DIFFERENTIAL INHIBITION OF HUMAN PROSTAGLANDIN ENDOPEROXIDE SYNTHASE-1 AND -2 BY NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

We have evaluated the selectivity in vitro of various conventional nonsteroidal anti-inflammatory drugs (NSAIDs) and new anti-inflammatory compounds (NS-398, L-745,337 and SC58125) in inhibiting the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase (PGHS)-1 and monocyte PGHS-2 in a human whole blood assay. The effects of the compounds towards the cyclooxygenase activity of monocyte PGHS-2 induced in response to lipopolysaccharide (LPS) was evaluated by measuring the levels of PGE_2 produced in plasma. The effects of the same inhibitors on platelet PGHS-1 activity were assessed by allowing 1-ml whole blood samples to clot at 37°C for 1 h in the presence of the compounds and measuring immunoreactive TXB_2 levels in serum. Under these experimental conditions, most compounds resulted equipotent towards the two isozymes. Differently, meloxicam, nimesulide and diclofenac were approximately 10- to 20-fold more potent in inhibiting the cyclooxygenase activity of monocyte PGHS-2 than platelet PGHS-1. L-745,337, NS-398 and SC58125 achieved selective inhibition of monocyte PGHS-2 (IC_{50} PGHS-1/IC_{50} PGHS-2: <100) and 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 common side-effects of NSAIDs are due primarily to their ability to affect the activity of PGHS-1.

Key words: Prostaglandin endoperoxide synthases; human blood monocytes; human platelets; nonsteroidal anti-inflammatory drugs; selective PGHS-2 inhibitors

INTRODUCTION

Monocytes/macrophages release pro-inflammatory mediators in response to tissue injury or infection. Prostaglandins (PGs) and thromboxane (TX)A_2, collectively termed prostanoids, are biologically active compounds that participate in both acute and chronic inflammation. They are formed by two successive metabolic steps: 1) arachidonic acid (AA) is released from membranous phospholipids by phospholipase A_2 (PLA_2) which consists of two forms, secretory (sPLA_2) (1) and cytosolic (cPLA_2) (2); 2) free AA is converted to PGG_2 and then to PGH_2 by the cyclooxygenase and peroxidase activities,
respectively, of the PGH synthase (PGHS, COX) protein, of which there are two isoforms, PGHS-1 and PGHS-2 (reviewed in ref. 3). PGHS-1 is constitutively expressed in most tissues and may undergo slow changes in levels of expression associated with cellular differentiation. PGHS-2 is undetectable in most mammalian tissues, but its expression can be induced rapidly (2—6 h) in response to growth factors, tumor promoters, hormones, bacterial endotoxin (lipopolysaccharide, LPS) and cytokines; in most cases the levels of the protein then decrease rapidly. However, post-transcriptional regulation can contribute to the magnitude and duration of PGHS-2 mRNA expression (4). The anti-inflammatory steroids (i.e. dexamethasone) (5) and IL-4 and IL-10 inhibit the expression of PGHS-2 but not PGHS-1 in LPS-stimulated monocytes/macrophages (6).

Prostanoid synthesis by PGHS-1 and -2 involves different arachidonate substrate pools coupled to different extracellular stimuli and different phospholipase systems (3). Although, PGHS-1 and PGHS-2 appear to be present in the same subcellular locations (endoplasmic reticulum and nuclear envelope), the activity of the constitutive enzyme occurs primarily in the endoplasmic reticulum whereas that of the inducible enzyme occurs both in the cytoplasm and over the surface of the nucleus. Thus, it has been hypothesized that the activity of cytosolic PGHS-2 is involved in increasing PGHS-1-dependent production of prostanoids that act extracellularly while nuclear PGHS-2 may operate to channel prostanoids to the nucleus (3). PGHS-2-dependent production of PGE₂ has been involved in matrix metalloproteinase (7) and IL-6 expression (8). PGHS-2 expression is required for endotoxin-induced prostanoid synthesis (9, 10) and PGHS-2 induction has been demonstrated in vivo under inflammatory conditions (11). The administration of selective PGHS-2 inhibitors to experimental animals suppresses prostanoid biosynthesis in inflamed tissues but not in the gastrointestinal tract (11—13). Thus, it has been suggested that the anti-inflammatory actions of nonsteroidal anti-inflammatory drugs (NSAIDs) are due to inhibition of PGHS-2, whereas the toxic effects on the stomach and kidney are due to inhibition of the constitutive isoenzyme, PGHS-1 (14).

The aim of our study was to evaluate pharmacological inhibition of monocyte PGHS-2 induced in human whole blood in response to LPS by conventional NSAIDs and novel anti-inflammatory drugs.

**MATERIALS AND METHODS**

**Materials**

[^H]-PGE₂ and[^H]-TXB₂ (200-250 Ci/mmol) were from Du Pont de Nemours GmbH (Bad Homburg, Germany). 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, USA). PGE$_2$, TXB$_2$, nimesulide, valeryl salicylate and 6-MNA (6-methoxy-2-naphthyl acetic acid, the active metabolite of nabumetone) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-PGE$_2$ and anti-TXB$_2$ sera were obtained in our laboratory and their characteristics have been described previously (9, 17). Heparin, LPS derived from Escherichia Coli 026:B6, sodium salicylate and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO, USA). L-745,337 was kindly provided by Merck Frosst (Pointe Claire-Dorval, Quebec, Canada) through the courtesy of Dr. A.W. Ford-Hutchinson. NS-398 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). SC58125 was a gift of Dr. P. Isakson (Searle, St Louis, MO, USA). Indomethacin, meloxicam, piroxicam, naproxen, diclofenac, ibuprofen, flurbiprofen were provided by Dr. M. Pairet (Dr. Karl Thomae GmbH, Germany). S-ketoprofen was provided by Laboratorios Menarini (Barcelona, Spain). Rabbit polyclonal antibodies prepared against PGHS-2 peptide (C)-NASSRSGLDDINPTVLLK, which is only present in the carboxyl-terminal (amino acid sequence 580-598) of human PGHS-2 were obtained as described recently (16). Specific rabbit polyclonal antibodies directed against PGHS-1 were a gift from Dr. W.L. Smith (Department of Biochemistry, Michigan State University, USA).

Whole blood assay to evaluate the effects of cyclooxygenase inhibitors on monocyte PGHS-2 activity and platelet PGHS-1 activity in vitro

Heparinized 1-ml whole blood samples were drawn from healthy volunteers (2F, 7M; aged 25-50 yr) 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 different compounds in the presence of LPS (10 mg/ml). Immunoreactive PGE$_2$ levels were measured in plasma by a specific radioimmunoassay as an index of the cyclooxygenase activity of LPS-induced monocyte PGHS-2 (Fig. 1, panel A). The effects of the same inhibitors on platelet PGHS-1 activity were assessed by allowing 1-ml 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 TXB$_2$ levels in serum by a specific radioimmunoassay (Fig. 1, panel B).

Isolation of monocytes from human blood

Mononuclear cells were separated from heparinized whole blood or leukocyte concentrates (buffy coat, obtained from the Blood Bank) by Ficoll-Paque as described by Boyum (15). After centrifugation (400 x 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 0.05M 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 x 10$^5$cells/ml) and their viability (<96%) examined by trypan blue exclusion. The cell suspension was constituted by approximately 90% monocytes. Isolated monocytes were incubated in CDMEM for 24 hr at 37°C in 5% CO$_2$-humidified atmosphere both in the absence and in the presence of LPS (10 mg/ml). The purity of preparations of isolated monocytes was assessed by forward and right-angle scatter measurements using flow cytometry (Coulter, Hialeah, FL, USA).
Fig. 1. Whole blood assay to evaluate the effects of cyclooxygenase inhibitors on monocyte PGHS-2 activity (panel A) and platelet PGHS-1 activity (panel B) in vitro.

Effects of SC58125 on PGE_2 and PGHS isozyme biosynthesis by LPS stimulated monocytes

Isolated monocytes (2 x 10^6 cells/ml) were incubated for 24 h at 37°C with LPS (10 mg/ml) both in the absence and in the presence of SC58125 and the biosynthesis of PGE_2 and PGHS isozymes evaluated.

Western blot analysis

Isolated monocytes (2 x 10^6 cells/ml) were lysed and 10 mg of proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and immunoblotting techniques using rabbit polyclonal antibodies directed against the carboxyl-terminal portion of human PGHS-2 (16) and against PGHS-1 as previously described (9).
Analyses of PGE$_2$ and TXB$_2$

PGE$_2$ and TXB$_2$ were measured by previously described and validated radioimmunoassays (9, 17). 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 1:20 to 1:20,000. We used 4,000 dpm of $[^3H]$PGE$_2$ or $[^3H]$TXB$_2$ and specific rabbit anti-PGE$_2$ and -TXB$_2$ sera diluted 1:120,000 and 1:100,000, respectively. The least detectable concentration was 1—2 pg/ml.

Statistical analysis

Data generated from different experiments were expressed as mean±SD. The sigmoidal dose-response curves were analyzed with ALLFIT, a basic computer program for simultaneous curve-fitting based on a four parameter logistic equation (18).

RESULTS

At 24 h of incubation with 10 mg/ml of LPS, PGE$_2$ production averaged $10,480\pm 4,643$ pg/ml (mean±SD, $n = 7$). Under these experimental conditions, the contribution of platelet PGHS-1 activity to whole blood prostanoid production in response to LPS was suppressed by pretreating the subjects with aspirin (300 mg, 48 h before sampling).

We used the measurement of PGE$_2$ produced in whole blood in response to LPS stimulation for 24 h as an index of the cyclooxygenase activity of monocyte PGHS-2 (Fig. 1, panel A) and evaluated the inhibitory effects of various conventional NSAIDs and new anti-inflammatory compounds in vitro. These effects were compared with the inhibition of the cyclooxygenase activity of platelet PGHS-1 by assessing the production of TXB$_2$ during whole blood clotting for 60 minutes (Fig. 1, panel B). Serum immunoreactive TXB$_2$ averaged $357\pm 200$ ng/ml (mean±SD, $n = 7$).

As shown in the table, under these experimental conditions, a number of conventional NSAIDs (ibuprofen, naproxen, S-ketoprofen, flurbiprofen, indomethacin and piroxicam) and 6-MNA, the active metabolite of nabumetone, resulted equipotent towards the two isozymes. The ratio of IC$_{50}$ for PGHS-1 and IC$_{50}$ for PGHS-2 ranged between 0.50 and 3.1 but ALLFIT analysis of the sigmoidal dose-response curves for inhibition of the two isozymes by the different compounds did not show any statistically significant difference. Sodium salicylate inhibited both isozymes with IC$_{50}$ of approximately 1.5 mM. In contrast, valeryl salicylate resulted a selective inhibitor of platelet PGHS-1. Meloxicam, nimesulide and diclofenac were approximately 10- to 20-fold more potent in inhibiting the cyclooxygenase activity of monocyte PGHS-2 than platelet PGHS-1. L-745,337, NS-398 and SC58125 achieved selective inhibition of monocyte PGHS-2 (IC$_{50}$ ratio: < 100).
As shown in Fig. 2, in isolated human monocytes stimulated for 24 h with 10 mg/ml of LPS, SC58125 at concentrations that completely suppressed the production of PGE\(_2\) did not affect the induction of PGHS-2 as analyzed by Western blot using specific antibodies directed against a unique aminoacid sequence present in human PGHS-2 (16). Similarly the constitutive expression of PGHS-1 was not affected to any detectable extent.

Table 1. Inhibition of human whole blood PGHS-1 and PGHS-2 activities by cyclooxygenase inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_{50}) for PGHS-1 (\mu)M</th>
<th>IC(_{50}) for PGHS-2 (\mu)M</th>
<th>IC(<em>{50}) for PGHS-1/IC(</em>{50}) for PGHS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valeryl Salicylate</td>
<td>1058 ± 400</td>
<td>&gt; 4500</td>
<td>&lt; 0.24</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>9.2 ± 4.2</td>
<td>18.3 ± 5.4</td>
<td>0.50</td>
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<tr>
<td>Naproxen</td>
<td>15.6 ± 8</td>
<td>28 ± 12</td>
<td>0.56</td>
</tr>
<tr>
<td>S-Ketoprofen</td>
<td>0.11 ± 0.04</td>
<td>0.18 ± 0.06</td>
<td>0.61</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>0.90 ± 0.27</td>
<td>0.90 ± 0.25</td>
<td>1.00</td>
</tr>
<tr>
<td>Sodium Salicylate</td>
<td>1528 ± 304</td>
<td>1481 ± 282</td>
<td>1.03</td>
</tr>
<tr>
<td>6-MNA*</td>
<td>278 ± 96</td>
<td>187 ± 96</td>
<td>1.49</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.53 ± 0.11</td>
<td>0.28 ± 0.05</td>
<td>1.90</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>2.9 ± 1.3</td>
<td>0.93 ± 0.50</td>
<td>3.10</td>
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<tr>
<td>Meloxicam</td>
<td>4.8 ± 0.44</td>
<td>0.43 ± 0.05</td>
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<tr>
<td>Nimesulide</td>
<td>9.2 ± 2</td>
<td>0.52 ± 0.06</td>
<td>17.70</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>0.34 ± 0.06</td>
<td>0.018 ± 0.003</td>
<td>18.90</td>
</tr>
<tr>
<td>SC58125</td>
<td>38.7 ± 25</td>
<td>0.27 ± 0.17</td>
<td>143.30</td>
</tr>
<tr>
<td>NS-398</td>
<td>16.8 ± 11</td>
<td>0.10 ± 0.03</td>
<td>168.00</td>
</tr>
<tr>
<td>L-745, 337</td>
<td>369 ± 61</td>
<td>1.5 ± 0.34</td>
<td>246.00</td>
</tr>
</tbody>
</table>

* 6-MNA, 6-methoxy-2-naphthyl acetic acid, the activite metabolite of Nabumetone
DISCUSSION

We have recently demonstrated monocyte PGHS-2-dependent formation of the pro-inflammatory PGE$_2$ and the vasoconstrictor and platelet agonist 8-epi-PGF$_{2a}$ formation in human whole blood in response to LPS (19). The induction of monocyte PGHS-2 in response to LPS is a simple model that is suitable for evaluating the extent of PGHS-2 inhibition both in vitro and after oral administration of NSAIDs in humans (9). Evaluation of drug selectivity towards the two PGHS isozymes in vitro showed that a number of conventional NSAIDs are equipotent towards PGHS-1 and PGHS-2. In contrast, NS-398, L-745,337 and SC58125, novel anti-inflammatory and analgesic agents that do not produce gastrointestinal lesions following oral administration to experimental animals (11—13), resulted <100-fold more potent towards monocyte PGHS-2 than platelet PGHS-1. At variance with dexamethasone, which inhibits the biosynthesis of prostanoids by suppressing the induction of PGHS-2, L-745, 337, NS-398 (20) and SC58125 (in the present study) selectively block its cyclooxygenase activity. These aryl-methyl-sulphonamide derivatives are weak competitive inhibitors of PGHS-1 but inhibit PGHS-2 in a slow, time-dependent process that seems to be due to the penetration of the phenyl-sulphonamide moiety into a second internal pocket extending off the NSAID binding site of the inducible enzyme (21—23). These compounds inhibit PGHS-1 in a simple competitive manner, probably because of lack of this secondary pocket in PGHS-1 (24).

The development of highly selective inhibitors of the cyclooxygenase activity of PGHS-2, such as L-745, 337, SC58125 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 common side-effects of NSAIDs are due primarily to their ability to affect the activity of PGHS-1.

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