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Effects of the novel anti-inflammatory compounds, *N*-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulphonamide (NS-398) and 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone (L-745,337), on the cyclo-oxygenase activity of human blood prostaglandin endoperoxide synthases

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1 We have evaluated the selectivity of ketoprofen and two novel nonsteroidal anti-inflammatory drugs, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulphonamide (NS-398) and 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone (L-745,337), in inhibiting the cyclo-oxygenase activity of prostaglandin endoperoxide synthase-2 (PGHS-2) *vs* PGHS-1 in human blood monocytes and platelets, respectively.

2 Heparinized whole blood samples were drawn from healthy volunteers pretreated with aspirin, 300 mg 48 h before sampling, to suppress the activity of platelet PGHS-1 and incubated at 37°C for 24 h with increasing concentrations of the test compounds in the presence of lipopolysaccharide (LPS, 10 $\mu g\ ml^{-1}$). Immunoreactive PGE_2 levels were measured in plasma by a specific radioimmunoassay as an index of the cyclo-oxygenase activity of LPS-induced monocyte PGHS-2.

3 The effects of the same inhibitors on platelet PGHS-1 activity were assessed by allowing whole blood samples, drawn from the same subjects in aspirin-free periods, to clot at 37°C for 1 h in the presence of the compounds and measuring immunoreactive thromboxane B_2 (TXB_2) levels in serum by a specific radioimmunoassay.

4 Under these experimental conditions, ketoprofen enantioselectively inhibited the cyclo-oxygenase activity of both PGHS-1 and PGHS-2 with equal potency (IC_{50} ratio: approx. 0.5 for both enantiomers), while L-745,337 and NS-398 achieved selective inhibition of monocyte PGHS-2 (IC_{50} ratio: >150). L-745,337 and NS-398 did not affect LPS-induced monocyte PGHS-2 biosynthesis to any detectable extent.

5 We conclude that L-745,337 and NS-398 are selective inhibitors of the cyclo-oxygenase activity of human monocyte PGHS-2. These compounds may provide adequate tools to test the contribution of this novel pathway of arachidonate metabolism to human inflammatory disease.

Keywords: Prostaglandin endoperoxide synthases; human blood monocytes; human platelets; L-745,337; NS-398; ketoprofen

Introduction

The conversion of arachidonic acid to prostaglandin H_2 (PGH_2) is catalysed by prostaglandin endoperoxide synthase (PGHS) which exhibits both cyclo-oxygenase and peroxidase activities (DeWitt, 1991). PGH_2 is further metabolized by other enzymes to various prostanoids (prostaglandins, prostacyclin and thromboxane A_2). Two isozymes of PGHS are known, referred to as PGHS-1 and PGHS-2 (Smith, 1992). PGHS-1 is a constitutive enzyme present in almost all cell types (Simmons *et al.*, 1991). Thus, PGHS-1 is the major isoform of gastrointestinal tissue (DeWitt & Smith, 1988). Prostanoid production by PGHS-1 is involved in physiological functions such as vascular homeostasis, control of kidney function and gastric cytoprotection (Smith, 1992). PGHS-2 is induced in a more restricted cell-specific fashion by mitogenic and inflammatory stimuli (Kujubu *et al.*, 1991; Fletcher *et al.*, 1992; O'Banion *et al.*, 1992; Lee *et al.*, 1992; O'Sullivan *et al.*, 1992a,b; Hempel *et al.*, 1994; Patrignani *et al.*, 1994).

The cyclo-oxygenase activity of PGHS is inhibited by aspirin and related nonsteroidal anti-inflammatory drugs (NSAIDs) (Vane, 1971). Inhibition of prostanoid formation by NSAIDs is the basis for their therapeutic actions as well as their side-effects. In fact, it has been suggested that the anti-inflammatory action of NSAIDs is due to inhibition of PGHS-2, whereas the toxic effects on the stomach and kidney are due to inhibition of the constitutive isozyme, PGHS-1 (Vane, 1994). Since PGHS-1 and PGHS-2 are structurally distinct proteins with only 60% homology (Jones *et al.*, 1993), the development of drugs that selectively inhibit the cyclo-oxygenase activity of PGHS-2 might lead to a new generation of anti-inflammatory drugs with increased tolerability (Vane, 1994). Recently, new anti-inflammatory agents such as *N*-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulphonamide (NS-398) and 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone (L-745,337) have been developed that produce fewer gastrointestinal lesions in experimental animals than conventional NSAIDs (Futaki *et al.*, 1993; Chan *et al.*, 1994; Chan *et al.*, 1995; Masferrer *et al.*, 1994). Thus, the aim of our study was to evaluate the selectivity of L-745,337 and NS-398 in comparison with ketoprofen in inhibiting the cyclo-oxygenase activity of PGHS-2 *vs* PGHS-1 in human blood monocytes and platelets, respectively. The whole blood assay which we have recently developed (Patrignani *et al.*, 1994) has distinct advantages *vis-a-vis* other assay systems (Meade *et al.*, 1993; Mitchell *et al.*, 1993): (1) it can be used *in vitro* in the pre-clinical assessment of inhibitor molecules as well as *ex vivo* during phase I/II studies; (2) it compares clinically relevant target cells; (3) it takes into account the variable degree of protein binding of different inhibitors.

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A preliminary account of this study was presented at the Clinical Research Meeting (San Diego, U.S.A., May 5–8, 1995) and published in abstract form (Patrignani *et al.*, 1995).

Methods

Subjects

Nine healthy volunteers (2 female and 7 male subjects; aged 25–50 years) were studied on several occasions. Informed consent was obtained from each subject. Peripheral venous blood samples were drawn between 10 h 00 min and 12 h 00 min, before and 48 h after the oral administration of aspirin 300 mg.

PGHS-2 induction in whole blood

One ml aliquots of peripheral blood samples containing 10 iu of sodium heparin were incubated both in the absence and in the presence of LPS (10 μg ml^{-1}) for 4 and 24 h at 37°C as described by Patrignani *et al.* (1994). The contribution of platelet PGHS-1 was suppressed by pretreating the subjects with aspirin 300 mg 48 h before sampling. Plasma was separated by centrifugation (10 min at 2,000 r.p.m.) and kept at −30°C until assayed for PGE_2.

PGHS-1 activity in whole blood

Peripheral blood samples were drawn from the same donors when they had not taken any NSAIDs during the 2 weeks preceding the study. One ml aliquots of whole blood were immediately transferred into glass tubes and allowed to clot at 37°C for 60 min. Serum was separated by centrifugation (10 min at 3,000 r.p.m.) and kept at −30°C until assayed for TXB_2. Whole blood TXB_2 production was measured as a reflection of maximally stimulated cyclo-oxygenase activity of platelet PGHS-1 by endogenously formed thrombin (Patrono *et al.*, 1980).

Isolation of monocytes from human blood

Mononuclear cells were separated from whole blood containing sodium heparin (10 iu ml^{-1}) by Ficoll-Paque as described by Boyum (1968). After centrifugation (400 *g* for 40 min at room temperature), lympho-monocytes layered at the gradient interface. Mononuclear cells were carefully removed, washed three times and resuspended in DMEM buffered with 0.05 M HEPES, pH 7.4, supplemented with 0.5% heat-inactivated FCS and 4 mM L-glutamine. This will be referred to as complete medium (CDMEM). Aliquots of 10 ml were seeded into plastic Petri dishes and incubated at 37°C in 5% CO_2-humidified atmosphere for 60 min. The adherent cells were recovered by gently scraping with a rubber policeman, resuspended in CDMEM (2×10^6 cells ml^{-1}) and their viability (>96%) examined by trypan blue exclusion. The purity of the preparation of isolated monocytes was assessed by forward and right-angle scatter measurements using flow cytometry (Coulter, Hialeah, FL, U.S.A.). The cell suspension contained approximately 90% monocytes.

Effects of cyclo-oxygenase inhibitors

L-745,337 was dissolved in methanol (0.0001-25 mg ml^{-1}) and 10 μl aliquots of these solutions were pipetted directly into test tubes to give a final concentration of 0.001 to 250 μg ml^{-1} (0.0026–639 μM) of whole blood or monocyte suspension. The methanol was evaporated and 1 ml aliquots of whole blood or isolated monocyte suspension (2×10^6 cells ml^{-1}) were added. NS-398 was dissolved in DMSO (0.00015-15 mg ml^{-1}) and 2 μl of this solution was added to 1 ml aliquots of whole blood to give a final concentration of 0.0003–30 μg ml^{-1} (0.00095–95 μM). **S**- and **R**-ketoprofen were dissolved in NaCl (0.9%). The effects of these inhibitors on PGHS-2 activity were studied by incubating each compound at five to six different concentrations with multiple heparinized whole blood samples in the presence of LPS (10 μg ml^{-1}) for 4 and 24 h. Moreover, we studied the effects of L-745,337 and NS-398 on the biosynthesis of PGE_2, PGHS-1 and PGHS-2 by isolated human monocytes (2×10^6 cells ml^{-1}) incubated with LPS (10 μg ml^{-1}) for 24 h. The effects on PGHS-1 activity were evaluated by incubating each drug at five to six different concentrations with multiple whole blood samples that were allowed to clot at 37°C for 60 min.

Analyses of TXB_2 and PGE_2

PGE_2 and TXB_2 concentrations were measured by previously described and validated radioimmunoassays (Ciabattoni *et al.*, 1979; Patrignani *et al.*, 1982). Unextracted serum and plasma samples as well as cell culture media were diluted in the standard diluent of the assay (0.02 M phosphate buffer, pH 7.4) and assayed in a volume of 1.5 ml, at a final dilution of 1:50–1:20,000. We used 4,000 d.p.m. of [^{3}H]-PGE_2 or [^{3}H]-TXB_2 and specific rabbit anti-PGE_2 or anti-TXB_2 sera diluted 1:120,000 or 150,000, respectively. The minimum detectable concentration was 1 pg ml^{-1} for both assays. Thus, the detection limit of the assays was 0.05 ng ml^{-1} of sample.

Western Blot analysis

Isolated monocytes (2×10^6 cells) were lysed in phosphate-buffered saline (pH 7.4) containing 0.5% Nonidet P-40 and 1 mM phenylmethylsulphonylfluoride, 0.2 mM leupeptin, 50 μM pepstatin A and 0.01% EDTA for 30 min at 4°C. The lysate was heated at 100°C for 5 min in the presence of 63 mM Tris-HCl, pH 6.8, 4% (wt/vol) sodium dodecyl sulphate (SDS), 20% (vol/vol) glycerol, 2% mercaptoethanol and 2 mg ml^{-1} of bromophenol blue. The samples were adjusted for equal amounts of proteins and 10 μg of protein lysate was analysed by polyacrylamide gel electrophoresis in the presence of SDS for 90 min at 20 mA using a mini-gel vertical apparatus (Bio-Rad Laboratories). A 9% separating gel and a 4% stacking gel were used. The resolved proteins were transferred electrophoretically to nitrocellulose membranes as described by Towbin *et al.* (1979). The membranes were saturated with a solution of 5% fat-free dried milk in phosphate-buffered saline:0.05% Tween-20 (PBS-Tween-20), followed by incubation with either rabbit polyclonal antiserum (1:750 dilution) directed against the carboxyl-terminal portion of human PGHS-2 or polyclonal anti-PGHS-1 serum (1:1,000 dilution) for 2 h at room temperature. The membranes were extensively washed with PBS-Tween-20 and then incubated with a biotinylated anti-rabbit IgG diluted 1:2,000 for 1 h at room temperature and the blot was developed with streptavidin-peroxidase.

Materials

[^{3}H]-PGE_2 and [^{3}H]-TXB_2 (200–250 Ci mmol^{-1}) were from Du Pont de Nemours GmbH (Bad Homburg, Germany). PGE_2 and TXB_2 were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Anti-PGE_2 and anti-TXB_2 sera were obtained in our laboratory and their characteristics have been described previously (Ciabattoni *et al.*, 1979; Patrignani *et al.*, 1982). Ficoll-Paque was obtained from Pharmacia Biotech (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY, U.S.A.). Heparin, L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), LPS derived from *Escherichia coli* 026:B6, biotinylated anti-rabbit IgG, streptavidin-peroxidase, phenylmethylsulphonyl fluoride, leupeptin, pepstatin A, Nonidet P-40, dimethyl sulphoxide (DMSO) and dexamethasone were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). L-745,337 was kindly provided by Merck Frosst (Pointe Claire-Dorval, Quebec, Canada) through the courtesy of Drs A.W. Ford-

Hutchinson and C.-C. Chan. **S**- and **R**-ketoprofen (2-[3-benzoylphenyl]propionic acid) were provided by Laboratorios Menarini (Barcelona, Spain) through the courtesy of Dr D. Mauleòn. NS-398 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Rabbit polyclonal antibodies prepared against PGHS-2 peptide (C)-NASSSRSGLDDINPTVLLK, which is only present in the carboxyl-terminal (amino acid sequence 580–598) of human PGHS-2 (Hla and Neilson, 1992; Jones *et al.*, 1993) were obtained as described recently (Habib *et al.*, 1993). Specific rabbit polyclonal antibodies directed against PGHS-1 were a gift from Dr W.L. Smith (Department of Biochemistry, Michigan State University).

Statistical analysis

Results generated from 3–6 different experiments are expressed as mean ± s.d.. The sigmoidal dose-response curves were analysed with ALLFIT, a basic computer programme for simultaneous curve-fitting based on a four parameter logistic equation (De Lean *et al.*, 1978).

Results

We measured PGE_2 production in heparinized whole blood, incubated with LPS (10 $\mu g\ ml^{-1}$) for 24 h, as a reflection of the cyclo-oxygenase activity of monocyte PGHS-2 (Patrignani *et al.*, 1994). The contribution of platelet cyclo-oxygenase activity was suppressed selectively by pretreating the subjects with aspirin (300 mg 48 h before sampling). Plasma immunoreactive PGE_2 averaged 23.7 ± 13 ng ml^{-1} (mean ± s.d., n = 11). Moreover, we measured TXB_2 production after clotting of whole blood obtained from healthy subjects not exposed to aspirin, as a reflection of the cyclo-oxygenase activity of platelet PGHS-1 (Patrono *et al.*, 1980). Serum immunoreactive TXB_2 averaged 357 ± 200 ng ml^{-1} (mean ± s.d., n = 11). The IC_{50} values for inhibition of the cyclo-oxygenase activity of monocyte PGHS-2 and platelet PGHS-1 are detailed in Table 1. Both L-745,337 and NS-398 selectively inhibited the cyclo-oxygenase activity of monocyte PGHS-2 at concentrations < 1% of those required to achieve comparable inhibition of platelet PGHS-1. The dose-response curves for the inhibition of PGHS-1 and PGHS-2 by NS-398 are shown in Figure 1. At 1 μM NS-398, the cyclo-oxygenase activity of PGHS-2 was almost completely inhibited whereas PGHS-1 retained about 80% of its activity. NS-398 and L-745,337 reduced PGE_2 production in whole blood incubated with LPS for 4 and 24 h with comparable potency (data not shown).

In contrast, ketoprofen enantioselectively inhibited both enzymes with comparable potency (Table 1). **S**-ketoprofen was approximately 70 and 90 fold more potent than the **R** enantiomer in inhibiting PGHS-1 and PGHS-2, respectively.

Isolated human monocytes respond to LPS (10 $\mu g\ ml^{-1}$) with a time-dependent increase in PGE_2 production that correlates with the accumulation of PGHS-2 (Patrignani *et al.*, 1994). At 24 h of incubation, PGE_2 production was 4.4 ± 4.3 ng/10^6 cells (mean ± s.d., n = 6). NS-398 and L-745,337 incubated with isolated human monocytes in the

Table 1 Inhibition of human whole blood PGHS-1 and PGHS-2 activities by cyclo-oxygenase inhibitors

Inhibitor	*PGHS-1*	*PGHS-2*	*IC_{50} (PGHS-1)* / *IC_{50} (PGHS-2)*
	IC_{50} (μM)		
NS-398	16.8 ± 11.8	0.103 ± 0.028	163
L-745,337	367.7 ± 60.5	1.5 ± 0.34	245
S-ketoprofen	0.11 ± 0.04	0.18 ± 0.06	0.61
R-ketoprofen	7.5 ± 0.73	20.6 ± 0.45	0.36

All values are mean ± s.d. (n = 4–6).

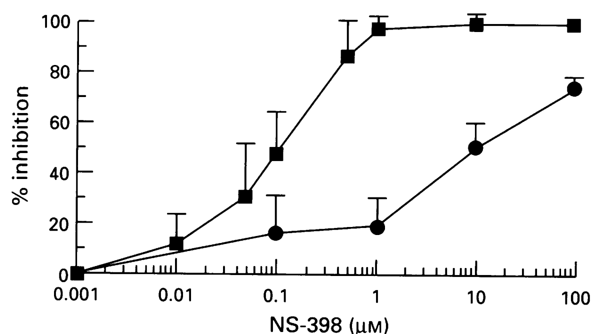

Figure 1 Dose-response curves for inhibition of human whole blood PGHS-1 and PGHS-2 by NS-398. Increasing concentrations of NS-398 (0.0095–95 μM) were incubated with 1 ml heparinized whole blood samples (drawn from healthy volunteers pretreated with 300 mg of aspirin 48 h before sampling) in the presence of LPS (10 $\mu g\ ml^{-1}$) for 24 h, and plasma PGE_2 levels were assayed as a reflection of monocyte PGHS-2 activity (■). NS-398 was also incubated with 1 ml whole blood samples (drawn from the same donors when they had not taken any NSAIDs during the 2 weeks preceding the study) allowed to clot for 1 h, and serum thromboxane B_2 (TXB_2) levels were measured as a reflection of platelet PGHS-1 activity (●). Results are depicted as percentage inhibition (mean ± s.d.) from four separate experiments.

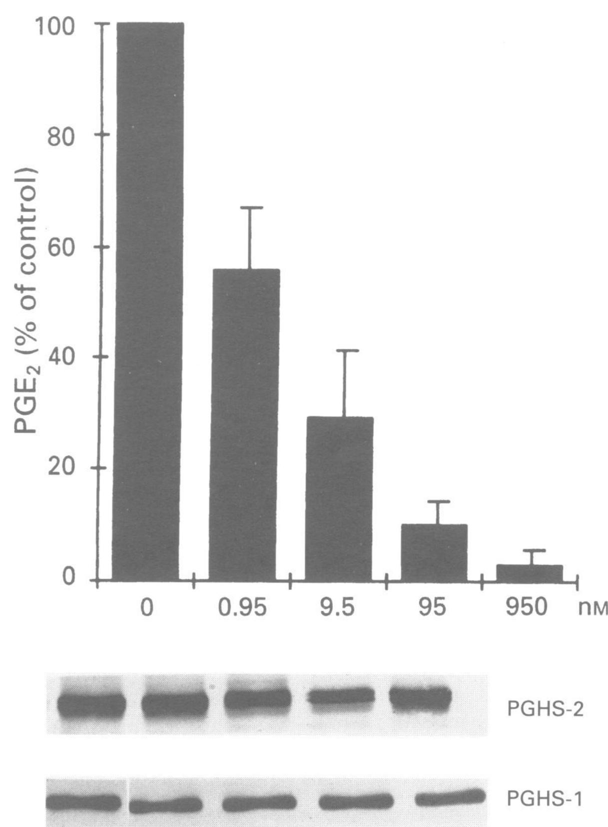

Figure 2 Inhibitory effects of NS-398 on PGE_2 production and PGHS isozyme biosynthesis by LPS-stimulated human monocytes. Increasing concentrations of NS-398 (0.95–950 nM) were incubated with 1 ml of human monocyte suspensions in the presence of LPS (10 $\mu g\ ml^{-1}$) for 24 h at 37°C. Supernatants were assayed for PGE_2 by a specific RIA technique while monocytes were lysed and proteins were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques using rabbit antibodies directed against PGHS-1 or the carboxyl-terminal of PGHS-2. Equal amounts of proteins (10 μg) were loaded in all lanes. Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG and streptavidin-peroxidase. This figure is representative of 3 experiments.

presence of LPS (10 μg ml^{-1}) for 24 h inhibited PGE_2 production with an IC_{50} of 1.5 ± 0.5 and 46.4 ± 22 nM (mean ± s.d., $n = 3-6$), respectively (Figures 2 and 3).

As shown in Figures 2 and 3, NS-398 and L-745,337 at concentrations that completely suppressed the production of PGE_2 did not affect LPS-induced biosynthesis of monocyte PGHS-2 as analysed by Western blot using specific antibodies directed against a unique aminoacid sequence present in human PGHS-2 (Habib *et al.*, 1993). Similarly, the constitutive expression of PGHS-1 was not affected to any detectable extent.

Discussion

Different methods have been developed to study the effects of NSAIDs on the cyclo-oxygenase activity of PGHS isozymes *in vitro*. Thus, inhibition of the cyclo-oxygenase activity of PGHS-1 and PGHS-2 has been evaluated in purified enzyme systems, in microsomal membranes from cos-1 cells transfected with the murine (Meade *et al.*, 1993) or human isozymes (Laneuville *et al.*, 1994), cultured cells selectively expressing PGHS-1 and PGHS-2 and in cultured intact cells, such as bovine aortic endothelial cells and endotoxin-activated J774.2 macrophages (Mitchell *et al.*, 1993). In many such systems arachidonate concentrations are used that are artificially high

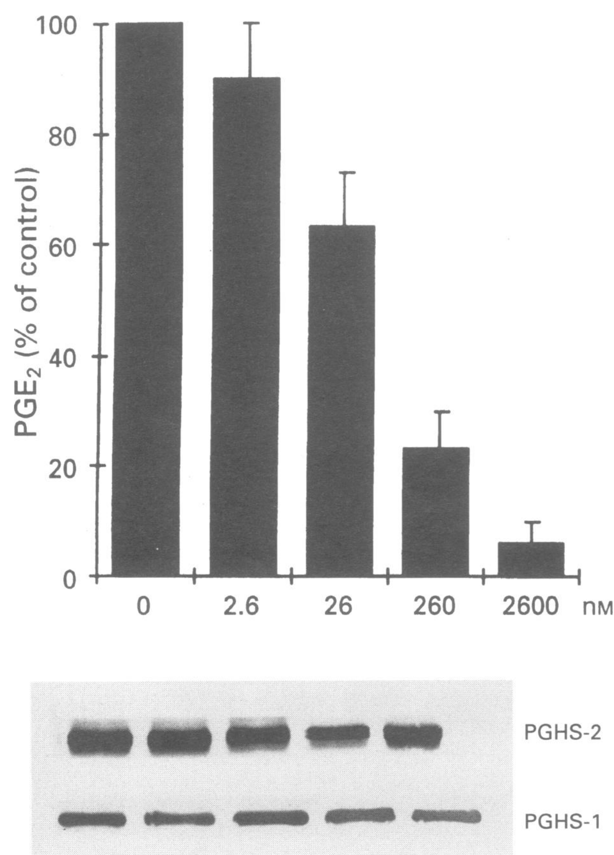

Figure 3 Inhibitory effects of L-745,337 on PGE_2 production and PGHS isozyme biosynthesis by LPS-stimulated human monocytes. Increasing concentrations of L-745,337 (2.6–2600 nM) were incubated with 1 ml of human monocyte suspensions in the presence of LPS (10 μg ml^{-1}) for 24 h at 37°C. Supernatants were assayed for PGE_2 by a specific RIA technique while monocytes were lysed and proteins were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques using rabbit antibodies directed against PGHS-1 or the carboxyl-terminal of PGHS-2. Equal amounts of proteins (10 μg) were loaded in all lanes. Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG and streptavidin-peroxidase. This figure is representative of 6 experiments.

compared with those occurring in intact cells. Moreover, measurement of instantaneous inhibition of cyclo-oxygenase activity may underestimate the potency of some NSAIDs which are time-dependent inhibitors (Laneuville *et al.*, 1994). Thus, measuring prostaglandin production in intact cells that selectively express PGHS-2 using arachidonate released from endogenous lipid stores would be a more suitable screening method for selective inhibitors than measuring instantaneous inhibition of PGHS-2 in a recombinant system (Laneuville *et al.*, 1994).

We have recently described a relatively simple model of human PGHS-2 expression in LPS-stimulated whole blood (Patrignani *et al.*, 1994). This method is suitable for evaluating the extent of PGHS-2 inhibition both *in vitro* and after oral dosing of NSAIDs in man. We demonstrated that LPS stimulated blood cells to produce large amounts of PGE_2 in a time-dependent fashion that correlated with the accumulation of a protein doublet of approximately 72 kDa in monocytes, as detected by Western blot using antibodies directed against a synthetic PGHS-2-specific aminoacid sequence. Differently, PGHS-1 expressed in unstimulated monocytes was not affected by the incubation with LPS up to 24 h. Dexamethasone, a selective inhibitor of PGHS-2 expression in monocytes/macrophages (Lee *et al.*, 1992; O'Banion *et al.*, 1992; Kutchera *et al.*, 1993) profoundly suppressed PGE_2 production in LPS-stimulated whole blood both at 4 and 24 h (Patrignani *et al.*, 1994). The contribution of platelet PGHS-1 to cyclo-oxygenase activity measured in whole blood was suppressed selectively by treating the subjects with aspirin 300 mg *in vivo* (Patrono *et al.*, 1985). Aspirin was administered 48 h before sampling because of previous evidence that the return of intact platelet protein and enzyme activity after a single dose of aspirin is only detectable after a 2-day lag, most likely reflecting acetylation by aspirin of PGHS-1 in megakaryocytes (Roth *et al.*, 1975; Patrono *et al.*, 1980).

To test the biochemical selectivity of various NSAIDs towards PGHS-2 and PGHS-1, in the present study, we compared their inhibitory effects on PGE_2 production by LPS-stimulated whole blood at 4 and 24 h and on TXB_2 production by platelets during 1 h whole blood clotting. The production of TXB_2 in this setting has been previously demonstrated to represent an index of the cyclo-oxygenase activity of platelet PGHS-1 in response to endogenously formed thrombin (Patrono *et al.*, 1980; 1985; Patrignani *et al.*, 1982). NS-398 and L-745,337 are novel anti-inflammatory and analgesic agents that do not produce gastrointestinal lesions following oral administration to experimental animals (Futaki *et al.*, 1993; Masferrer *et al.*, 1994; Chan *et al.*, 1994; 1995). Thus, NS-398, at doses that completely inhibited PGE_2 synthesis in the fluid exudate that is formed following carrageenan injection into a subcutaneous air pouch (ED_{50}: 0.3 mg kg^{-1}), did not inhibit PGE_2 synthesis in the stomach, nor were gastric lesions observed when NS-398 was administered at 30 mg kg^{-1} to rats (Masferrer *et al.*, 1994). NS-398 was reported to inhibit selectively the activity of both recombinant and purified PGHS-2 without affecting PGHS-1. Baculovirus-expressed recombinant murine PGHS-2 was inhibited with an IC_{50} of 30 nM while PGHS-1 activity was not affected at concentrations as high as 100 μM (Masferrer *et al.*, 1994). In purified enzymes (PGHS-1 and PGHS-2 from ram seminal vesicles and sheep placenta, respectively), NS-398 selectively inhibited the cyclo-oxygenase activity of PGHS-2 (IC_{50}: 3.8 μM) (Futaki *et al.*, 1994) but was approximately 100 fold less potent than with the recombinant enzyme. In the present study, we have shown that NS-398 inhibited the cyclo-oxygenase activity of platelet PGHS-1 and monocyte PGHS-2 of human whole blood *in vitro* with IC_{50} of 17 *vs* 0.1 μM, respectively. Variable IC_{50} values for inhibition of the cyclo-oxygenase activity of PGHS-2 by NS-398 *in vitro* most likely reflect the different experimental conditions used in the various assays systems. In our system, the effects of inhibitors are evaluated in intact cells expressing the human isozyme in the presence of plasma proteins which by binding the compounds may influence their concentration at the active

site of the enzyme. Thus, NS-398 and L-745,337 were approximately 70 and 30 fold more potent, respectively, in inhibiting the cyclo-oxygenase activity of PGHS-2 when assessed in isolated monocytes than in whole blood.

One potential limitation of our experimental approach is related to the instability of some compounds in whole blood over prolonged incubation at 37°C. However, when the inhibitory effects are compared at 4 and 24 h of incubation, any time-dependent inactivation of the inhibitor should become apparent. This was not the case for either NS-398 or L-745,337 (data not shown).

Similarly to NS-398, L-745,337 was approximately a 200 fold better inhibitor of the cyclo-oxygenase activity of monocyte PGHS-2 than platelet PGHS-1. In human osteosarcoma cells selectively expressing PGHS-2 (Chan *et al.*, 1995), L-745,337 inhibited PGE_2 production in response to exogenous arachidonic acid with an IC_{50} (23 nM) quite similar to that in human monocytes (45 nM) in the present study. In contrast, in arachidonic acid challenged U-937 cells expressing PGHS-1, the compound did not affect the production of PGE_2 at concentrations up to 10 μM (Chan *et al.*, 1995).

Ketoprofen, a conventional NSAID, inhibited both enzymes with equal potency. Similarly to the inhibition of the constitutive enzyme, inhibition of monocyte PGHS-2 showed enantioselectivity.

Unlike dexamethasone which inhibits the biosynthesis of prostanoids by suppressing the induction of PGHS-2, L-745,337 and NS-398 selectively block its cyclo-oxygenase activity. Copeland *et al.* (1994) have recently demonstrated that NS-398 and DuP 697, compounds of the general form of aryl methyl sulphonamides and aryl methyl sulphonyls, irreversibly inactivate PGHS-2 in a time-dependent fashion while showing minimal reversible inhibition of PGHS-1. They suggested that binding of these inhibitors to PGHS-2 induces a slow structural transition of the enzyme that results in its selective inactivation (Copeland *et al.*, 1994).

In conclusion, the development of anti-inflammatory compounds that are selective inhibitors of the cyclo-oxygenase activity of PGHS-2, such as L-745,337 and NS-398, may provide adequate tools to test the contribution of this novel pathway of arachidonate metabolism to human inflammatory disease and to verify the hypothesis that the unwanted side-effects of NSAIDs are due to their ability to affect the activity of PGHS-1 (Vane, 1994). The method of PGHS isozyme inhibition assessment *in vitro* we have used in the present study allows evaluation of the clinical pharmacology of the same compounds during phase I/II studies.

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