2-I^{579} to E^{598}, Cys extended) coupled to KLH protein.

Small-Scale Expression of COX-2 in Sf9 Cells. We plated 3 x 10^6 Sf9 cells into each of twenty five 100 mm tissue culture dishes containing 7 mL Graces medium (Gibco-BRL) supplemented with 10 % FCS. After 30 min, 100 mL of virus stock (1.4 x 10^8 pfu/mL) was added to each plate. The infected cells were incubated at 27°C for 48 hr, then were collected and counted (1.04 x 10^8 cells).

For time-course studies, multiple plates were prepared as described above and were harvested daily for analysis by Western blotting. To determine the effect of tunicamycin on COX-2

glycosylation, several plates were prepared and were grown in medium supplemented with 0, 0.5, 2.0, or 5.0 μg/mL tunicamycin (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Cellular Fractionation. Infected cells from a 250 mL culture were pelleted by centrifugation, then were resuspended in 14 mL Buffer A (100 mM $KHPO_4$, pH 7.2, 0.3 mM diethyldithiocarbamate, 1 mM EDTA). The suspension was sonicated 3 x 2 sec on ice, and the sonicates were spun at 10,000x*g* for 10 min in a polypropylene centrifuge tube. The pellet was resuspended in 5 mL Buffer A, was homogenized in a Dounce homogenizer, and was stored at -80°C as the 10,000x*g* pellet fraction. The supernatant was then spun at 200,000x*g* for 45 min. The high-speed supernatant was collected, and the precipitated microsomes were resuspended in 10 mL Buffer A and were homogenized on ice.

Large-Scale Preparation of Microsomes. Sf9 cells (1L) in log-phase were infected with recombinant virus at an m.o.i. between 1 and 5. After 72 hr, microscopic examination of a culture sample revealed that most of the insect cells appeared infected. The cells were transferred to conical bottles and pelleted at 4°C. The cell pellet was resuspended in 30 mL Buffer A, and the cells were sonicated 3 x 5 sec on ice. The lysate was spun at 1,000x*g* for 10 min, and the pellet was discarded. The supernatant was then spun at 150,000x*g* for 45 min at 4°C. The supernatant was discarded, and the waxy pellet was resuspended in 20 mL Buffer A and was homogenized for 1 min (30 strokes) on ice in a Dounce homogenizer with a tight-fitting pestle. The microsomes were aliquoted in 2 mL fractions and stored at -80°C.

Cyclooxygenase Activity. The enzyme preparation was reconstituted with 10 μM hematin on ice for 15 min. An aliquot (50 μL) of this preparation was added to 0.4 mL reaction buffer (0.1 M TrisCl (pH 7.5), 2 mM phenol and 2 mM glutathione) and incubated at room temperature for 5 min with shaking. In certain experiments, the microsomal suspension was preincubated with 3 mM aspirin or 0.1 mM indomethacin for 5 min. Addition of [^{14}C]-arachidonic acid (50 or 100 μM, final concentration) started the reaction. The arachidonic acid substrate was prepared by mixing the ethanolic solutions of the radioactive arachidonic acid (New England Nuclear, Boston, MA) with unlabelled arachidonic acid (Biomol, Springhouse, PA) and then diluting with the reaction buffer. A constant amount of radioactivity (200,000 dpm) was added to each incubation. The addition of 0.2 mL cold 0.1 N HCl and cooling on ice stopped the reaction. [^{3}H]PGE_2 (10,000 dpm)(New England Nuclear, Boston, MA) was added to the incubation mixture immediately before stopping the incubation to correct for extraction recovery. The cold reaction mixture was extracted twice with 1 mL fractions of ethyl acetate. The combined organic layers were dried with Na_2SO_4 and then concentrated in a stream of dry nitrogen. The extract was spotted on a Whatman LK6D plate and was developed using the organic phase of ethyl acetate: isooctane: water: acetic acid (11:5:10:2). The radioactive bands were quantitated on an AMBIS Scanner (Ambis Systems, San Diego, CA). Products were identified by comigration with commercial standards (Biomol, Springhouse, PA).

In studies comparing inhibitor potencies, one minute incubations were performed using 10 μM arachidonic acid as described above. Inhibitors were dissolved in DMSO with the final DMSO concentration being 1% in the incubation. Microsomal COX-2 (50 μg) was added to start the reaction. After stopping the incubation with HCl, we measured PGE_2 using [^{125}I]-PGE_2 and an anti-PGE_2 antibody (PerSeptive Diagnostics, Cambridge MA) (12). A parallel experiment was performed using [^{14}C]-arachidonic acid as substrate. The products were extracted and analyzed as described above.

Peroxidase Activity. Peroxidase activity was measured in a colorimetric assay with a dual-beam spectrophotometer (SLM/Aminco DW 2000) using N,N,N',N'-tetramethylphenylenediamine (TMPD)(500 μM) and H_2O_2 (500 μM) as substrates as described by Kulmacz (13).

Materials. Sheep COX-2 was purchased from Cayman Chemical Co. (Ann Arbor, MI). BW 755C was prepared by CIBA Chemistry Department. ETYA was purchased from Biomol (Biomol, Springhouse, PA). Unless otherwise specified, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

We performed Western blots to determine the optimal time after infection to harvest COX-2. As shown in Figure 1, the recombinant baculovirus human COX-2 gave an intense band of immune reactivity that co-migrated with sheep COX-2. Preimmune serum failed to detect this band. The band was not detected in uninfected cells or in cells infected by a recombinant baculovirus with the COX-2 coding sequence in an antisense orientation. Expression of COX-2 protein peaked at 48 hr after infection and remained constant through 120 hr. The COX-2 peroxidase activity followed a similar time course (data not shown). For subsequent work, cells were harvested at 72 hr, and the enzymatic activity was assessed.

Cyclooxygenase activity of subcellular fractions from infected Sf9 cells was assayed using a radiotracer extent assay. We found 60% of the cyclooxygenase activity in the 10,000x*g* pellet and 40% in the 200,000x*g* fraction. No activity was observed in the 200,000x*g* supernatant. The activity of 10,000x*g* fraction was 2.1 μmoles PG (extent) in 12 mg total protein, while the 200,000x*g* fraction contained 1.2 μmoles PG in 12 mg total protein. The distribution of COX-2 activity coincides with that reported by Shimokawa and Smith for the expression of COX-1 in a baculovirus/Sf9 expression system (14). For subsequent work we prepared a membrane fraction from the infected Sf9 cells by centrifuging the 1,000x*g* supernatant for 1 hr at 100,000x*g* and collecting this pellet.

The product profile for the recombinant human COX-2 activity is shown in Figure 2A. The identity of the radioactive metabolites was determined by the comigration of prostaglandin standards in adjacent lanes. As indicated, the major products were PGF_2, PGE_2, PGD_2 and a metabolite with an R_f of 0.9 which comigrates with PGH_2. This latter metabolite represented 50% of the total prostanoid products. COX-2 microsomes pretreated with 100 μM indothemacin formed none of the

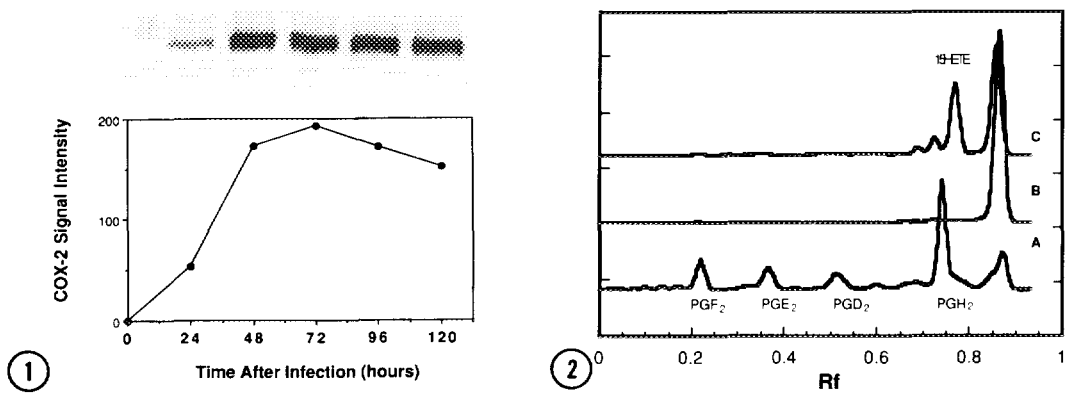

Figure 1. Time course of recombinant human COX-2 expression in Sf9 cells. Equal volumes of cell lysates were subjected to Western blotting using an antipeptide antibody specific for COX-2 at various times after infection by BVCOX-2.

Figure 2. Prostaglandin formation by recombinant human COX-2. COX-2 (200 μg) was incubated with 50 μM [^{14}C]-arachidonic acid for 5 min. Standard prostaglandins were co-chromatographed in adjacent lanes. (A) COX-2 was preincubated for 5 min with 1% ethanol carrier before the addition of substrate. (B) COX-2 was preincubated with 100 μM indomethacin for 5 min before the addition of substrate. (C) COX-2 was preincubated with aspirin (3 mM) for 5 min before the addition of substrate.

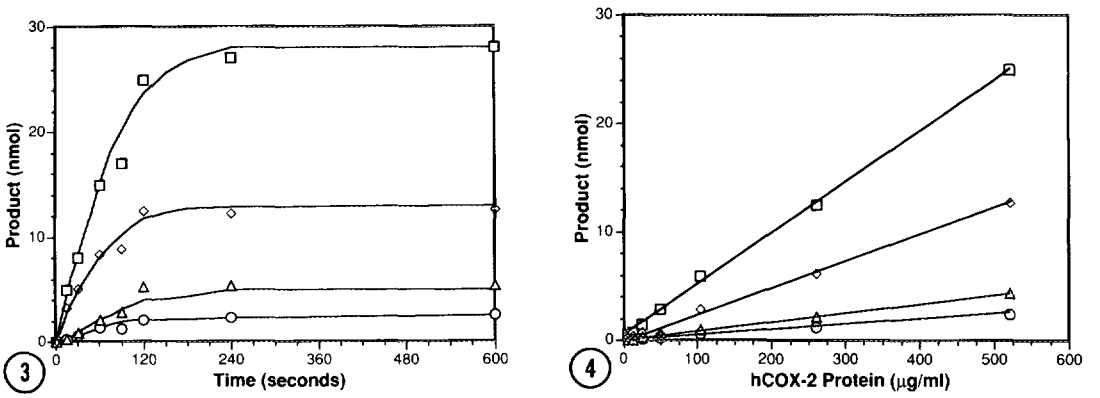

Figure 3. Time course for the formation of products by recombinant human COX-2. Microsomal preparation of COX-2 (100 µg protein) is incubated with 100µM [^{14}C]-arachidonic acid. Product analysis (PGF_2, O; PGE_2, Δ; PGH_2, ◇; total prostaglandins, □) is performed by quantitation of the radioactivity for each of the peaks on the TLC plate.

Figure 4. The dependence of protein levels on product formation by recombinant human COX-2. Microsomal preparation of COX-2 is incubated with 100 µM [^{14}C]-arachidonic acid for 5 min. Product analysis (PGF_2, O; PGE_2, Δ; PGH_2, ◇; total prostaglandins, □) is performed by quantitation of the radioactivity for each of the peaks on the TLC plate.

prostanoids (Figure 2B). Enzyme not reconstituted with heme or acid-treated before the addition of arachidonic acid showed no cyclooxygenase activity. Pretreatment with 3 mM aspirin caused a disappearance of PGF_2, PGE_2, and PGD_2, reduction in PGH_2, and appearance of a new product which comigrated with 15-HETE (Figure 2C). This "aspirin shift" in product formation appears to be a unique characterisitic of COX-2 (9,15).

Prostaglandin production followed the typical time course (16), increasing over the first 60 sec and reaching a plateau by 2 min (Figure 3). The extent of synthesis at 5 min, instead of velocity, was selected as a measure of COX-2 levels since reaction extent represents a reliable index of cyclooxygenase protein level in a heterogeneous matrix (15). Activity increased in a linear manner with protein concentration between 0.05 and 0.5 mg protein/mL assay (Figure 4).

In addition to displaying a cyclooxygenase activity, COX-2 possesses a peroxidase activity. The peroxidase activity in the microsomal preparations increased as a function of both H_2O_2 and TMPD concentration. Optimal peroxidase velocity was observed with 0.5 mM H_2O_2 and 0.5 mM TMPD (data not shown). Our findings with the recombinant human COX-2 are similar to those previously reported for ovine seminal vesicle COX-1(13).

These results confirmed that the radioactive products were dependent on cyclooxygenase activity. The formation of the high R_f metabolite (PGH_2) was not seen with ovine microsomal COX-1 or -2 (data not shown); the higher proportion of PGH_2 from enzyme expressed in insect cells may be due to lesser amounts of PGH_2 isomerases in the insect cell microsomes. Comparing the reaction products, we find that the ratio of the individual metabolites to the total products formed remained constant after 60 sec incubation (Figure 3). PGF_2, PGE_2 and PGH_2 represented 10%, 20% and 50%, respectively, of the total prostaglandin products. PGD_2 production was equivalent to PGE_2 production (data not shown). The proportions also remained constant as total protein concentration increased, PGE_2 and PGH_2 being 20% and 50%, respectively, of total prostanoids (Figure 4).

Table 1. Inhibition of recombinant human COX-2 by NSAIDs. A comparison between analytical techniques to measure product formation. Microsomal preparation of COX-2 (100 μg protein) was incubated with 10 μM of either unlabeled or [^{14}C]-arachidonic acid for 1 min. The incubation was started with the addition of the enzyme.

NSAIDs	IC_{50} μM	
	Radiotracer	RIA
BW 755C	20	10
Diclofenac	40	40
Etodalac	60	70
ETYA	25	15
Ibuprofen	35	40
6-Methylnaphthylacetic acid	100	80
Naproxen	60	50

The lack of dependence of the product profile on time or on COX-2 protein concentration suggested that any one of the metabolites could serve as an indicator of COX-2 activity. We tested this hypothesis by comparing the potencies of inhibitors when determined either by the radiotracer technique where all the products were measured or by a radioimmunoassay for PGE_2. The results for eight non-steroidal anti-inflammatory drugs are shown in Table 1. The IC_{50} values are similar for the two techniques, and the rank orders of potencies are identical (BW755c > diclofenac > 6-methoxynaphthylacetic acid). Moreover, the shapes of the dose response curves are identical for the two techniques (data not shown).

Since COX-1 requires N-linked glycosylation for proper activity (17), we sought to confirm that the baculoviral expression system produces glycosylated enzyme. When the infected Sf9 cells were cultured in tunicamycin, the apparent M_r changed from approximately 75 kD to 68 kD (data not shown). Moreover, microsomes from tunicamycin treated cells displayed less than 1% of the specific activity of control preparations. These findings, coupled with the fact that the untreated cells showed a broad triplet on the well-resolved Western blots and the treated cells gave a more sharply resolved single band, convinces us that the recombinant human COX-2 has extensive N-linked glycosylation.

Thus, the recombinant human COX-2 produced in insect cells was shown to reproduce all salient features of the enzyme from native sources. On scale-up, a typical 1.0 L preparation yields cyclooxygenase activity of 240 nmoles prostanoids produced • mg^{-1} protein in a 1 min extent assay and peroxidase activity of 450 nmole H_2O_2 consumed • min^{-1} • mg^{-1} protein. Based on this value, the

expression system described would yield more than 20 Units (μmoles/min) cyclooxygenase activity per liter cells.

In summary, we have devised an efficient method to prepare recombinant human COX-2 by heterologous expression in insect cells. The recombinant protein has heme-dependent cyclooxygenase and peroxidase activities as previously described for ovine prostaglandin H synthase (COX-1) (18). The cyclooxygenase properties of recombinant human COX-2 parallel those described for chick (15) and mouse (9) COX-2. Despite the formation of at least four metabolites, the enzyme forms cyclooxygenase products in fixed proportion; therefore, measurement of any single product serves as an indicator of cyclooxygenase activity. With this material, the inhibition of the human COX-2 isozyme can be investigated.

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