Selective induction of cyclo-oxygenase-2 activity in the permanent human endothelial cell line HUV-EC-C: biochemical and pharmacological characterization

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1 Cyclo-oxygenase (COX), the enzyme responsible for the conversion of arachidonic acid (AA) to prostaglandin H₂ (PGH₂), exists in two forms, termed COX-1 and COX-2 which are encoded by different genes. COX-1 is expressed constitutively and is known to be the site of action of aspirin and other non-steroidal anti-inflammatory drugs. COX-2 may be induced by a series of pro-inflammatory stimuli and its role in the development of inflammation has been claimed.

2 Endothelial cells are an important physiological source of prostanoids and the selective induction of COX-2 activity has been described for finite cultures of endothelial cells, but not for permanent endothelial cell lines.

3 The HUV-EC-C line is a permanent endothelial cell line of human origin. We have determined the COX activity of these cells under basal conditions and after its exposure to two different stimuli, phorbol 12-myristate 13-acetate (PMA) and interleukin-1 β (IL-1 β).

4 Both PMA and IL-1 β produced dose- and time-dependent increases of the synthesis of the COX-derived eicosanoids. These increases were maximal after the treatment with 10 nM PMA for 6 to 9 h. Under these conditions, the main eicosanoid produced by the cells was PGE₂.

5 The increase of COX activity by PMA or IL-1 β correlated with an increase of the enzyme's apparent V_{max}, whilst the affinity for the substrate, measured as apparent K_m, remained unaffected.

6 Treatment of the cells with PMA induced a time-dependent increase in the expression of both COX-1 and COX-2 mRNAs. Nevertheless, this increase was reflected only as an increase of the COX-2 isoenzyme at the protein level.

7 The enzymatic activity of the PMA-induced COX was measured in the presence of a panel of enzyme inhibitors, and the IC₅₀ values obtained were compared with those obtained for the inhibition of human platelet COX activity, a COX-1 selective assay. Classical non-steroidal anti-inflammatory drugs (NSAIDs) inhibited both enzymes with varying potencies but only the three compounds previously shown to be selective COX-2 inhibitors (SC-58125, NS-398 and nimesulide) showed higher potency towards the COX of PMA-treated HUV-EC-C.

8 Overall, it appears that the stimulation of the HUV-EC-C line with PMA selectively induces the COX-2 isoenzyme. This appears to be a suitable model for the study of the physiology and pharmacology of this important isoenzyme, with a permanent endothelial cell line of human origin.

Keywords: Inducible cyclo-oxygenase; COX-2; endothelial cell line; HUV-EC-C; phorbol 12-myristate 13-acetate; interleukin-1; human platelets; COX-1; NSAID; nimesulide; SC-58125; NS-398

Introduction

Cyclo-oxygenase (COX), also termed prostaglandin-H synthase, is the key enzyme involved in the biosynthesis of prostanoids from arachidonic acid (AA), Wlodawer & Samuelson, 1973; Smith *et al.*, 1991) and octadecanoids from linoleic acid (Hamberg & Samuelsson, 1967; Kадuce *et al.*, 1989; Camacho *et al.*, 1995). COX catalyses the oxidative transformation of AA into prostaglandin H₂ (PGH₂), the common precursor for physiologically important prostaglandins and thromboxane A₂ (TXA₂) (Wlodawer & Samuelsson, 1973; Smith *et al.*, 1991; Smith, 1992). Prostanoids are inflammation, thrombosis and vascular tone modulators and they are classic targets for therapeutic intervention (Flower, 1974). The isomerization of PGH₂ to PGE₂, PGD₂, and PGF_{2 α} may be spontaneous or enzymatically catalysed (Nugteren & Christ-Hazelhof, 1980; Smith, 1992) whereas the formation of PGI₂ and TXA₂ from PGH₂ is catalysed by specific enzymes termed PGI₂-synthase

and TXA₂-synthase, respectively (Haurand & Ullrich, 1985; Hecker & Ullrich, 1989; Tanabe & Ullrich, 1995). COX is expressed in most cell types and tissues (De Witt, 1991; Smith, 1992; O'Neill & Ford-Hutchinson, 1993; Goppelt-Strube, 1995). Nevertheless, specific prostanoids are formed in different tissues due to the differentially expressed secondary enzymes (Tanabe & Ullrich, 1995; Urade *et al.*, 1995).

COX is the site of action of aspirin and a wide range of other non-steroidal anti-inflammatory drugs (NSAIDs). Inhibition of prostanoid biosynthesis can account for both the therapeutic and side effects associated with the administration of NSAIDs (Vane, 1971). Studies in recent years have indicated that two isoforms of COX exist, encoded by separate genes, referred to as COX-1 and COX-2 (Yokoyama & Tanabe, 1989; Fletcher *et al.*, 1992). Human COX-2 shares about 60% identity with COX-1 (Jones *et al.*, 1993). COX-1 is constitutively expressed in most tissues (De Witt, 1991; O'Neill & Ford-Hutchinson, 1993; Goppelt-Strube, 1995) and in blood monocytes and platelets (Funk *et al.*, 1991; Hla & Neilson, 1992). COX-1 has been assigned as responsible for 'house-

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keeping' prostanoid biosynthesis when agonist-activated mobilization of esterified AA from membrane phospholipids occurs. In contrast, COX-2 expression is induced by serum, growth factors, phorbol esters, cytokines, hormones and bacterial toxins, and is over-expressed in the inflammatory process (Maier *et al.*, 1990; Kujubu *et al.*, 1991; 1993; Lee *et al.*, 1992; Sirois & Richards, 1992; Sirois *et al.*, 1992; O'Sullivan *et al.*, 1992a, b; O'Bannon *et al.*, 1992; Jones *et al.*, 1993). It has been postulated that COX-2-derived prostanoids may play a role in inflammation and other pathological situations. Expression of COX-2 is inhibited by glucocorticoids, probably by a post-transcriptional mechanism (Lee *et al.*, 1992; O'Bannon *et al.*, 1992; Kujubu & Herschmann, 1992; Masferrer *et al.*, 1992), and this partially contributes to the anti-inflammatory activity exhibited by these compounds.

While differential regulation and/or tissue specific location of COX-1 and COX-2 has been extensively studied, the catalytic-structural differences between these proteins are still not well-known (Kulmacz *et al.*, 1994; Bakovic & Dunford, 1994; Lacomte *et al.*, 1994; Laneuville *et al.*, 1995; Capdevila *et al.*, 1995; Kulmacz & Wang, 1995; Kurumbail *et al.*, 1996; McKeeve *et al.*, 1996). Despite this fact, a growing amount of data indicate that selective inhibition of COX-2 is possible (Meade *et al.*, 1993; Barnett *et al.*, 1994; Mitchell *et al.*, 1994; Copeland *et al.*, 1994; Quellet & Percival, 1995; Camacho *et al.*, 1995). COX-2 selective inhibitors could have therapeutic advantages compared with the classic non selective compounds, especially regarding gastric side-effects (Arai *et al.*, 1993; Spangler, 1993; Masferrer *et al.*, 1994; Seibert *et al.*, 1994; Klein *et al.*, 1994; Chan *et al.*, 1995). In addition, experimental use of COX-2 selective NSAIDs will also contribute to our understanding of the physiological role of the two forms of COX.

Different cell types have been used to study the selectivity of NSAIDs on COX-1 and COX-2 *in vitro*, including fibroblasts, endothelial cells and cos-1 cells transfected with murine COX-1 or COX-2 (Meade *et al.*, 1993). Human endothelial cells are particularly relevant because the vascular endothelium is an important physiological source of prostaglandins at sites of inflammation. *In vitro*, stimulated endothelial cells produce PGI₂, PGF_{2α} and PGE₂ as the main eicosanoids under different experimental conditions. Minor amounts of PGD₂, 12-hydroxy-heptadecatrienoic acid (HHT), 15-hydroxy-eicosatetraenoic acid (15-HETE) and 11-HETE are also produced (Marcus *et al.*, 1978; Alhenc-Gelas *et al.*, 1982; Hopkins *et al.*, 1984; Kühn *et al.*, 1985; Zavoico *et al.*, 1989), all of which are derived from COX activity (López *et al.*, 1993). Finite cultures of endothelial cells from human umbilical vein (HUVEC) are the most widely used tool to study endothelial cell physiology. Nevertheless, the use of HUVEC as a model for quantitative studies involving COX, such as screening tests, has serious disadvantages. A large individual variability in COX activity is found, making it a time consuming procedure because of the great number of primary cultures that are necessary. The purpose of the present work was to characterize the metabolic profile of AA via the COX pathway and the selective induction and expression of COX-2 in a permanent human endothelial cell line (HUV-EC-C).

Methods

HUV-EC-C culture and treatment with IL-1β and PMA

The HUV-EC-C is a permanent endothelial cell line derived from the vein of a normal human umbilical cord (ATCC CRL 1730). Cells were grown in F12K medium containing 10% foetal bovine serum (FBS) and supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 u ml⁻¹ penicillin, 100 u ml⁻¹ streptomycin, 100 µg ml⁻¹ heparin and 50 µg ml⁻¹ endothelial cell growth supplement (ECGS). Cells in confluent state were maintained without heparin and ECGS for 48 h before the addition (or not) of the desired amount of

IL-1β or phorbol-12-myristate-13-acetate (PMA). Experiments were performed with HUV-EC-C passage 19-27.

Preparation of platelet suspensions

Platelets were isolated from human peripheral blood obtained from healthy donors who denied taking any non-steroidal anti-inflammatory drugs (NSAIDs) during at least the previous week. The blood was anticoagulated with 2 mg ml⁻¹ sodium EDTA and centrifuged at 180 g for 10 min at room temperature to obtain a platelet-rich plasma. Then, the platelet-rich plasma was centrifuged at 2,000 g for 20 min at 4°C to obtain a platelet pellet. Cells were washed twice with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ (GIBCO-BRL) and resuspended to 5 × 10⁷ cells ml⁻¹ with Hank's balanced salt solution (HBSS, GIBCO-BRL).

Determination of the metabolic profile of AA and COX-activity in HUV-EC-C

After the desired period of time of cell exposure to the indicated concentration of IL-1β and PMA, cells were incubated at 37°C in the presence of 0.5 ml Hank's F12K containing 10 mM HEPES and the desired concentration of [¹⁴C]-AA in 5 µl of ethanol. At the indicated periods of time, the reactions were stopped by adding 1 N HCl to yield pH 3 followed by one volume of cold methanol. Samples were kept at -80°C until analysis. High performance liquid chromatographic (h.p.l.c.) analysis of eicosanoids was performed as previously described (Solá *et al.*, 1992). Briefly, samples were injected into a reverse phase column (Ultrasphere-ODS 4 × 250 µm, Beckman) and the composition of the mobile phase was 100% of A (acetonitrile: water: acetic acid 33:67:0.01) for the first 16 min after the injection. Afterwards, the percentage of B (acetonitrile: water: acetic acid 90:10:0.01) was increased linearly until it reached 65% 26 min after injection, and then maintained until min 45. Mobile phase flow rate was 1 ml min⁻¹. When HETEs had to be analysed the samples were injected into the column and eluted isocratically with methanol/water/trifluoroacetic acid/triethylamine (75:25:0.1:0.05), flow rate 1 ml min⁻¹.

COX-1 and -2 specific mRNA analysis

Cells were incubated in the presence or absence of 10 nM PMA for the indicated periods of time. Total RNA was isolated by phenol-chloroform extraction and isopropanol precipitation as described by Chomczynski & Sacchi (1987). The specific mRNA levels were estimated by means of a quantitative RT-PCR protocol. One µg of total RNA was reverse-transcribed into cDNA by incubating with 50 units of murine leukemia virus reverse transcriptase in a reaction buffer containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM MgCl₂, 2.5 µM random hexamers, 20 u RNAsin, 1 mM dNTPs in a final volume of 20 µl. The reaction mixture was incubated for 30 min at 42°C and then was stopped by heating for 5 min at 99°C and cooled for 5 min at 5°C. The primers used for COX-1 and COX-2 were 5'-TGCCCAGCTCCTGGCCCCGCG-CTT-3' (sense) and 5'-GTGCATCAACACAGGCGCCTC-TTC-3' (antisense) and 5'-TTCAAATGAGATTGTGGGAA-AATTGCT-3' (sense) and 5'-AGATCATCTCTGCCTGAG-TATCTT-3' (antisense), respectively (Hla & Neilson, 1992). The RNA for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was amplified and used as the internal control. The sense and antisense primers used for GAPDH were 5'-CCACCCATGGCAAATTCCATGGCA-3' and 5'-TCTA-GACGGCAGGTCAGGTCCACC-3', respectively (Maier *et al.*, 1990). The PCR was carried out in a DNA Thermal Cycler 480 (Perkin Elmer) with a reaction mixture (100 µl) containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1 µM sense/antisense primers, 2.5 u Taq polymerase and 4 µCi [³H]-dCTP. Serial half dilutions of the cDNA were done in order to prove linearity. Twenty seven cycles were performed as follows, 1.5 min 94°C, 1.5 min 58°C, 1.5 min

72°C, for all the samples. The products of the amplification were separated by electrophoresis in a 1.5% w/v Low Melting Point Agarose gel, containing ethidium bromide. To eliminate the excess of [^3H]-dCTP and to avoid excessive background, the running buffer was replaced by a fresh one when the loading dye had migrated half way through the gel. The bands were visualized and cut under u.v. light with a circular template and melted by heating to 70°C. Complete digestion of the gel pieces was performed by incubating the pieces in a water bath at 50°C for 1 h with 1 ml of the tissue solubilizer NCS-II. Afterwards, 10 ml of scintillation cocktail were added and the radioactivity was monitored in a β -counter (LS-3800, Beckman, San Ramón, CA). The radioactivity was normalized with respect to GAPDH.

Western-blot analysis of COX-1 and COX-2

Cells were incubated in the presence or absence of 10 nM PMA for the indicated periods of time. To solubilize COX proteins, cells were scraped, sonicated and incubated for 1 h at 4°C with extraction buffer (50 mM Tris-HCl pH 8.0 containing 10 mM EDTA, 1 mM PMSF and 1% v/v Tween-20) with gentle shaking. Cell extracts were centrifuged at 10,000 g for 5 min and were then boiled for 3 min in Laemmli sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol. Protein concentration of the supernatants was determined using the DC Protein Assay (Bio-Rad), a colorimetric assay for protein measurement of samples solubilized in detergent. The samples were adjusted by dilution to have an equal amount of protein for each condition, and 20 μg of protein lysate were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) (SDS-PAGE). An 8% w/v resolving gel and a 4% w/v stacking gel were used. The resolved proteins were electrotransferred onto nitrocellulose membranes as described by Towbin *et al.* (1979). Nonspecific antibody binding to the nitrocellulose was prevented by incubating the filter overnight at 4°C with block solution (3% w/v bovine serum albumin, 20 mM Tris, pH 7.5, 150 mM NaCl and 0.01% v/v Tween 20). Blocked membranes were incubated for 3 h at room temperature with either rabbit polyclonal antiserum against ovine COX-1 (PG20, 1:1000 dilution) or rabbit polyclonal antiserum against a peptide corresponding to a distinct carboxyl-terminal region of human COX-2 (PG27, 1:1000 dilution). The membranes were extensively washed with 20 mM Tris, pH 7.5, 150 mM NaCl and 0.01% v/v Tween 20. Immunoreaction and detection were performed by using an ECL Chemiluminescence Western blotting Kit (rabbit) following the manufacturer's instructions, and bands were visualized after exposure to Hyperfilm ECL.

Inhibition of COX-1 activity in human platelets

A 0.2 ml aliquot of platelets suspension ($5 \times 10^7 \text{ ml}^{-1}$) were preincubated in the presence or absence of drugs during 15 min at 37°C. AA 50 μM was then added and incubations were continued for a further 15 min. Then, tubes were placed on ice and centrifuged at 2,000 g for 10 min at 4°C. The production of TXB₂ was measured in the supernatants by an enzyme-immunoassay system (BIOTRAK, Amersham). The inhibitory effects on the COX-1 activity were evaluated by incubating each drug at five to six different concentrations with triplicate determinations. IC₅₀ values were obtained by non-linear regression by use of InPlot, GraphPad Software on an IBM computer.

Inhibition of COX-2 activity in the HUV-EC-C line

HUV-EC-C expresses selectively COX-2 isoenzyme after treatment with PMA (this paper). HUV-EC-C (2×10^4 per well) were seeded onto 96-well plates and made quiescent by removing the growth factor for 48 h before the initiation of the experiments. Quiescent cells were treated with 10 nM PMA for 6 h at 37°C to induce the COX-2 isoenzyme. Cultured medium

was then changed and cells were incubated in the presence or absence of drugs for 30 min at 37°C. AA 50 μM was then added, and the cells were incubated for a further 30 min. The production of PGF₂ in response to AA was measured in the supernatants by an enzyme-immunoassay system (BIOTRAK, Amersham). The inhibitory effects on the COX-2 activity were evaluated by incubating each drug with five or six different concentrations in triplicate. IC₅₀ values were obtained as mentioned above.

Drugs

Non-commercial available drugs (nimesulide, NS-398: (N-[2-cyclohexyloxy-4-nitrophenyl] methansulphonamide), SC-58125: (1-[(4-methylsulphonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)pyrazole, ketorolac) were synthesized by the Department of Chemical Synthesis of Laboratorios Almirall. Stock solutions (10^{-3} M) of the drugs were dissolved in 50% dimethylsulphoxide or water, and further dilutions were done with F12 K medium (for COX-2 assay) or HBSS (for COX-1 assay). Drug vehicle, at the concentrations employed, did not affect enzyme activities.

Figure 1 Representative chromatograms from samples of HUV-EC-C (a) untreated (control) and treated with (b) 10 u ml⁻¹ interleukin-1 β (IL-1 β) or (c) 20 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. Cells were incubated at 37°C with 25 μM [^{14}C]-arachidonic acid (AA) for 20 min. Peak identification of each eicosanoid was performed by coelution with reference standards.

Materials

Foetal bovine serum, phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were purchased from GIBCO-BRL (France). F12K medium, heparin, endothelial cell growth supplement (ECGS), arachidonic acid, phorbol 12-myristate 13-acetate (PMA) and all other compounds were obtained from Sigma-Aldrich Química S.A. (Spain). Human recombinant IL-1 β 50000 u μg^{-1} , purity >98% was from Boehringer Mannheim (GmbH, Germany). Solvents were h.p.l.c. grade from Sharlau S.A. (Barcelona, Spain). Random hexamers and Rnasin (GeneAmp RNA kit), ethidium bromide and Taq polymerase were from Perkin Elmer (Brachburg, NJ, U.S.A.). dNTPs were from Epicentre Technologies (Madison, WI, U.S.A.). Low Melting Point Agarose was supplied by Gibco BRL (Paisley, Scotland). The rest of the electrophoresis reagents were from Bio-Rad Laboratories (Spain). Nitrocellulose membranes were obtained from Schleicher & Schüll (Germany). Rabbit polyclonal antisera against COX-1 (PG 20) and COX-2 (PG 27) were from Oxford Biomedical Research, Inc. (U.S.A.).

COX-1 (ram seminal vesicles) and COX-2 (sheep placenta) electrophoresis standards were from Cayman Chemical Company (U.S.A.). [^{14}C]-arachidonic acid ([^{14}C]-AA), 55–58 mCi mmol^{-1} , [^3H]-dCTP 48–71 Ci mmol^{-1} , tissue solubilizer NCS-II, enzyme-immunoassay kits for PGE $_2$ and TXB $_2$ determinations, Hyperfilm ECL and the ECL chemiluminescence kit were purchased from Amersham Ibérica (Madrid, Spain). The scintillation cocktail (Ecoscint H) was from National diagnostics (Atlanta, GA, U.S.A.).

Results

H.p.l.c. analysis of eicosanoids produced by the HUV-EC-C line when cells were incubated with exogenous [^{14}C]-AA showed that both control and stimulated cells produced PGF $_{2\alpha}$, PGE $_2$ and 12-hydroxy-heptadecatrienoic acid (HHT) as the main eicosanoids, whereas PGD $_2$, 6-keto-PGF $_{1\alpha}$, 15-hydroxy-eicosatetraenoic acid (15-HETE) and 11-HETE were produced in minor amounts (Figure 1). Treatment of HUV-EC-C cells with PMA resulted in a higher production of the

Figure 2 Biosynthesis of eicosanoids ((a) 6-keto-PGF $_{1\alpha}$, (b) PGE $_2$, (c) 12-hydroxy-heptadecatrienoic acid (HHT), (d) PGF $_{2\alpha}$, (e) PGD $_2$ and (f) hydroxy-eicosatetra-enoic acids (HETEs)) by HUV-EC-C as a function of IL-1 β concentration. Cells were incubated for 24 h at 37°C in the presence of 0, 1, 2, 5, 10 and 20 μml^{-1} IL-1 β . Afterwards, cells were incubated with 25 μM [^{14}C]-AA for 20 min, and then eicosanoids were analysed by h.p.l.c. Data are shown as the mean, $n=4$; vertical lines show s.e.mean.

above mentioned eicosanoids compared to cells treated with IL-1 β or untreated cells. PGE₂ was the most synthesized prostanoid after PMA-stimulation of HUV-EC-C cells. The HETEs were quantitated together, since they resolved poorly with the chromatographic technique used.

Biosynthesis of all eicosanoids was increased several fold in a concentration-dependent fashion after exposure of HUV-EC-C to IL-1 β (Figure 2) and PMA (Figure 3). PMA enhanced the production of all eicosanoids much more than IL-1 β , except for PGF_{2 α} . PMA induced a maximum eicosanoid biosynthetic activity at 10 nM and higher concentrations produced lower amounts of COX products. A lower, although significant increase in the production of AA metabolites was also observed when IL-1 β was used as the stimulus. The maximum biosynthetic activity with IL-1 β was observed at 10 u ml⁻¹. Indomethacin inhibited the formation of all eicosanoids in a concentration-dependent manner (Figure 4), which indicated a COX origin for all of them. Therefore, COX activity, expressed as [¹⁴C]-AA transformed through COX pathway, was evaluated as the sum of all the aforementioned compounds.

COX activity was measured as a function of time of exposure to either IL-1 β or PMA. Figure 5 shows that COX activity achieved a maximum 6 to 9 h after addition of both stimuli and remained elevated for at least 24 h.

The effect of varying the AA concentration on enzyme activity was measured in control, IL-1 β and PMA-treated cells (Figure 6). In previous time-course assays, a linear relationship between COX-derived eicosanoids formation and incubation time was found for the first 10 min of incubation with the substrate and this incubation time was chosen for kinetic experiments. Apparent K_m values for AA of 12.2 ± 0.65 , 10.9 ± 1.7 , and 7.0 ± 1.4 μ M (mean $\pm$ s.e.mean, $n=4$) were obtained for the control, IL-1 β - and PMA-treated cells, respectively. The apparent V_{max} values obtained in the same assays were 481.9 ± 20.25 , 919.7 ± 30.6 and 2577.4 ± 127.3 pmol/10⁶ cells/10 min (mean $\pm$ s.e.mean, $n=4$), respectively, for the same above-mentioned conditions.

Overall, the effect of PMA on COX activity was quantitatively more significant than that observed with IL-1 β . Therefore, PMA was chosen as the preferred stimulus and was used

Figure 3 Biosynthesis of eicosanoids ((a) 6-keto-PGF_{1 α} , (b) PGE₂, (c) HHT, (d) PGF_{2 α} , (e) PGD₂ and (f) HETEs) by HUV-EC-C as a function of phorbol 12-myristate 13-acetate (PMA) concentration. Cells were incubated for 24 h at 37°C in the presence of 0, 1, 5, 10, 20 and 50 nM PMA. Afterwards, cells were incubated with 25 μ M [¹⁴C]-AA for 20 min and then eicosanoids were analysed by h.p.l.c. Data are shown as the mean, $n=4$; vertical lines show s.e.mean.

Figure 4 Effect of indomethacin concentration on the synthesis of (a) prostaglandins (●), HHT (■); (b) 11-HETE (▲) and 15-HETE (◆). PMA treated-HUV-EC-C were preincubated for 5 min with several concentrations of indomethacin before 25 μ M [14 C]-AA addition. Afterwards, cells were incubated for another 20 min. Data are shown as the mean, $n=4$; vertical lines show s.e.mean. For abbreviations used see legends of Figures 2 and 3.

Figure 5 Time-course response of the COX activity in HUV-EC-C treated with IL-1 β or PMA. Cells were incubated for 0, 1, 3, 6, 9 and 24 h in the presence of 5 μ l ml^{-1} IL-1 β (●) or 10 nM PMA (■). COX activity is expressed as pmol [14 C]-AA transformed through COX pathway by 10^6 cells after incubation with 25 μ M substrate for 20 min. Data are shown as the mean, $n=4$; vertical lines show s.e.mean. For abbreviations used see legends of Figures 2 and 3.

to study whether the increase of eicosanoids observed was related to the *de novo* synthesis of COX-1 and/or COX-2 isoenzymes.

The effect of PMA on the transcription of both COX-1 and COX-2 genes was determined by RT-PCR as described in Methods. Results in Figure 7 show that after PMA treatment there was a significant increase (7 fold) in specific mRNA levels of COX-2 in a time-dependent manner, whereas COX-1 specific mRNA levels were increased moderately (2 fold). The specific mRNA levels for both isoenzymes reached a maximum between 6 to 9 h after exposure to the stimuli. Afterwards, a slight decrease in the levels of both COX-1 and COX-2 mRNA was observed.

The Western blot analysis of the COX isoforms present in the HUV-EC-C line is shown in Figure 8. We measured the

Figure 6 Effect of substrate concentration on COX activity in control (▲), PMA-treated (■) and IL-1 β -treated (●) HUV-EC-C. Cells were incubated with several concentrations of [14 C]-AA (5, 10, 25, 50 and 100 μ M) for 10 min. Insert: Woolf plot, where S indicates AA concentration (μ M) and v the enzyme activity (pmol/ 10^6 cells 10 min $^{-1}$). Data are shown as the mean, $n=4$; vertical lines show s.e.mean.

time-course of COX-1 (A) and COX-2 (B) protein expression by incubating HUV-EC-C with or without 10 nM PMA for various time intervals. In the absence of PMA, the antibody against COX-1 (PG 20) recognized a weak band of approximately 70 kDa corresponding to the migration of purified COX-1 from ram seminal vesicles (A, lanes a and g, respectively), whereas the antibody against COX-2 peptide (PG 27) did not immunoreact with any protein band of the unstimulated cells (B, lane a). In contrast, after PMA treatment there was a significant increase of COX-2 protein expression in a time-dependent manner (B, lanes b to f), whereas the weak protein band corresponding to COX-1 was not modified (A, lanes b to f). COX-2 expression reached a maximum level after 6 to 8 h of PMA exposure (B, lanes d and e), followed by a decrease at 24 h (B, lane f). The antibody against COX-2 peptide (PG 27) recognized a protein doublet of approximately 70 kDa corresponding to the migration of purified COX-2 from sheep placenta and did not detect the purified COX-1 from ram seminal vesicles (B, lanes h and g, respectively). We determined the specificity of the antisera used in the above experiments by using solubilized COX-1 from membranes of human platelets, a cell type that is unable to synthesize inducible proteins. The rabbit polyclonal antiserum against human COX-2 (PG27) did not immunoreact with the human platelet COX-1 whereas the antiserum against ovine COX-1 (PG20) did crossreact with the human platelet COX-1 (data not shown).

We tested the effect of known NSAIDs and new selective COX-2 inhibitors on PMA-induced COX-2 activity in the HUV-EC-C line (Table 1). IC_{50} values of COX-2 inhibition were compared with those obtained for the inhibition of human platelet-dependent TXB_2 production, a COX-1 selective assay. In these models, ketorolac was the most potent inhibitor of platelet COX and showed the greatest selectivity for COX-1. Indomethacin was a potent inhibitor of COX-1 but was only 3 times more potent against COX-1 than COX-2. Aspirin was 13 times more active against COX-1, but was less potent than indomethacin on either assay. Ibuprofen was also an inhibitor of COX-1 and was 10 fold weaker towards COX-2 activity.

The sulphonamide, NS-398, and the pyrazole, SC-58125, were the most potent inhibitors of COX-2 and also presented the highest selectivity for COX-2. Another sulphonamide, nimesulide, also showed COX-2 selectivity but was 10 times less potent than NS-398 at inhibiting COX-2.

Figure 7 Specific COX-1 and COX-2 mRNA expression normalized to GAPDH in HUV-EC-C as a function of time of exposure to PMA. Cells were incubated for 0, 1, 3, 6, 9 and 24 h in the presence of 10 nM PMA. (a) Specific COX-1 (●) and COX-2 (■) mRNA levels normalized to GAPDH levels are represented. Data are the mean value of two independent experiments, vertical lines indicate the limits of the individual values obtained. (b) Representative gel photographs from PCR samples corresponding to the indicated periods of time of treatment with PMA are shown.

Discussion

We have found that the pattern of AA metabolism by the permanent endothelial cell line, HUV-EC-C and of the HUVEC are quite similar although PGI₂ (evaluated as 6-keto-PGF_{1α}) was not the main prostanoid produced by the HUV-EC-C line. Under different experimental conditions, including endogenous AA and addition of exogenous AA, HUVEC produce PGI₂, PGF_{2α} and PGE₂ as the main eicosanoids and minor amounts of PGD₂, HHT, 15-HETE and 11-HETE

Figure 8 Selective expression of COX-2 isoenzyme in PMA-treated HUV-EC-C. Quiescent confluent HUV-EC-C were incubated with 10 nM PMA for 0 (lane a), 1 (lane b), 3 (lane c), 6 (lane d), 8 (lane e) and 24 h (lane f). For each condition, equal amounts of proteins (20 µg) were resolved by SDS-PAGE on 8% acrylamide gels, transferred to nitrocellulose membranes, and immunoblotted either with anti-COX-1 (PG-20) (A) or anti-COX-2 (PG-27) (B) antisera, followed by enhanced chemiluminescence detection. Electrophoresis standards of COX-1 (from ram seminal vesicles, 0.3 µg, lane g) and COX-2 (from sheep placenta, 0.3 µg, lane h) were used as a reference. Similar results were obtained with cell extracts from two different batches of cells.

Table 1 Inhibition of HUV-EC-C PMA-induced COX-2 activity and human platelet COX-1 activity by COX inhibitors

Compound	IC ₅₀ for COX-1 (µM)	IC ₅₀ for COX-2 (µM)	Ratio COX-2/COX-1
Aspirin	1.2±0.3	15.8±3.9	13
Indomethacin	0.05±0.02	0.15±0.06	3
Ketorolac	0.0014±0.0003	0.14±0.09	100
Ibuprofen	0.34±0.3	3.8±1.8	11
Nimesulide	12.5±3.1	0.4±0.09	0.03
NS-398	28.9±16.1	0.04±0.02	0.001
SC-58125	13.3±1.9	0.07±0.05	0.006

Results are expressed as mean±s.e.mean of the IC₅₀ values obtained from three independent experiments. Assay conditions are described in Methods. The ratio of the IC₅₀ values for the compounds on COX-2 relative to COX-1 is given in the far right column.

(Marcus *et al.*, 1978; Alhenc-Gelas *et al.*, 1982; Kühn *et al.*, 1985; Zavoico *et al.*, 1989; Hopkins *et al.*, 1984), all of them derived from COX activity (López *et al.*, 1993). The HUV-EC-C line, produced PGE₂ and PGF_{2α} as the main prostanoids thereby exhibiting an AA metabolic profile similar to that found in microvessel-derived endothelial cells (Bull *et al.*, 1991; Stanimirovic *et al.*, 1993; Szczepanski *et al.*, 1994). As shown in HUVEC (López *et al.*, 1993), the formation of HETEs by the HUV-EC-C line was inhibited by indomethacin in a concentration-dependent manner, which suggests that COX is the enzyme responsible (Hecker *et al.*, 1987).

IL-1β and PMA treatment of the HUV-EC-C line increased the biosynthesis of prostanoids from exogenous AA. Moreover, COX activity was higher after treatment with PMA than with IL-1β in HUV-EC-C. In HUVEC it has been clearly shown that both PMA and IL-1 increased the synthesis of prostanoids (Maier *et al.*, 1990; Habib *et al.*, 1993; Jones *et al.*, 1993), although the induction of COX activity was greater with IL-1α than in the presence of PMA (Jones *et al.*, 1993). This discrepancy could be explained by the fact that Jones and colleagues measured COX activity in HUVEC only by the production of 6-keto-PGF_{1α}, and it may be that prostanoids, such as PGE₂, increased more than PGI₂ by the action of PMA.

Low levels of COX-1 transcripts are expressed in quiescent HUV-EC-C line while COX-2 transcripts were undetectable. PMA stimulation of HUV-EC-C increased significantly the mRNA levels of COX-2 whereas only a modest increase in COX-1 mRNA was observed. Interestingly, only COX-2 protein was enhanced after the exposure to the phorbol ester and the expression of COX-2 mRNA and protein remained evident after 24 h. These data suggest that in the HUV-EC-C line, (i) only the COX-1 gene is expressed by quiescent cells; (ii) PMA induces the COX-2 gene to a much higher extent than the COX-1 gene; (iii) PMA induces only the expression of the COX-2 protein; and (iv) in PMA-treated cells there is a correlation between COX activity, COX-2 mRNA levels and COX-2 protein mass. Taken together, these data indicate that PMA selectively induces a functionally active COX-2 protein in the HUV-EC-C line.

The expression of COX-1 mRNA without modification of COX-1 protein has also been observed in mouse NIH3T3 cells stimulated by serum (De Witt & Meade, 1993), in EGV-6 cells (a tracheal epithelial cell line) stimulated with PMA (Kitzler *et al.*, 1995) and in HUVEC stimulated with PMA (Habib *et al.*, 1993) although Xu *et al.* (1996) showed that COX-1 protein is also enhanced by PMA in HUVEC. These results suggest a post-transcriptional regulation of COX-1 expression in the HUV-EC-C line and/or a specific induction of nonfunctional, aberrantly spliced COX-1 mRNA, as has been observed in EGV-6 cells (Kitzler *et al.*, 1995).

Unstimulated HUV-EC-C express undetectable levels of the COX-2 mRNA and protein. In contrast, there are several studies that suggest that HUVEC express significant levels of COX-2 mRNA and protein under basal conditions (Hla & Neilson, 1992; Jones *et al.*, 1993; Camacho *et al.*, 1995). Furthermore, in HUV-EC-C the effect of PMA on COX-2 expression, both at the mRNA and protein levels, persisted for a longer time than in HUVEC (Hla & Neilson, 1992; Habib *et al.*, 1993).

It is currently believed that the unwanted side-effects of NSAIDs are due to inhibition of COX-1 and the therapeutic effects are due to their action on the COX-2 isoenzyme (Vane & Botting, 1995). Drugs which have the highest potency for inhibition of COX-2 and a more favourable COX-2/COX-1 activity ratio may have potent anti-inflammatory activity with fewer side effects in the stomach and kidney. Conversely to the classical NSAIDs, COX-2 selective inhibitors such as nimesulide, NS-398, SC-58125 among others are anti-inflammatory and analgesic agents that do not produce gastrointestinal lesions following oral administration (Arai *et al.*, 1993; Davis & Brogden, 1994; Seibert *et al.*, 1994). This is likely to be due to their potency and relative selectivity towards inhibition of COX-2 isoenzyme.

To test the biochemical selectivity of various NSAIDs towards COX-1 and COX-2, we compared their ability to inhibit TXB₂ production by platelets and PGE₂ production by PMA-stimulated HUV-EC-C respectively. In these models, aspirin, indomethacin, ibuprofen and ketorolac inhibited preferentially COX-1 as opposed to COX-2. Ketorolac showed the highest potency for inhibition of COX-1 followed by indomethacin, ibuprofen and aspirin, which order of potency correlates with their tendency to cause gastric damage (Lanza, 1989; Bateman, 1994; Langman *et al.*, 1994). Mitchell *et al.* (1994), using non-human cultured cells as a source of COX-1 (bovine aortic endothelial cells) and COX-2 (mouse macrophages), described higher COX-2/COX-1 ratios for aspirin (166), indomethacin (60) and ibuprofen (15) than those obtained in this study. However, Klein *et al.* (1994) using human platelets as a source of COX-1 and IL-1 stimulated rat mesangial cells as a source of COX-2 obtained a COX-2/COX-1 ratio for indomethacin of 5.9 which was very close to the value obtained here. Surprisingly, when the whole human blood system described by Patrignani *et al.* (1994) was used to measure COX-1 and COX-2 activities, indomethacin was twice as potent as an inhibitor of COX-2 than COX-1. Thus, it appears that the COX selectivity of the drugs is very dependent on the assay system used.

NS-398 showed the lowest COX-2/COX-1 ratio, followed by SC-58125 and nimesulide. NS-398 has been shown to inhibit selectively the activity of purified sheep COX-2 (Futaki *et al.*, 1994), recombinant murine COX-2 (Masferrer *et al.*, 1994) and recombinant human COX-2 (Gierse *et al.*, 1995), with COX-2/COX-1 ratios >0.038, >0.0003 and >0.001, respectively. Recently, Panara *et al.* (1995) have found that NS-398 is a COX-2 selective inhibitor with a ratio of 0.006 in platelets (COX-1) and LPS-stimulated monocytes (COX-2) from human whole blood. In the present study, we have shown that in human cell models NS-398 has a COX-2/COX-1 ratio of 0.001, identical to that obtained by Gierse *et al.* (1995). Similar to NS-398, SC-58125 was approximately a 200 fold more potent as an inhibitor of COX-2 activity in PMA-stimulated HUV-EC-C than platelet COX-1. Seibert *et al.* (1994) obtained similar results with murine recombinant COX-1 (IC₅₀ >10 μM) and COX-2 (IC₅₀ = 0.05 μM). Finally, nimesulide showed a higher COX-2/COX-1 ratio than NS-398 and SC-58125 in our cell models. These results are consistent with those described by Barnett *et al.* (1994) with human recombinant COX-1 and COX-2.

At present, controversial findings regarding the selectivity of standard NSAIDs have been described in the literature (Battistini *et al.*, 1994; Griswold & Adams, 1996), which has hampered progress in this field. It has been suggested that cellular models are more likely to reflect the selectivity exhibited by the compounds in whole animal models and in clinical practice (Mitchell *et al.*, 1994). An ideal COX-2 model should couple the use of cells from human origin with the ease and reproducibility that permanent cell lines provide. Such a model will be of benefit in the study of COX-2 physiology as well as in the screening of selective COX-2 inhibitors.

Our results show that the HUV-EC-C line has a very low basal COX activity and that the dramatic increase in activity seen when stimulated with PMA is likely to reflect selective COX-2 expression. The main eicosanoid formed by HUV-EC-C, PGE₂, increased at least 10 fold in concentration in cells treated with 10 nM PMA, when compared with controls. Therefore, evaluation of the formation of PGE₂ by ELISA in PMA stimulated HUV-EC-C is a suitable experimental strategy to determine the action of NSAIDs on COX-2 activity.

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