British Journal of Pharmacology (1996) 118, 1285-1293

Induction of prostaglandin endoperoxide synthase-2 in human monocytes associated with cyclo-oxygenase-dependent F_2-isoprostane formation

[1]Paola Patrignani, Giovanna Santini, Maria R. Panara, Maria G. Sciulli, Anita Greco, Maria T. Rotondo, Maria di Giamberardino, †Jacques Maclouf, *Giovanni Ciabattoni & Carlo Patrono

Departments of Pharmacology, University of Chieti "G. D'Annunzio" School of Medicine, 66013 Chieti; *Catholic University School of Medicine, 00168 Rome, Italy, and †I.F.R. Biologie de la Circulation-Lariboisière, Institut National de la Santé et de la Recherche Médicale, Unité 348, Hopital Lariboisière, 75475 Paris, France

1 The isoprostane 8-epi-prostaglandin (PG)$F_{2\alpha}$ is produced by free radical-catalyzed peroxidation of arachidonic acid. It may also be formed as a minor product of the cyclo-oxygenase activity of platelet PGH synthase (PGHS)-1. We investigated 8-epi-$PGF_{2\alpha}$ production associated with induction of the human monocyte PGHS-2 and its pharmacological modulation.

2 Heparinized whole blood samples were drawn from healthy volunteers, 48 h following oral dosing with aspirin 300 mg to suppress platelet cyclo-oxygenase activity. One ml aliquots were incubated with lipopolysaccharide (LPS: 0.1-50 $\mu g\ ml^{-1}$) for 0-24 h at 37°C. PGE_2 and 8-epi-$PGF_{2\alpha}$ were measured in separated plasma by radioimmunoassay and enzyme immunoassay techniques.

3 Levels of both eicosanoids were undetectable (i.e. $<60\ pg\ ml^{-1}$) at time 0. LPS induced the formation of PGE_2 and 8-epi-$PGF_{2\alpha}$ in a time- and concentration-dependent fashion, coincident with the induction of PGHS-2 detected by Western blot analysis of monocyte lysates. After 24 h at 10 $\mu g\ ml^{-1}$ LPS, immunoreactive PGE_2 and 8-epi-$PGF_{2\alpha}$ averaged $10,480 \pm 4,643$ and $295 \pm 140\ pg\ ml^{-1}$ (mean ± s.d., $n=6$), respectively.

4 Dexamethasone and 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone (L-745,337), a selective inhibitor of the cyclo-oxygenase activity of PGHS-2, reduced PGE_2 and 8-epi-$PGF_{2\alpha}$ production in response to LPS.

5 Isolated monocytes produced PGE_2 and 8-epi-$PGF_{2\alpha}$ in response to LPS (10 $\mu g\ ml^{-1}$) in a time-dependent fashion. Monocyte PGE_2 and 8-epi-$PGF_{2\alpha}$ production was largely prevented by dexamethasone (2 μM) and cycloheximide (10 $\mu g\ ml^{-1}$) in association with suppression of PGHS-2 but not of PGHS-1 expression.

6 We conclude that the induction of PGHS-2 in human monocytes is associated with cyclo-oxygenase-dependent generation of the vasoconstrictor and platelet-agonist 8-epi-$PGF_{2\alpha}$.

Keywords: Prostaglandin endoperoxide synthases; 8-epi-$PGF_{2\alpha}$; PGE_2; human blood monocytes; L-745,337; dexamethasone

Introduction

The F_2-isoprostanes are formed from arachidonic acid (AA) which undergoes peroxidation catalyzed by free radicals to yield arachidonyl radical intermediates. These are then transformed to a series of prostaglandin(PG) F_2-like compounds (Morrow *et al.*, 1990, 1994b). Unlike cyclo-oxygenase-derived prostanoids, F_2-isoprostanes are initially formed *in situ* on phospholipids, from which they are subsequently released preformed, presumably by phospholipases (Morrow *et al.*, 1992). They circulate in plasma and are excreted in urine (Morrow *et al.*, 1994a). 8-epi-$PGF_{2\alpha}$ is an abundant F_2-isoprostane formed *in vivo* in man (Morrow *et al.*, 1994a). The administration of nonsteroidal antiinflammatory drugs (NSAIDs) to healthy volunteers did not significantly affect the urinary excretion of 8-epi-$PGF_{2\alpha}$ (Wang *et al.*, 1995). This result supports its proposed formation by a cyclo-oxygenase-independent mechanism.

Prostaglandin endoperoxide synthase (PGHS) is the first enzyme in the pathway leading from AA to prostanoids [PGs and thromboxane(TX)s]. PGHS is a bifunctional enzyme which exhibits both cyclo-oxygenase and peroxidase activities. It catalyzes the conversion of AA to PGG_2 and PGG_2 to PGH_2 (DeWitt, 1991). Two isoforms of PGHS have been identified. PGHS-1 is a constitutive enzyme present in almost all cell types whereas PGHS-2 has a restricted tissue distribution and is expressed at a very low basal level but is highly inducible in response to mitogenic and inflammatory stimuli (Smith & DeWitt, 1995).

In monocytes/macrophages, lipopolysaccharide (LPS) and pro-inflammatory cytokines stimulate the production of prostanoids by inducing the expression of PGHS-2 gene (O'Sullivan *et al.*, 1992; O'Banion *et al.*, 1992; Patrignani *et al.*, 1994; Hempel *et al.*, 1994; Reddy & Herschman, 1994). PGHS-1, constitutively present in these cells cannot utilize endogenous AA released from membrane stores in response to different extracellular signals (Reddy & Herschman, 1994).

The biosynthesis of PGHS-2 is inhibited by glucocorticoids (O'Banion *et al.*, 1992; Kujubu & Herschman, 1992; Lee *et al.*, 1992). Moreover, selective inhibitors of the cyclo-oxygenase activity of PGHS-2 have been developed (Masferrer *et al.*, 1994; Chan *et al.*, 1994; Seibert *et al.*, 1994). We have recently demonstrated that NS-398 and L-745,337, derivatives of aryl methyl sulphonamides, are approximately 200 fold better inhibitors of the cyclo-oxygenase activity of monocyte PGHS-2 than platelet PGHS-1 (Panara *et al.*, 1995).

Recently, Praticò *et al.* (1995) have demonstrated that 8-epi-$PGF_{2\alpha}$ may also be formed as a minor product of the cyclo-oxygenase activity of platelet PGHS-1. This observation is

[1]Author for correspondence at: Cattedra di Farmacologia I, Università di Chieti "G. D'Annunzio", Palazzina delle Scuole di Specializzazione, Via dei Vestini, 31, 66013 Chieti, Italy.

consistent with the schema of formation of 8-epi-$PGF_{2\alpha}$ via the corresponding endoperoxide by a biomimetic cyclization proposed by Corey *et al.* (1984). Moreover, Hecker *et al.* (1987) demonstrated that 8-epi-$PGF_{2\alpha}$ is a product of the ram seminal vesicle PGHS.

We have investigated whether 8-epi-$PGF_{2\alpha}$ formation is associated with the induction of PGHS-2 in human monocytes in response to LPS. We studied the pharmacological modulation of monocyte 8-epi-$PGF_{2\alpha}$ formation by inhibitors of PGHS-2 biosynthesis (dexamethasone and cycloheximide) and a selective inhibitor of the cyclo-oxygenase activity of PGHS-2, 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone (L-745,337) (Chan *et al.*, 1994; Panara *et al.*, 1995).

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

Methods

Subjects

Nine healthy volunteers (5 female and 4 male subjects; aged 23–50 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 i.u. of sodium heparin were incubated both in the absence and in the presence of LPS (0.1–50 $\mu g\ ml^{-1}$) for 0–24 h at 37°C as recently described (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 700 *g*) and kept at −80°C until assayed for PGE_2 or immediately processed for the measurement of 8-epi-$PGF_{2\alpha}$.

Isolation of mononuclear cells and polymorphonuclear leukocytes (PMNs) 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 (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 approximately 90% monocytes, as confirmed by nonspecific esterase staining and positivity by immunofluorescence for anti-CD14 monoclonal antibodies. The adherent cells were recovered by gently scraping with a rubber policeman, resuspended in CDMEM (3×10^6 cells ml^{-1}) and their viability (>96%) examined by trypan blue exclusion. The non-adherent cells, were replated twice in plastic Petri dishes for 60 min in order to obtain a cell suspension consisting predominantly of lymphocytes (>90%) and then resuspended in CDMEM (3×10^6 cells ml^{-1}). PMNs were separated from erythrocytes by dextran sedimentation (Boyum, 1968). After washing the cells twice with phosphate-buffered saline, contaminating erythrocytes were lysed with 0.83% ammonium chloride. The remaining cells were washed and resuspended in CDMEM (3×10^6 cells ml^{-1}). This preparation contained 98% PMNs of greater than 98% viability as determined by trypan blue exclusion. Isolated monocytes, lymphocytes and PMNs were incubated in CDMEM for 0–24 h at 37°C in 5% CO_2-humidified atmosphere both in the absence and in the presence of LPS (10 $\mu g\ ml^{-1}$). The purity of preparations of isolated monocytes, PMNs and lymphocytes was assessed by forward and right-angle scatter measurements using flow cytometry (Coulter, Hialeah, FL, U.S.A.).

Effects of L-745,337 and dexamethasone on the biosynthesis of PGE_2 and 8-epi-$PGF_{2\alpha}$ by LPS-stimulated whole blood

L-745,337 (0.0026–26 mM) and dexamethasone (0.2 mM) were dissolved in methanol and 10 μl aliquots of these solutions were pipetted directly into test tubes to give a final concentration in whole blood or monocyte suspensions of 0.026–260 μM of L-745,337 or 2 μM of dexamethasone. The methanol was evaporated and 1 ml aliquots of whole blood were added. The effects of these inhibitors on the formation of PGE_2 and 8-epi-$PGF_{2\alpha}$ were studied by incubating the compounds with multiple heparinized whole blood samples in the presence of LPS (10 $\mu g\ ml^{-1}$) for 24 h.

Effects of dexamethasone and cycloheximide on the biosynthesis of PGE_2 and 8-epi-$PGF_{2\alpha}$ and PGHS isozymes by LPS-stimulated human isolated monocytes

Dexamethasone (2 μM) and cycloheximide (10 $\mu g\ ml^{-1}$) were incubated with human isolated monocyte suspensions (3×10^6 cells ml^{-1}) for 24 h at 37°C with LPS (10 $\mu g\ ml^{-1}$). The levels of PGE_2 and 8-epi-$PGF_{2\alpha}$ were measured in the culture medium while PGHS isozyme mass was evaluated in monocyte lysates by Western blot.

Reversed-phase high performance liquid chromatography (r.p.-h.p.l.c.) of eicosanoids formed by LPS-stimulated whole blood or isolated monocytes

[^{3}H]-8-epi-$PGF_{2\alpha}$ (3,000 d.p.m.) was added to 6 ml of plasma obtained from whole blood samples incubated for 24 h with LPS or to 3 ml of monocyte culture medium for recovery evaluation. After adjusting the pH to 4–4.5 with formic acid, prostanoids were extracted from plasma or monocyte culture medium on C-18 Sep-Pak cartridges and eluted with 10 ml of ethyl acetate. BHT (0.005%) was added to plasma prior to lipid extraction. After evaporation of the ethyl acetate to dryness, the extracts were reconstituted with 200 μl of methanol-water (1:1 vol/vol) and injected into a Nova-Pak C-18 column of a Beckman System Gold h.p.l.c. and eluted with a solvent system of acetonitrile-water-glacial acetic acid (23:77:0.1 vol/vol) at a flow rate of 1 ml min^{-1} and u.v. absorption at 195 nm. Authentic 6-keto-$PGF_{1\alpha}$, 8-epi-$PGF_{2\alpha}$, TXB_2, $PGF_{2\alpha}$, 8-epi-PGE_2 and PGE_2 eluted with retention times of 11.5, 29, 36, 49.1 and 58.3 min, respectively; 1 ml fractions were collected, evaporated to dryness, and reconstituted with 250 μl of phosphate buffer (0.02 M, pH 7.4). Radioactivity in h.p.l.c. fractions corresponding to the retention time of [^{3}H]-8-epi-$PGF_{2\alpha}$ was measured by scintillation counting to determine recovery (60±5%). All fractions were evaluated for the content of 8-epi-$PGF_{2\alpha}$-like immunoreactivity by radioimmunoassay using two different anti-8-epi-$PGF_{2\alpha}$ sera (No. 9 and No. 1).

Analyses of PGE_2 and 8-epi-$PGF_{2\alpha}$

PGE_2 and 8-epi-$PGF_{2\alpha}$ concentrations were measured by previously described and validated RIAs (Ciabattoni *et al.*, 1979; Wang *et al.*, 1995). Unextracted plasma samples and cell culture media as well as r.p.-h.p.l.c. fractions 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 1:20–1:500. We used 4,000 d.p.m. of [^{3}H]-PGE_2 or 3,000 d.p.m. of [^{3}H]-8-epi-$PGF_{2\alpha}$ and specific rabbit anti-PGE_2 or two different anti-8-epi-$PGF_{2\alpha}$ sera (No. 9 and No. 1) diluted 1:120,000, 1:100,000 and 1:200,000, respectively. The least detectable concentration was 1–2 pg ml^{-1} for all assays.

Thirteen different samples that were analyzed by RIA for the content of 8-epi-PGF$_{2\alpha}$ were also quantified by enzyme immunoassay (EIA) by a previously described procedure (Wang *et al.*, 1995) employing acetylcholinesterases from the electric eel coupled to 8-epi-PGF$_{2\alpha}$ as the tracer.

Western blot analysis

Isolated monocytes, lymphocytes and PMNs (3×10^6 cells) were lysed in phosphate-buffered saline (pH 7.4) containing 0.5% Nonidet P-40, 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} bromophenol blue. The samples were adjusted for equal amounts of proteins and 10 μg of protein lysate were analyzed 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$_2$ (200 Ci mmol^{-1}) was from DuPont de Nemours GmbH (Bad Homburg, Germany) while [^{3}H]-8-epi-PGF$_{2\alpha}$ (25 Ci mmol^{-1}) was from DuPont NEN (Boston, MA, U.S.A.). TXB$_2$, 6-keto-PGF$_{1\alpha}$, PGE$_2$ and 8-epi-PGF$_{2\alpha}$ were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). 8-epi-PGE$_2$ was kindly provided by Dr L.J. Roberts II (Division of Clinical Pharmacology, Vanderbilt University). Anti-PGE$_2$ and anti-8-epi-PGF$_{2\alpha}$ sera were obtained in our laboratory and their characteristics have been described previously (Ciabattoni *et al.*, 1979; Wang *et al.*, 1995). 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, monoclonal antibodies to human CD14, streptavidin-peroxidase, phenylmethylsulphonyl fluoride, leupeptin, pepstatin A, Nonidet P-40, dextran 60, dimethyl sulphoxide (DMSO), dexamethasone, cyclohexamide, bovine serum albumin, Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)], Tween 20 and butylated hydroxytoluene (BHT) 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 Dr A.W. Ford-Hutchinson. 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 & 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 Univesity). C-18 Sep-Pak cartridges and Nova-Pack C-18 column (4 μm; 0.39 x 15 cm) were purchased from Waters Associates (Milford, MA, U.S.A.).

Statistical analysis

Data generated from different experiments were expressed as mean ± s.d. Statistical comparisons were made by Student's unpaired *t* test and by analysis of variance and significant differences between treatments were determined by Student's Newman-Keuls test. The sigmoidal dose-response curves were analyzed with ALLFIT, a basic computer programme for simultaneous curve-fitting based on a four parameter logistic equation (De Lean *et al.*, 1978).

Results

As shown in Figure 1, incubation of heparinized whole blood with LPS (10 μg ml^{-1}) resulted in a time-dependent enhancement of the formation of PGE$_2$ and 8-epi-PGF$_{2\alpha}$. At 24 h of incubation, PGE$_2$ and 8-epi-PGF$_{2\alpha}$ production was significantly increased *vs* saline control. PGE$_2$ and 8-epi-PGF$_{2\alpha}$

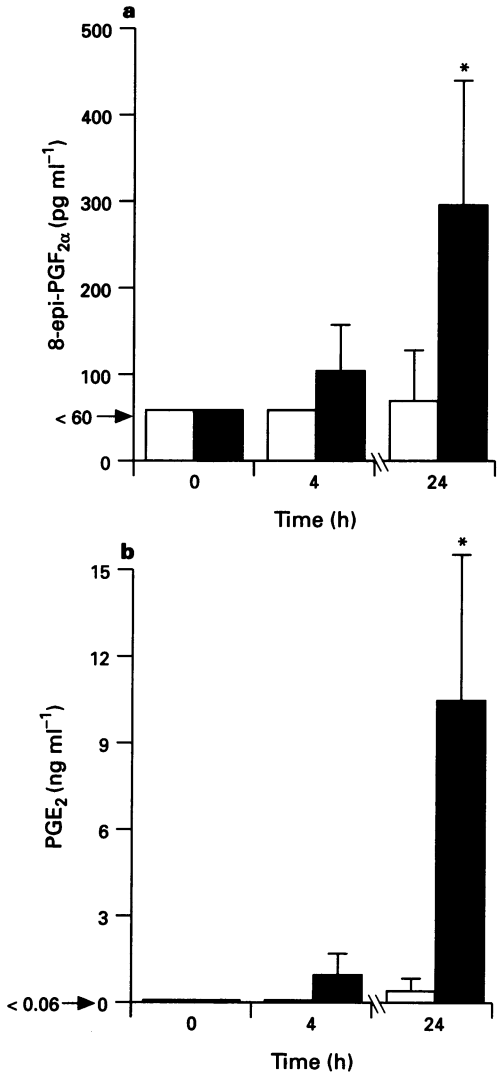

Figure 1 Time-course of 8-epi-PGF$_{2\alpha}$ and PGE$_2$ production by human whole blood incubated with LPS or saline. Duplicate 1 ml aliquots of heparinized whole blood samples drawn from healthy volunteers pretreated with aspirin (300 mg 48 h prior to sampling) were incubated both in the absence (saline, □) and in the presence of LPS (10 μg ml^{-1}, ■) for 0 to 24 h at 37°C. At the indicated times plasma was separated by centrifugation (10 min at 700 *g*) and assayed for 8-epi-PGF$_{2\alpha}$ (a) and PGE$_2$ (b) by specific RIA techniques. Values are reported as mean ± s.d. from 6 separate experiments; LPS *versus* saline, $^*P < 0.05$.

averaged 10,480±4,643 and 295±140 pg ml^{-1} (mean±s.d., $n=6$), respectively. 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). The induction of PGE_2 and 8-epi-$PGF_{2\alpha}$ production by LPS was dose-dependent (Figure 2).

In order to characterize the nature of 8-epi-$PGF_{2\alpha}$-like immunoreactivity detected by a specific anti-8-epi-$PGF_{2\alpha}$ serum (No. 9) in LPS-stimulated whole blood samples, [^{3}H]-8-epi-$PGF_{2\alpha}$ was added to plasma samples which were then subjected to extraction and r.p.-h.p.l.c. separation. 8-epi-$PGF_{2\alpha}$-like immunoreactivity and radioactivity were measured in all h.p.l.c. fractions. As shown in Figure 3, 8-epi-$PGF_{2\alpha}$-like immunoreactivity and [^{3}H]-8-epi-$PGF_{2\alpha}$ co-eluted with the u.v. adsorption (195 nm) profile of authentic 8-epi-$PGF_{2\alpha}$. Moreover, no detectable 8-epi-$PGF_{2\alpha}$-like immunoreactivity was found in other r.p.-h.p.l.c. fractions coincident with the retention times of other potentially cross-reacting eicosanoids, including 8-epi-PGE_2. The levels of 8-epi-$PGF_{2\alpha}$ measured in a plasma sample by direct radioimmunoassay and after extraction and r.p.-h.p.l.c. separation were 0.9 and 1.1 ng ml^{-1}, respectively.

To evaluate whether LPS-induced production of PGE_2 and 8-epi-$PGF_{2\alpha}$ by whole blood was dependent on newly synthesized PGHS-2 expressed by blood cells, we studied the effects of dexamethasone that has been reported to inhibit PGHS-2 biosynthesis (O'Banion *et al.*, 1992; Kujubu & Herschman, 1992; Lee *et al.*, 1992). Dexamethasone (2 μM) suppressed PGE_2 and 8-epi-$PGF_{2\alpha}$ production in LPS-stimulated whole blood by more than 80% (Table 1).

Further evidence for the involvement of the cyclo-oxygenase activity of PGHS-2 in 8-epi-$PGF_{2\alpha}$ production in response to LPS was obtained by investigating the effects of L-745,337, a selective inhibitor of PGHS-2 (Chan *et al.*, 1994; Panara *et al.*, 1995). As shown in Figure 4, L-745,337 reduced LPS-induced PGE_2 and 8-epi-$PGF_{2\alpha}$ formation in whole blood with virtually identical dose–response curves.

To characterize the cellular source of PGE_2 and 8-epi-$PGF_{2\alpha}$ production in whole blood, we studied their production by human isolated monocytes, lymphocytes and PMNs. PGE_2

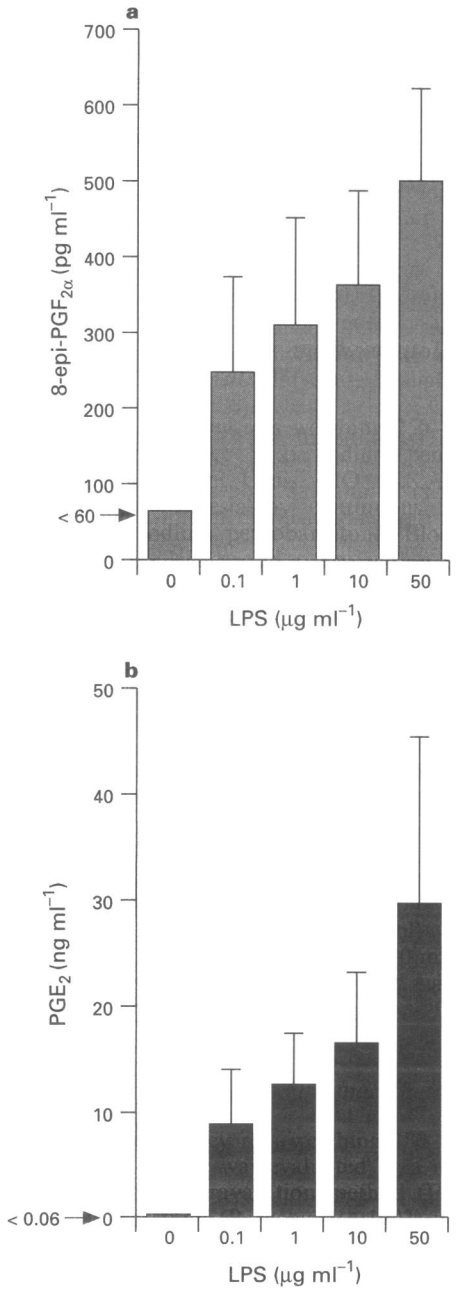

Figure 2 Dose-response curves for 8-epi-$PGF_{2\alpha}$ and PGE_2 production in human whole blood incubated with LPS. Duplicate 1 ml aliquots of heparinized whole blood samples drawn from healthy volunteers pretreated with aspirin (300 mg 48 h prior to sampling) were incubated with LPS (0.1–50 μg ml^{-1}) for 24 h at 37°C. Plasma was separated by centrifugation (10 min at 700 *g*) and assayed for 8-epi-$PGF_{2\alpha}$ (a) and PGE_2 (b) by specific RIA techniques. Values are reported as mean±s.d. from 3 separate experiments.

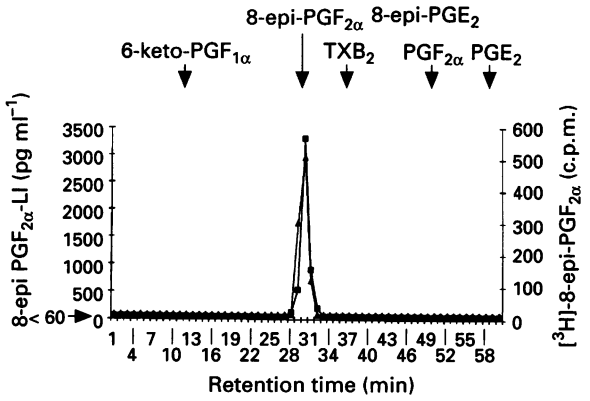

Figure 3 Co-elution of [^{3}H]-8-epi-$PGF_{2\alpha}$ and 8-epi-$PGF_{2\alpha}$-like immunoreactivity(LI) extracted from plasma with the u.v. absorption (195 nm) profile of authentic 8-epi-$PGF_{2\alpha}$ by r.p.-h.p.l.c.: 3,000 d.p.m. of [^{3}H]-8-epi-$PGF_{2\alpha}$ and 0.005% BHT were added to a plasma sample (6 ml), obtained from whole blood incubated for 24 h with LPS. After C-18 Sep-Pak extraction, eluted prostanoids were subjected to separation by r.p.-h.p.l.c. using the solvent system acetonitrile/water/glacial acetic acid (23:77:0.1, vol/vol), a flow rate of 1 ml min^{-1} and u.v. absorption at 195 nm. Radioactivity (■) and 8-epi-$PGF_{2\alpha}$-LI (▲) were evaluated in the h.p.l.c. fractions. The arrows indicate the retention times of authentic standards.

Table 1 Effects of LPS and dexamethasone on PGE_2 and 8-epi-$PGF_{2\alpha}$ production in human whole blood incubated for 24 h at 37°C

Addition to whole blood	*PGE_2* (pg ml^{-1})	*8-epi-$PGF_{2\alpha}$* (pg ml^{-1})
None	<60	<60
LPS (10 μg ml^{-1})	10,000±5,000	374±176
LPS+dexamethasone (2 μM)	560±140*	<60*

Values are mean±s.d., $n=3$; LPS+dexamethasone *versus* LPS, *$P<0.05$.

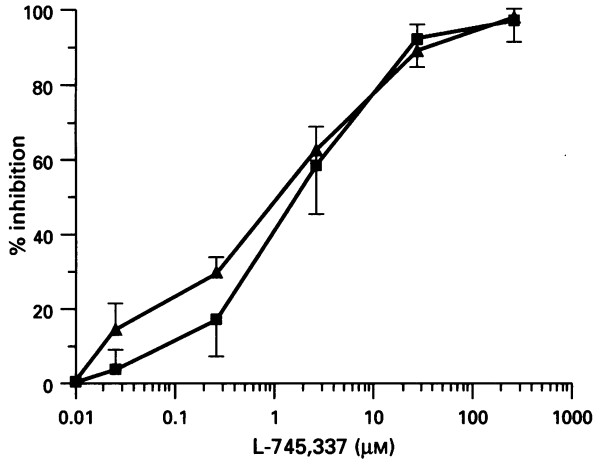

Figure 4 Inhibitory effects of L-745,337 on 8-epi-PGF$_{2\alpha}$ (▲) and PGE$_2$ (■) production by LPS-stimulated whole blood at 24 h. Increasing concentrations of L-745,337 (0.026–260 μM) were incubated with 1 ml heparinized whole blood samples in the presence of LPS (10 μg ml^{-1}) for 24 h at 37°C. Plasma was separated by centrifugation (10 min at 700 *g*) and assayed for 8-epi-PGF$_{2\alpha}$ and PGE$_2$ by specific RIA techniques. Values are reported as percentage inhibition (mean ± s.d.) from 3 separate experiments.

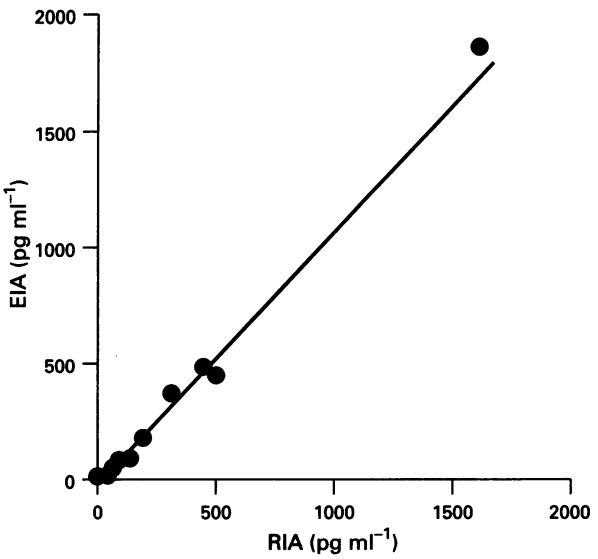

Figure 5 Correlation between the levels of 8-epi-PGF$_{2\alpha}$-like immunoreactivity measured by RIA and EIA in thirteen samples of monocyte culture medium. Supernatants of isolated monocytes collected under different experimental conditions with 8-epi-PGF$_{2\alpha}$-like immunoreactivity ranging from 10 to 1600 pg ml^{-1}, as detected by RIA, were also assayed by EIA as described in Methods. A highly significant correlation ($r = 0.985$, $P < 0.001$, $n = 12$) was found between the two sets of measurements, even when the sample with the highest concentration of 8-epi-PGF$_{2\alpha}$-like immunoreactivity was excluded.

and 8-epi-PGF$_{2\alpha}$ production was undetectable (< 20 pg per 10^6 cells) in human PMNs and lymphocytes. In contrast, PGE$_2$ and 8-epi-PGF$_{2\alpha}$ were produced by isolated monocytes in a time-dependent fashion. At 24 h of incubation with LPS (10 μg ml^{-1}), the production of PGE$_2$ and 8-epi-PGF$_{2\alpha}$ averaged 5,700 ± 2,800 and 214 ± 144 pg per 10^6 cells (mean ± s.d., $n = 3$), respectively.

To validate the measurements of immunoreactive 8-epi-PGF$_{2\alpha}$ in monocyte culture medium, we compared the concentrations detected by two anti-8-epi-PGF$_{2\alpha}$ sera showing different cross-reactivities with other eicosanoids (Wang *et al.*, 1995) both before and after extraction and r.p.-h.p.l.c. separation. The level of 8-epi-PGF$_{2\alpha}$ measured by direct radioimmunoassay in the culture medium of monocytes exposed to LPS was 1.3 ng ml^{-1} with both antisera. This value was virtually identical to those measured using antisera No. 1 and No. 9 in the h.p.l.c. fractions corresponding to the retention time of authentic 8-epi-PGF$_{2\alpha}$ (1.4 and 1.3 ng ml^{-1}, respectively). Exogenous 8-epi-PGF$_{2\alpha}$ (1.25 ng ml^{-1}) added to CDMEM was recovered quantitatively (90 ± 7%) when assayed by direct radioimmunoassay or after extraction and h.p.l.c. separation using the two different anti-8-epi-PGF$_{2\alpha}$ sera, thus indicating the stability of 8-epi-PGF$_{2\alpha}$ under these experimental conditions as well as the absence of cross-reacting material(s) in the culture medium. Moreover, we compared the levels 8-epi-PGF$_{2\alpha}$-like immunoreactivity measured by RIA and EIA in thirteen different samples of monocyte culture medium encompassing a 150 fold range of concentrations. As shown in Figure 5, a highly significant linear correlation ($r = 0.997$, $P < 0.001$) was found between the two sets of measurements.

Prostanoid formation in response to LPS increased in parallel with the mass of a monocyte protein doublet analyzed by Western blot (Patrignani *et al.*, 1994) using antibodies directed against the carboxyl-terminal portion of human PGHS-2 (Habib *et al.*, 1993). In contrast, the use of specific anti-PGHS-1 antibodies showed that PGHS-1 was present in unstimulated monocytes and was not affected by incubation with LPS up to 24 h (Patrignani *et al.*, 1994).

To demonstrate further that the production of PGE$_2$ and 8-epi-PGF$_{2\alpha}$ by isolated monocytes was dependent on the induction of PGHS-2 in response to LPS, we studied the effects of dexamethasone and cycloheximide. As shown in Figure 6, after 24 h of incubation with LPS (10 μg ml^{-1}), dexamethasone (2 μM) significantly reduced (by approximately 80%) the production of both PGE$_2$ and 8-epi-PGF$_{2\alpha}$. Cycloheximide (10 μg ml^{-1}) completely suppressed both PGE$_2$ and 8-epi-PGF$_{2\alpha}$ production (Figure 7). The induction of the monocyte PGHS-2 protein was correspondingly suppressed by dexamethasone and cycloheximide (Figures 6 and 7). Dexamethasone and cycloheximide had no detectable effect on the expression of monocyte PGHS-1 (Figures 6 and 7).

Discussion

Oxidative stress and lipid peroxidation have been suggested to play a role in a wide spectrum of disorders including atherosclerosis, ischaemia-reperfusion injury, inflammatory diseases, cancer and aging (Halliwell & Grootveld, 1987).

The formation of isoprostanes in lipid bilayers by a free radical-catalyzed mechanism involving peroxidation of AA may contribute in an important way to alterations in fluidity and integrity of cellular membranes. One of these compounds is 8-epi-PGF$_{2\alpha}$ which has attracted considerable attention because of its biological activities. 8-epi-PGF$_{2\alpha}$ is a potent vasoconstrictor (Takahashi *et al.*, 1992; Banerjee *et al.*, 1992) that induces DNA synthesis in vascular smooth muscle cells through the interaction with receptors that are distinct from but closely related to TXA$_2$/PGH$_2$ receptors (Fukunaga *et al.*, 1993). Several lines of evidence suggest that in human platelets, 8-epi-PGF$_{2\alpha}$ acts as a partial agonist of TXA$_2$/PGH$_2$ receptors (Morrow *et al.*, 1992; Praticò *et al.*, 1994; Yin *et al.*, 1994): (1) it induces shape change, but not aggregation and the release reaction, that is blocked by the TXA$_2$/PGH$_2$ receptor antagonist SQ 29548; (2) it inhibits TXA$_2$-dependent aggregation induced by platelet agonists; (3) it potentiates the reversible primary aggregation induced by low-doses of ADP.

Quantification of 8-epi-PG isomers in urine and plasma may provide stable markers of oxidative reactions in human subjects, as they possess longer half-lives than hydroperoxides and/or reactive oxygen species. The urinary excretion of 8-epi-PGF$_{2\alpha}$ significantly correlated with increasing age (Wang *et al.*, 1995). Higher plasma levels of free and esterified F$_2$-isoprostanes and increased urinary excretion of 8-epi-PGF$_{2\alpha}$ have

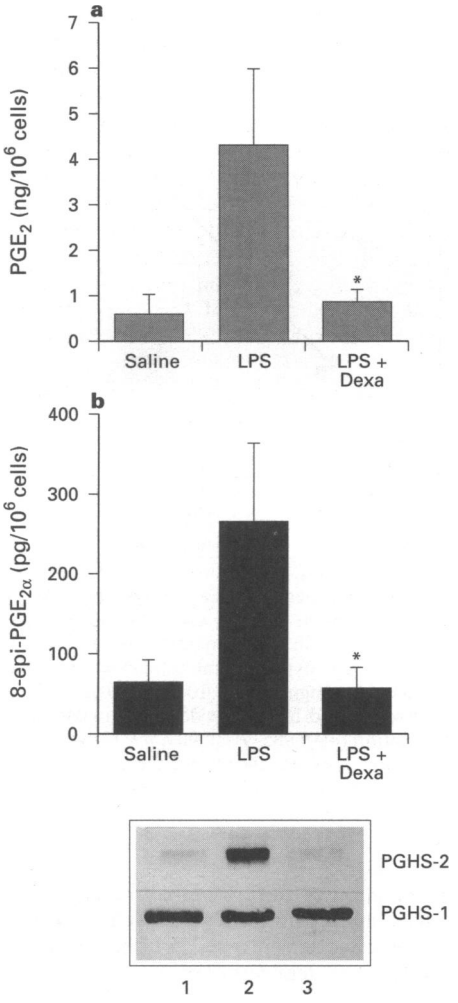

Figure 6 Inhibitory effects of dexamethasone on 8-epi-PGF$_{2\alpha}$ and PGE$_2$ production and PGHS isozyme biosynthesis by LPS-stimulated human monocytes: 1 ml human monocyte suspension (3×10^6 cells) was incubated with saline or LPS ($10\,\mu g\,ml^{-1}$) for 24 h at 37°C both in the absence and in the presence of dexamethasone (Dexa, 2 μM). Supernatants were assayed for PGE$_2$ (a) and 8-epi-PGF$_{2\alpha}$ (b) by specific RIA techniques while monocytes were lysed and proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques using rabbit antibodies directed against PGHS-1 or the carboxyl-terminal of PGHS-2 (c) Equal amounts of proteins ($10\,\mu g$) were loaded in all lanes (lane 1, saline; lane 2, LPS; lane 3, LPS + dexamethasone). Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG and streptavidin-peroxidase. This panel is representative of 3 experiments. Dexamethasone + LPS *versus* LPS, $*P<0.05$.

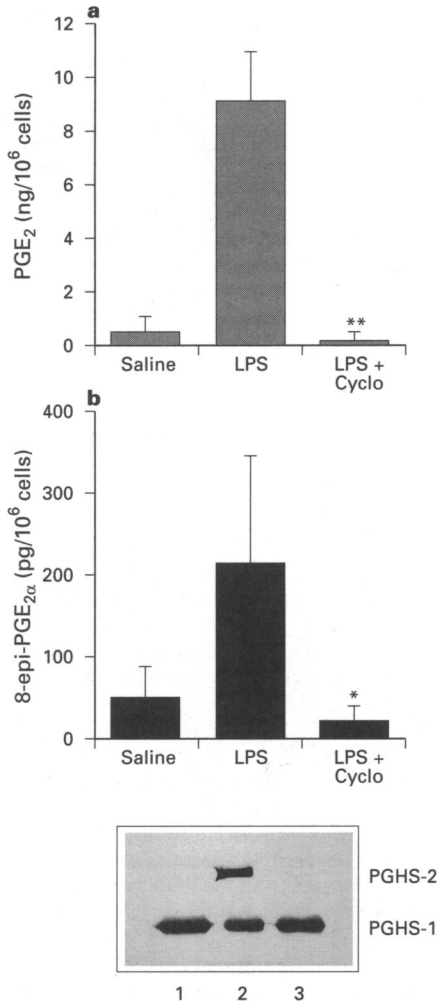

Figure 7 Inhibitory effects of cycloheximide on 8-epi-PGF$_{2\alpha}$ and PGE$_2$ production and PGHS isozyme biosynthesis by LPS-stimulated human monocytes: 1 ml human monocyte suspension (3×10^6 cells) was incubated with saline or LPS ($10\,\mu g\,ml^{-1}$) for 24 h at 37°C both in the absence and in the presence of cycloheximide (Cyclo, $10\,\mu g\,ml^{-1}$). Supernatants were assayed for PGE$_2$ (a) and 8-epi-PGF$_{2\alpha}$ (b) by specific RIA techniques while monocytes were lysed and proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques using rabbit antibodies directed against PGHS-1 or the carboxyl-terminal of PGHS-2 (c) Equal amounts of proteins ($10\,\mu g$) were loaded in all lanes (lane 1, saline; lane 2, LPS; lane 3, LPS + cycloheximide). Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG and streptavidin-peroxidase. This panel is representative of 3 experiments. Cycloheximide + LPS *versus* LPS, $*P<0.05$, $**P<0.01$.

been demonstrated in smokers (Wang *et al.*, 1995; Morrow *et al.*, 1995). This supports the hypothesis that the pathogenesis of diseases induced by cigarette smoking involves oxidative modification of important biological molecules *in vivo*.

In addition to a cyclo-oxygenase-independent mechanism of formation, recent evidence suggests that 8-epi-PGF$_{2\alpha}$ can be produced as a minor product of the activity of platelet PGHS-1 in response to collagen, thrombin and AA (Praticò *et al.*, 1995). Activated platelets may generate 8-epi-PGF$_{2\alpha}$ and TXB$_2$ in a molar ratio of approximately 1 : 1,000 (Wang *et al.*, 1995; Praticò *et al.*, 1995).

A second inducible enzyme endowed with cyclo-oxygenase activity has been discovered (Kujubu & Herschman, 1992; Lee *et al.*, 1992). PGHS-2 gene can be expressed in different cell types in response to pro-inflammatory and mitogenic stimuli (Smith & DeWitt, 1995). We have recently characterized the induction and pharmacological modulation of PGHS-2 in human whole blood stimulated with LPS and provided evidence to suggest that circulating monocytes represent the major cellular source of inducible prostanoid biosynthesis in this system (Patrignani *et al.*, 1994). Thus, we were interested in testing the hypothesis that PGHS-2 induction in blood monocytes in response to LPS may provide a novel mechanism of F$_2$-isoprostane formation. Our results support this hypothesis by showing that: (a) LPS induced the formation of 8-epi-PGF$_{2\alpha}$ in human whole blood in a time-dependent fashion, coincident with the induction of PGHS-2 detected by Western blot analysis of monocyte lysates; (b) L-745,337, a selective inhibitor of PGHS-2, reduced LPS-induced PGE$_2$ and 8-epi-PGF$_{2\alpha}$ formation in whole blood with virtually identical dose–response curves; (c) inhibition of monocyte 8-epi-PGF$_{2\alpha}$ formation by dexamethasone and cycloheximide was associated with reduced expression of PGHS-2 but not PGHS-1.

8-epi-PGF$_{2\alpha}$ formation in plasma obtained from LPS-sti-

mulated whole blood and in culture media from LPS-stimulated human monocytes was measured by a RIA technique that we have recently developed and validated (Wang *et al.*, 1995). We addressed limitations inherent to immunological detection of 8-epi-PGF$_{2\alpha}$ in biological fluids containing a mixture of potentially cross-reacting eicosanoids by several independent criteria. Firstly, we used two different anti-8-epi-PGF$_{2\alpha}$ sera (No. 1 and No. 9) with a different pattern of cross-reactivities towards other arachidonate metabolites, including 8-epi-PGE$_2$ (7.7 and 0.1%, respectively: Wang *et al.*, 1995). The finding of identical concentrations of 8-epi-PGF$_{2\alpha}$-like immunoreactivity with the two antisera makes it unlikely that this reflected the true level of 8-epi-PGF$_{2\alpha}$ plus a variable proportion of other cross-reacting metabolites. Moreover, the use of antiserum No. 9 in EIA was previously validated by comparison with NICI-GC/MS for the detection of 8-epi-PGF$_{2\alpha}$ in human urine, which contains a complex array of primary eicosanoids and their enzymatic metabolites (Wang *et al.*, 1995). That direct RIA of 8-epi-PGF$_{2\alpha}$ in the culture medium of isolated monocytes or plasma did not overestimate (because of non-specific interferences or cross-reacting material) or underestimate (because of protein binding or degradation) the actual level of this eicosanoid is also indicated by the results of r.p.-h.p.l.c. and recovery studies, respectively. Thus, 8-epi-PGF$_{2\alpha}$-like immunoreactivity extracted from plasma or monocyte culture medium had a chromatographic behaviour indistinguishable from that of authentic 8-epi-PGF$_{2\alpha}$ (either labelled or unlabelled) and the total amount of immunoreactivity detected in the r.p.-h.p.l.c. fractions corresponding to the 8-epi-PGF$_{2\alpha}$ peak was within a 10–20% fluctuation of that measured in the unextracted samples. Most importantly, no detectable immunoreactivity was found in r.p.-h.p.l.c. fractions outside the retention time of authentic 8-epi-PGF$_{2\alpha}$ (Figure 3).

At 4 and 24 h of incubation of whole blood or isolated monocytes with LPS (10 μg ml^{-1}), PGE$_2$ and 8-epi-PGF$_{2\alpha}$ were produced in a molar ratio of approximately 7 : 1 and 30 : 1, respectively. Between 4 and 24 h, PGE$_2$ production increased by approximately 10 fold while 8-epi-PGF$_{2\alpha}$ rose by approximately 3 fold. The absence of parallelism in the synthesis of 8-epi-PGF$_{2\alpha}$ and PGE$_2$ is likely to reflect the different mechanism of synthesis and release of classical prostaglandins *vs* isoprostanes (Morrow *et al.*, 1992). Moreover, changes in the subcellular localization of PGHS-2 during its biosynthesis (Morita *et al.*, 1995) might contribute to time-dependent changes in the spectrum of cyclo-oxygenase products.

The contribution of platelet PGHS-1 to prostanoid formation in whole blood was suppressed selectively by pretreating the blood donors with aspirin *in vivo* 48 h before sampling (Patrono *et al.*, 1980). Platelets constitutively express PGHS-1 but cannot synthesize PGHS-2 in response to LPS (Hla & Neilson, 1992; Funk *et al.*, 1991). Aspirin acetylates platelet PGHS-1 (Roth *et al.*, 1975), thereby causing irreversible loss of its cyclo-oxygenase activity which persists for the platelet lifespan (7–10 days).

Support for a PGHS-2-dependent source of PGE$_2$ and 8-epi-PGF$_{2\alpha}$ formation in whole blood in response to LPS was provided by the use of dexamethasone, an inhibitor of PGHS-2 but not of PGHS-1 biosynthesis (O'Banion *et al.*, 1992; Kujubu & Herschman, 1992; Lee *et al.*, 1992). Dexamethasone almost completely suppressed the formation of both PGE$_2$ and 8-epi-PGF$_{2\alpha}$ in LPS-stimulated whole blood. Since dexamethasone might affect prostanoid biosynthesis also by inhibiting the release of AA from membrane phospholipids (Flower, 1988) and isoprostane release is dependent on phospholipase activity (Morrow *et al.*, 1992), we studied the effects of L-745,337, a selective inhibitor of the cyclo-oxygenase activity of human monocyte PGHS-2 (Panara *et al.*, 1995). L-745,337 inhibited PGE$_2$ and 8-epi-PGF$_{2\alpha}$ production in LPS-stimulated whole blood with similar dose–response curves (Figure 4), thus suggesting that 8-epi-PGF$_{2\alpha}$ formation is dependent on the cyclo-oxygenase activity of PGHS-2 in these circumstances.

Studies on isolated monocytes, lymphocytes and PMNs demonstrated that only monocytes responded to LPS by releasing PGE$_2$ and 8-epi-PGF$_{2\alpha}$ in a time-dependent fashion. The ability of monocytes to form PGE$_2$ and 8-epi-PGF$_{2\alpha}$ in response to LPS was dependent on the biosynthesis of a protein of approximately 72 kDa analyzed by Western blot using antibodies directed against a synthetic PGHS-2 specific peptide (Habib *et al.*, 1993). The protein was undetectable in unstimulated monocytes and in LPS-stimulated lymphocytes and PMNs. Cycloheximide and dexamethasone reduced both PGE$_2$ and 8-epi-PGF$_{2\alpha}$ formation and these effects were associated with the suppression of PGHS-2 but not PGHS-1 biosynthesis. Overall, these results suggest that the formation of 8-epi-PGF$_{2\alpha}$ by LPS-stimulated monocytes involves the cyclo-oxygenase activity of the inducible PGHS-2 but not of the constitutive PGHS-1. In accordance with our results, Reddy & Herschman (1994) have recently demonstrated that PGHS-2 expression is necessary for endotoxin-induced prostanoid biosynthesis in macrophages. PGHS-1 present in these cells cannot utilize AA released in response to endotoxin stimulation.

The mechanism of formation of 8-epi-PGF$_{2\alpha}$ is unlikely to be dependent on the generation of free radicals associated with the catalytic activity of PGHS-2 and consequently *via* the formation of peroxyl radical isomers. The formation of 8-epi-PGF$_{2\alpha}$ as a prostaglandin was recently demonstrated by the activity of platelet PGHS-1 in response to collagen, thrombin and AA (Praticò *et al.*, 1995). This is consistent with the biomimetic schema proposed by Corey *et al.* (1984).

The biosynthesis of 8-epi-PGF$_{2\alpha}$ in a PGHS-1 dependent fashion appears to contribute negligibly to the global formation of this prostanoid *in vivo*, as suggested by the failure of NSAIDs to depress urinary 8-epi-PGF$_{2\alpha}$ excretion in healthy subjects (Wang *et al.*, 1995) as well as in patients with acute ischaemic stroke (Ciabattoni *et al.*, 1995), a condition associated with episodic platelet activation (van Kooten *et al.*, 1994). However, verification of the relative contribution of 8-epi-PGF$_{2\alpha}$ formation by a PGHS-2-dependent mechanism *vis-a-vis* that catalyzed by free radicals is relevant to the use of plasma or urinary 8-epi-PGF$_{2\alpha}$ measurements as an index of lipid peroxidation in pathophysiological settings of monocyte activation.

Recent evidence for the existence of TXA$_2$/PGH$_2$ receptors in human monocytes coupled through activation of phospholipase C to increased intracellular calcium concentrations (Allan & Halushka, 1994), together with the demonstration of F$_2$-isoprostane receptors in vascular smooth muscle cells distinct from but closely related to TXA$_2$/PGH$_2$ receptors (Fukunaga *et al.*, 1993), provide a rationale for studying the effects of this eicosanoid on monocyte function. Moreover, PGHS-2-dependent F$_2$-isoprostane biosynthesis within the mitogenic and inflammatory milieu of atherosclerotic plaques (Ross, 1986) might transduce some of the effects of inflammation on vascular dysfunction.

In conclusion, we have demonstrated that 8-epi-PGF$_{2\alpha}$ can be formed by activated monocytes in a PGHS-2-dependent fashion. Thus, the assumption that isoprostane biosynthesis *in vivo* reflects non-enzymatic peroxidation of arachidonic acid requires validation in the setting of pathological conditions associated with monocyte activation. Furthermore, inhibition of the biosynthesis of the vasoconstrictor and mitogenic 8-epi-PGF$_{2\alpha}$ may integrate the spectrum of pharmacological effects of selective PGHS-2 inhibitors.

This study was supported by grants from Consiglio Nazionale delle Ricerche, Progetto Finalizzato FATMA, SP8 (93.00712.PF41 and 94.00627.PF41) and a BIOMED grant from the European Union (BMH1-CT93-1533).

References

ALLAN, C.J. & HALUSHKA, P.V. (1994). Characterization of human peripheral blood monocyte thromboxane A_2 receptors. *J. Pharmacol. Exp. Ther.*, **270**, 446–452.

BANERJEE, M., KANG, K.H., MORROW, J.D., ROBERTS II, L.J. & NEWMAN, J.H. (1992). Effects of a novel prostaglandin, 8-epi-$PGF_{2\alpha}$, in rabbit lung in situ. *Am.J. Physiol.*, **263**, H660–H663.

BOYUM, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest.*, **97**, 77–89.

CHAN, C.-C., GORDON, R., BRIDEAU, C., RODGER, I.W., LI, C.-S., PRASIT, P., TAGARI, P., ETHIER, D., VICKERS, P., BOYCE, S., RUPNIAK, N., WEBB, J., HILL, R. & FORD-HUTCHINSON, A.W. (1994). In vivo pharmacology of L-745,337: a novel non-steroidal antiinflammatory agent (NSAID) with an ulcerogenic sparing effect in rat and monkey stomach. *Can. J. Physiol. Pharmacol.*, **72** (Suppl.1), 266 (abstract)

CIABATTONI, G., PATRONO, C., VAN KOOTEN, F. & KOUDSTAAL, P.J. (1995). Dissociation of platelet activation and lipid peroxidation in acute ischemic stroke. *J. Investig. Med.*, **43**, 292A.

CIABATTONI, G., PUGLIESE, F., SPALDI, M., CINOTTI, G.A. & PATRONO, C. (1979). Radioimmunoassay measurement of prostaglandin E_2 and $F_{2\alpha}$ in human urine. *J. Endocrinol. Invest.*, **2**, 173–182.

COREY, E.J., SHIH, C., SHIN, N.-Y. & SHIMOJI, K. (1984). Preferential formation of 8-epi-$PGF_{2\alpha}$ via the corresponding endoperoxide by a biomimetic cyclization. *Tetrahedron Lett.*, **25**, 5013–5016.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.

DEWITT, D.L. (1991). Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim. Biophys. Acta*, **1083**, 121–134.

FLOWER, R.J. (1988). Eleventh Gaddum memorial lecture. Lipocortin and the mechanism of action of the glucocorticoids. *Br. J. Pharmacol.*, **94**, 987–1015.

FUKUNAGA, M., MAKITA, N., ROBERTS II, L.J., MORROW, J.D., TAKAHASHI, K. & BADR, K.F. (1993). Evidence for the existence of F_2-isoprostane receptors on rat vascular smooth muscle cells. *Am. J. Physiol.*, **264**, C1619–C1624.

FUNK, C.D., FUNK, L.B., KENNEDY, M.E., PONG, A.S. & FITZGERALD, G.A. (1991). Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.*, **5**, 2304–2312.

HABIB, A., CRÉMINON, C., FROBERT, Y., GRASSI, J., PRADELLES, P. & MACLOUF, J. (1993). Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. *J. Biol. Chem.*, **268**, 23448–23454.

HALLIWELL, B. & GROOTVELD, M. (1987). The measurement of free radical reactions in humans. Some thoughts for future experimentation. *FEBS Lett.*, **213**, 9–14.

HECKER, M., ULLRICH, V., FISCHER, C. & MEESE, C.O. (1987). Identification of novel arachidonic acid metabolites formed by prostaglandin H synthase. *Eur. J. Biochem.*, **169**, 113–123.

HEMPEL, S.L., MOMIK, M.M. & HUNNINGHAKE, G.W. (1994). Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.*, **93**, 391–396.

HLA, T. & NEILSON, K. (1992). Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7384–7388.

JONES, D.A., CARLTON, D.P., MCINTYRE, T.M., ZIMMERMAN, G.A. & PRESCOTT, S.M. (1993). Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J. Biol. Chem.*, **268**, 9049–9054.

KUJUBU, D.A. & HERSCHMAN, H.R. (1992). Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase gene. *J. Biol. Chem.*, **267**, 7991–7994.

LEE, S.H., SOYOOLA, E., CHANMUGAM, P., HART, S., SUN, W., ZHONG, H., LIOU, S., SIMMONS, D. & HWANG, D. (1992). Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.*, **267**, 25934–25938.

MASFERRER, J.L., ZWEIFEL, B.S., MANNING, P.T., HAUSER, S.D., LEAHY, K.M., SMITH, W.G., ISAKSON, P.C. & SEIBERT, K. (1994). Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3228–3232.

MORITA, I., SCHINDLER, M., REGIER, M.K., OTTO, J.C., HORI, T., DEWITT, D.L. & SMITH, W.L. (1995). Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J. Biol. Chem.*, **270**, 10902–10908.

MORROW, J.D., AWAD, J.A., BOSS, H.J., BLAIR, I.A. & ROBERTS II, L.J. (1992). Non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed in situ on phospholipids. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 10721–10725.

MORROW, J.D., FREI, B., LONGMIRE, A.W., GAZIANO, J.M., LYNCH, S.M., SHYR, Y., STRAUSS, W.E., OATES, J.A. & ROBERTS II, L.J. (1995). Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. *N. Engl. J. Med.*, **332**, 1198–1203.

MORROW, J.D., HILL, K.E., BURK, R.F., NAMMOUR, T.M., BADR, K.F. & ROBERTS II, L.J. (1990). A series of prostaglandin F_2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 9383–9387.

MORROW, J.D., MINTON, T.A., BADR, K.F. & ROBERTS II, L.J. (1994a). Evidence that the F_2-isoprostane, 8-epi-prostaglandin $F_{2\alpha}$, is formed in vivo. *Biochim. Biophys. Acta*, **1210**, 244–248.

MORROW, J.D., MINTON, T.A., MUKUNDAN, C.R., CAMPBELL, M.D., ZACKERT, W.E., DANIEL, V.C., BADR, K.F., BLAIR, I.A. & ROBERTS II, L.J. (1994b). Free-radical-induced generation of isoprostanes in vivo. Evidence for the formation of D-ring and E-ring isoprostanes. *J. Biol. Chem.*, **269**, 4317–4326.

MORROW, J.D., MINTON, T.A. & ROBERTS II, L.J. (1992). The F_2-isoprostane, 8-epi-prostaglandin $F_{2\alpha}$, a potent agonist of the vascular thromboxane/endoperoxide receptor, is a platelet thromboxane/endoperoxide receptor antagonist. *Prostaglandins*, **44**, 155–163.

O'BANION, M.K., WINN, V.D. & YOUNG, D.A. (1992). cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4888–4892.

O'SULLIVAN, M.G., HUGGINS JR, E.M., MEADE, E.A., DEWITT, D.L. & MCCALL, C.E. (1992). Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem. Biophys. Res. Commun.*, **187**, 1123–1127.

PANARA, M.R., GRECO, A., SANTINI, G., SCIULLI, M.G., ROTONDO, M.T., PADOVANO, R., DI GIAMBERARDINO, M., CIPOLLONE, F., CUCCURULLO F, PATRONO C. & PATRIGNANI, P. (1995). 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. *Br. J. Pharmacol.*, **116**, 2429–2434.

PATRIGNANI, P., PANARA, M.R., GRECO, A., FUSCO, O., NATOLI, C., IACOBELLI, S., CIPOLLONE, F., GANCI, A., CRÉMINON, C., MACLOUF, J. & PATRONO, C. (1994). Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. *J. Pharmacol. Exp. Ther.*, **271**, 1705–1712.

PATRIGNANI, P., PANARA, M.R., CIPOLLONE, F., GRECO, A., CIABATTONI, G. & PATRONO, C. (1995). Induction of prostaglandin endoperoxide synthase 2 in monocytes of human whole blood is associated with cyclooxygenase-dependent isoprostane formation. *J. Investig. Med.*, **43**, 335A.

PATRONO, C., CIABATTONI, G., PINCA, E., PUGLIESE, F., CASTRUCCI, G., DE SALVO, A., SATTA, M.A. & PESKAR, B.A. (1980). Low dose aspirin and inhibition of thromboxane B_2 production in healthy subjects. *Thromb. Res.*, **17**, 317–327.

PRATICÒ, D., LAWSON, J.A. & FITZGERALD, G.A. (1994). Cyclooxygenase-dependent formation of the isoprostane 8-epi-prostaglandin $F_{2\alpha}$. *Ann. N.Y. Acad. Sci.*, **744**, 139–145.

PRATICÒ, D., LAWSON, J.A. & FITZGERALD, G.A. (1995). Cyclooxygenase-dependent formation of the isoprostane 8-epi-prostaglandin $F_{2\alpha}$. *J. Biol. Chem.*, **270**, 9800–9808.

REDDY, S.T. & HERSCHMAN, H.R. (1994). Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J. Biol. Chem.*, **269,** 15473–15480.

ROSS, R. (1986). The pathogenesis of atherosclerosis. An update. *N. Engl. J. Med.*, **314,** 488–500.

ROTH, G.J., STANFORD, N. & MAJERUS, P.W. (1975). Acetylation of prostaglandin synthase by aspirin. *Proc. Natl. Acad. Sci. U.S.A.*, **72,** 3073–3076.

SEIBERT, K., ZHANG, Y., LEAHY, K., HAUSER, S., MASFERRER, J., PERKINS, W., LEE, L. & ISAKSON, P. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 12013–12017.

SMITH, W.L. & DEWITT, D.L. (1995). Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs. *Semin. Nephrol.*, **15,** 179–194.

TAKAHASHI, K., NAMMOUR, T.M., FUKUNAGA, M., EBERT, J., MORROW, J.D., ROBERTS II, L.J., HOOVER, R.L. & BADR, K.F. (1992). Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin $F_{2\alpha}$, in the rat. Evidence for interaction with thromboxane A_2 receptors. *J. Clin. Invest.*, **90,** 136–141.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.*, **76,** 4350–4354.

VAN KOOTEN, F., CIABATTONI, G., PATRONO, C., SCHMITZ, P.I.M., VAN GIJN, J. & KOUDSTAAL, P.J. (1994). Evidence for episodic platelet activation in acute ischemic stroke. *Stroke*, **25,** 278–281.

WANG, Z., CIABATTONI, G., CRÉMINON, C., LAWSON, J., FITZGERALD, G.A., PATRONO, C. & MACLOUF, J. (1995). Immunological characterization of urinary 8-epi-prostaglandin $F_{2\alpha}$, excretion in man. *J. Pharmacol. Exp. Ther.*, **275,** 94–100.

YIN, K., HALUSHKA, P.V., YAN, Y.T. & WONG, P.Y. (1994). Antiaggregatory activity of 8-epi-prostaglandin $F_{2\alpha}$ and other F-series prostanoids and their binding to thromboxane A_2/prostaglandin H_2 receptors in human platelets. *J. Pharmacol. Exp. Ther.*, **270,** 1192–1196.

(Received January 2, 1996
Revised March 11, 1996
Accepted March 19, 1996)