ELSEVIER

European Journal of Pharmacology 316 (1996) 65–72

ejp

Effects of flurbiprofen and flurbinitroxybutylester on prostaglandin endoperoxide synthases [1]

Giovanna Santini [a], Maria G. Sciulli [a], Maria R. Panara [a], Roberto Padovano [a], Maria di Giamberardino [a], Maria T. Rotondo [a], Piero Del Soldato [b], Paola Patrignani [a,*]

[a] *Department of Pharmacology I, University of Chieti 'G. D'Annunzio' School of Medicine, Palazzina delle Scuole di Specializzazione, via dei Vestini 31, 66013 Chieti, Italy*

[b] *Nicox Ltd., London, UK*

Received 1 July 1996; revised 6 August 1996; accepted 9 August 1996

Abstract

The aim of our study was to evaluate the selectivity of flurbiprofen and flurbinitroxybutylester for inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 vs. prostaglandin endoperoxide synthase-1 in human blood monocytes and platelets, respectively. In whole blood, flurbiprofen was approximately 10-fold more potent than flurbinitroxybutylester to inhibit the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 (IC_{50} μM: 0.90 ± 0.27 vs. 10.70 ± 5, mean $\pm$ S.D., $P < 0.05$). In contrast, the 2 compounds were equipotent to inhibit prostaglandin endoperoxide synthase-2 cyclooxygenase activity in whole blood (IC_{50} μM: 0.90 ± 0.25 vs. 0.80 ± 0.35) or isolated monocytes (IC_{50} μM: 0.03 ± 0.02 vs. 0.03 ± 0.02). Neither flurbiprofen nor flurbinitroxybutylester (0.28–112 μM) affected prostaglandin endoperoxide synthase isozyme expression by lypopolysaccharide-stimulated monocytes. In whole blood, flurbinitroxybutylester was slowly converted to flurbiprofen and this in turn could influence the extent of inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase-1. In conclusion, the addition of a nitroxybutyl moiety to flurbiprofen seems to reduce its capacity to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase-1. Whether this effect will result in a reduced risk of gastrointestinal toxicity remains to be studied in man.

Keywords: Prostaglandin endoperoxide synthase; Blood monocyte, human; Platelet, human; Flurbiprofen; Flurbinitroxybutylester

1. Introduction

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is limited by their untoward side-effects, mainly on the gastrointestinal tract (Soll et al., 1991). Different factors need to be considered when evaluating the toxicity of individual NSAIDs in humans (Bateman, 1996): (1) the potential local irritant effect of a particular pharmaceutical preparation; (2) the extent and variability of first-pass metabolism of the parent compound in the gut wall or liver; (3) the effect of disease state or age on clearance; and (4) the potential biological activity of metabolites. However, in experimental animals, it has been shown that gastric toxicity associated with NSAIDs is likely to involve inhibition of the cyclooxygenase activity of the constitutive enzyme, prostaglandin endoperoxide synthase-1 (Masferrer et al., 1994; Seibert et al., 1994; Chan et al., 1995).

Prostaglandin endoperoxide synthase is a bifunctional enzyme which exhibits both cyclooxygenase and peroxidase activities. It catalyzes the conversion of arachidonic acid to prostaglandin G_2 and of prostaglandin G_2 to prostaglandin H_2. Newly formed prostaglandin H_2 is subsequently converted to various biologically active prostanoids by specific isomerases (for a review, see Smith and DeWitt, 1995).

Two isoforms of prostaglandin endoperoxide synthase have been identified, prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2. Each isozyme is encoded by a different gene (Kraemer et al., 1992; Kujubu and Herschman, 1992). Prostaglandin en-

* Corresponding author. Tel.: (39-871) 355-775; Fax: (39-871) 355-718; e-mail: ppatrignani@unich.it

[1] A preliminary report of this study was presented at the First European Congress of Pharmacology (Milan, Italy, 16–19 June 1995) and published in abstract form (Santini et al., 1995, Pharmacol. Res. 31, 133).

PII S0014-2999(96)00640-1

doperoxide synthase-1 is a constitutive enzyme present in almost all cell types whereas prostaglandin endoperoxide synthase-2 has a restricted tissue distribution and is expressed at a very low basal level but is highly inducible in response to mitogenic factors and cytokines (O'Banion et al., 1991; Hla and Neilson, 1992; Jones et al., 1993). Prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2 are 60% identical at the amino-acid level. Those amino acids required for catalysis by prostaglandin endoperoxide synthase-1 are conserved in prostaglandin endoperoxide synthase-2 (Smith and DeWitt, 1995). However, subtle differences between the active site of the 2 isozymes are present as suggested by their different affinities toward various NSAIDs (Mitchell et al., 1993; Barnett et al., 1994; Laneuville et al., 1994; Patrignani et al., 1994; Grossman et al., 1995; Panara et al., 1995).

Highly selective inhibitors of the cyclooxygenase activity of human monocyte prostaglandin endoperoxide synthase-2 have recently been developed (Panara et al., 1995), yielding anti-inflammatory compounds effective in experimental animals without causing gastric lesions (Masferrer et al., 1994; Seibert et al., 1994; Chan et al., 1995). This is consistent with their ability to inhibit the activity of prostaglandin endoperoxide synthase-2 induced at sites of inflammation while sparing the activity of constitutive prostaglandin endoperoxide synthase-1 in the gastrointestinal tract (Masferrer et al., 1994; Seibert et al., 1994; Chan et al., 1995).

The addition of a nitroxybutylester group to flurbiprofen markedly reduces its gastrointestinal toxicity without altering its anti-inflammatory effectiveness when administered to experimental animals (Wallace et al., 1994a). One possible mechanism might involve the capacity of flurbinitroxybutylester to release nitric oxide (NO) in vivo (Wallace et al., 1994a). NO, similarly to prostaglandins, appears to play an important role in gastric mucosal defense (MacNaughton et al., 1989; Kitagawa et al., 1990; Lopez-Belmonte et al., 1993). However, the addition of a nitroxybutylester moiety to flurbiprofen might also affect its affinity for prostaglandin endoperoxide synthase isozymes. Thus, the aims of the present study were to investigate: (1) the selectivity of flurbinitroxybutylester and flurbiprofen to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 vs. prostaglandin endoperoxide synthase-1 in human blood monocytes and platelets, respectively; and (2) the stability of flurbinitroxybutylester and flurbiprofen in human whole blood.

2. Materials and methods

2.1. Subjects

Six healthy volunteers (3 female, 3 male; 23–50 years) were studied on several occasions. Informed consent was obtained from each subject. Peripheral venous blood samples were drawn between 10:00 and 12:00 h, before and 48 h after the oral administration of aspirin 300 mg. The volunteers had not taken any other NSAIDs or steroids during the 2 weeks preceding the study.

2.2. Materials

Ficoll-Paque was obtained from Pharmacia Biotech (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from Gibco Laboratories (Grand Island, NY, USA). Heparin, L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), lipopolysaccharide derived from *Escherichia coli* 026:B6, the calcium ionophore A23187, biotinylated anti-rabbit IgG, streptavidin-peroxidase were purchased from Sigma (St. Louis, MO, USA). Flurbiprofen and flurbinitroxybutylester were provided by Nicox (London, UK). Rabbit polyclonal antibodies prepared against prostaglandin endoperoxide synthase-2 peptide (C)-NASSSRSGLDDINPTVLLK, which is only present in the carboxyl-terminal (amino-acid sequence 580–598) of human prostaglandin endoperoxide synthase-2 (Hla and Neilson, 1992; Jones et al., 1993) were obtained as described recently (Habib et al., 1993). Specific rabbit polyclonal antibodies directed against prostaglandin endoperoxide synthase-1 were a gift from Dr. W.L. Smith (Department of Biochemistry, Michigan State University). Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA, USA).

2.3. Activity of prostaglandin endoperoxide synthase isozymes in whole blood

1-ml aliquots of peripheral blood samples containing 10 IU of sodium heparin were incubated both in the absence and in the presence of lypopolysaccharide (10 μg/ml) for 24 h at 37°C as recently described (Patrignani et al., 1994). The contribution of platelet prostaglandin endoperoxide synthase-1 was suppressed by pre-treating the subjects with aspirin 300 mg 48 h before sampling. Plasma was separated by centrifugation (10 min at $1600 \times g$) and kept at −30°C until assayed for the content of prostaglandin E_2 that was recently demonstrated to be an index of the cyclooxygenase activity of blood monocyte prostaglandin endoperoxide synthase-2 (Patrignani et al., 1994). Peripheral blood samples were drawn from the same donors who had not taken any NSAIDs during the 2 weeks preceding the study. 1-ml aliquots of whole blood were immediately transferred into glass tubes and allowed to clot at 37°C for 1 h. Serum was separated by centrifugation (10 min at $1600 \times g$) and kept at −30°C until assayed for thromboxane B_2. Whole-blood thromboxane B_2 production was measured as a reflection of maximally stimulated cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 by endogenously formed thrombin (Patrono et al., 1980). Plasma prostaglandin E_2 and serum tromboxane

B_2 were measured by specific and validated radioimmunoassay (RIA) techniques that have been described previously (Ciabattoni et al., 1979; Patrono et al., 1980).

2.4. Effects of flurbiprofen and flurbinitroxybutylester on prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2 activity in whole blood

Flurbiprofen and flurbinitroxybutylester were dissolved in dimethyl sulfoxide and 2 μl of these solutions was pipetted directly into test tubes to give a final concentration in whole blood of 0.028–164 μM. The effects of these inhibitors and dimethyl sulfoxide vehicle on prostaglandin endoperoxide synthase-1 activity were evaluated by incubating each drug at 5–6 different concentrations with multiple whole-blood samples that were allowed to clot at 37°C for 1 h. The effects on prostaglandin endoperoxide synthase-2 activity were studied by incubating each compound or dimethyl sulfoxide vehicle at 5–6 different concentrations with multiple heparinized whole-blood samples in the presence of lypopolysaccharide (10 μg/ml) for 24 h. The details of these procedures have been described previously (Patrignani et al., 1994).

2.5. Effects of flurbiprofen and flurbinitroxybutylester on the cyclooxygenase activity and expression of prostaglandin endoperoxide synthase-2 in isolated monocytes

Mononuclear cells were separated from heparinized human whole-blood or leukocyte concentrates (buffy coat, obtained from local blood collection centres) by Ficoll-Paque as described by Boyum (1968). Mononuclear cells were carefully removed, washed and resuspended in Dulbecco's modified Eagle medium (DMEM) buffered with 0.01 M Hepes, pH 7.4, supplemented with 0.5% heat-inactivated fetal calf serum 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 1 h. The adherent cells recovered by gently scraping with a rubber policeman were resuspended in CDMEM (2×10^6 cells/ml) and their viability (> 96%) was examined by Trypan blue exclusion. The purity of the preparation of isolated monocytes was assayed by forward and right-angle scatter measurement by flow cytometry (Coulter, Hialeah, FL, USA). The cell suspension was approximately 90% monocytes. The effects of flurbiprofen and flurbinitroxybutylester on the cyclooxygenase activity of monocyte prostaglandin endoperoxide synthase-2 were evaluated by incubating the isolated cells with increasing concentrations of the compounds (0.0028–2.8 μM) at 37°C for 24 h in the presence of lypopolysaccharide (10 μg/ml) and measuring immunoreactive prostaglandin E_2 in the supernatant. In some experiments, the effects of flurbiprofen and flurbinitroxybutylester (0.28–112 μM) on the expression of prostaglandin endoperoxide synthase-2 and prostaglandin endoperoxide synthase-1 were evaluated by a previously described Western blot technique (Patrignani et al., 1994). Briefly, 10 μg of protein lysate of monocytes were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis transferred to a nitrocellulose membrane and immunoblotted with either rabbit polyclonal antiserum (1:750 dilution) directed against the carboxyl-terminal portion of human prostaglandin endoperoxide synthase-2 or polyclonal anti-prostaglandin endoperoxide synthase-1 serum (1:1000 dilution). Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG (1:2000) and streptavidin-peroxidase.

In a different set of experiments, lymphomonocytes were seeded into 24-well plates (10×10^6/well) and incubated at 37°C in 5% CO_2-humidified atmosphere for 1 h. The non-adherent cells were removed; the remaining monolayer, made up of monocytes, was incubated in the presence of lypopolysaccharide (10 μg/ml) for 24 h at 37°C and the expression of prostaglandin endoperoxide synthase-2 was evaluated by Western blot. At the end of the incubation, the medium was replaced with fresh CDMEM (lypopolysaccharide-free) and monocytes were incubated with the calcium ionophore, A23187 (1 μg/ml), both in the absence and in the presence of flurbiprofen or flurbinitroxybutylester (0.0028–2.8 μM), for 1 h at 37°C. Prostaglandin E_2 levels were measured in the medium by RIA as an index of the cyclooxygenase activity of prostaglandin endoperoxide synthase-2.

2.6. Stability of flurbiprofen and flurbinitroxybutylester in whole blood

The inhibitory effects of flurbiprofen and flurbinitroxybutylester (0.28–28 μM) on the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 were evaluated after pre-incubation of the compounds at room temperature for 1 and 3 h. The production of TXB_2 in whole blood allowed to clot for 1 h at 37°C was assessed by RIA. The stability of flurbinitroxybutylester (28 μM) in whole blood was assessed by evaluating the concentrations of flurbiprofen and flurbinitroxybutylester by reversed-phase high performance liquid chromatography (RP-HPLC). Aliquots of 10 ml of serum or plasma samples were added to 190 ml of methanol/water (50:50, v/v) and injected directly into a Nova-Pak C_{18} column (Waters, Milford, MA, USA) of a Beckman System Gold HPLC. Flurbiprofen and flurbinitroxybutylester were separated by RP-HPLC using a mobile phase consisting of a gradient prepared from water:acetic acid (100:0.1, v/v) (solvent A) and acetonitrile:acetic acid (100:0.1, v/v) (solvent B) as follows: 0–15 min 40% B; 60 min 70% B. The flow rate was 1 ml/min. Absorbance was assessed at 250 nm. Synthetic flurbiprofen and flurbinitroxybutylester eluted with retention times of 11.4 and 44 min, respectively.

2.7. Statistical analysis

The data were expressed as mean ± S.D. Statistical comparisons were made by Student's unpaired *t*-test. The sigmoidal dose-response curves were analysed with Allfit, a basic computer program for simultaneous curve-fitting based on a 4-parameter logistic equation (De Lean et al., 1978).

3. Results

The measurement of prostaglandin E_2 production in heparinized whole blood incubated with lypopolysaccharide (10 μg/ml) for 24 h represents a reflection of the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 induced in circulating monocytes (Patrignani et al., 1994). Plasma prostaglandin E_2 averaged 9.1 ± 2.9 ng/ml (mean ± S.D., $n = 6$) under control conditions. The contribution of platelet cyclooxygenase activity was suppressed selectively by pre-treatment of the subjects with aspirin (300 mg 48 h before sampling). The measurement of thromboxane B_2 production in whole blood obtained from healthy subjects not exposed to aspirin and allowed to clot for 1 h at 37°C is an index of the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 in response to endogenously formed thrombin (Patrono et al., 1980). Serum thromboxane B_2 averaged 473 ± 211 ng/ml (mean ± S.D., $n = 6$) under control conditions. As shown in Table 1, flurbiprofen inhibited the cyclooxygenase activity of monocyte prostaglandin endoperoxide synthase-2 and platelet prostaglandin endoperoxide synthase-1 with comparable potency. In contrast, flurbinitroxybutylester was approximately 13-fold more potent towards the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 than of prostaglandin endoperoxide synthase-1 (Table 1, Fig. 1). The addition of a nitroxybutyl moiety to flurbiprofen was associated with a 10-fold reduction in its capacity to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase-1 (Table 1).

To ascertain whether the greater inhibitory effect of flurbinitroxybutylester on the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 than that of prostaglandin endoperoxide synthase-1 might have been a result

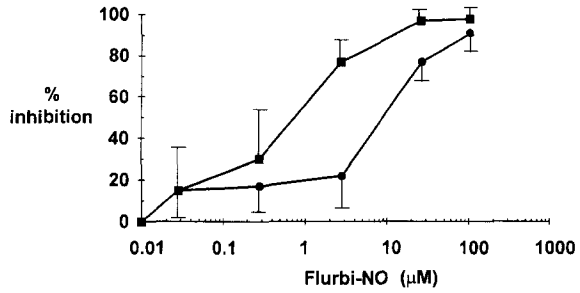

Fig. 1. Dose-response curves for inhibition of human whole-blood prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2 by flurbinitroxybutylester. Increasing concentrations of flurbinitroxybutylester (Flurbi-NO, 0.028–164 μM) were incubated with 1-ml aliquots of heparinized whole blood (drawn from healthy volunteers pre-treated with 300 mg of aspirin 48 h before sampling) in the presence of lypopolysaccharide (10 μg/ml) for 24 h, and plasma prostaglandin E_2 was assayed as a reflection of monocyte prostaglandin endoperoxide synthase-2 activity (■). Flurbinitroxybutylester 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 was measured as a reflection of platelet prostaglandin endoperoxide synthase-1 activity (○). Results are depicted as percentage inhibition and represent the means ± S.D. from 3 separate experiments.

of the longer time of incubation of the compound in the lypopolysaccharide-stimulated whole-blood assay (24 h) than in the whole-blood clotting assay (1 h), we investigated the effects of different times of pre-incubation in whole blood (1 and 3 h at room temperature) of flurbinitroxybutylester and flurbiprofen in whole blood on their capacity to inhibit the cyclooxygenase activity of thrombin-stimulated platelet prostaglandin endoperoxide synthase-1. Pre-incubation of flurbiprofen did not significantly affect the dose-response curve for inhibition of thromboxane B_2 production during whole-blood clotting (Table 2). In contrast, flurbinitroxybutylester pre-incubated for 3 h with whole blood was 4-fold more potent ($P < 0.05$) to inhibit the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 as compared to control conditions (no pre-incubation) (Table 2).

Since flurbinitroxybutylester is likely to be degraded to flurbiprofen by blood esterases, we analysed the stability of flurbinitroxybutylester in whole blood under different experimental conditions. Flurbinitroxybutylester (28 μM)

Table 1
Inhibition of human whole-blood prostaglandin endoperoxide synthase-1 (PGHS-1) and prostaglandin endoperoxide synthase-2 (PGHS-2) activities by flurbiprofen and flurbinitroxybutylester

Inhibitor	IC_{50} (μM)	
	PGHS-1	PGHS-2
Flurbiprofen	0.90 ± 0.27	0.90 ± 0.25
Flurbinitroxybutylester	10.70 ± 5 [a]	0.80 ± 0.35

All values are mean ± S.D. ($n = 3$–5).
IC_{50} for PGHS-1 vs. IC_{50} for PGHS-2. [a] $P < 0.05$.

Table 2
Effects of pre-incubation of flurbiprofen and flurbinitroxybutylester on inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase-1

Incubation conditions	IC_{50} (μM)	
	Flurbiprofen	Flurbinitroxybutylester
1 h at 37°C	0.70 ± 0.35	7.7 ± 3.3
1 h at room t° + 1 h at 37°C	0.90 ± 0.35	3.5 ± 1.40 [a]
3 h at room t° + 1 h at 37°C	0.50 ± 0.17	1.90 ± 0.70 [a]

All values are mean ± S.D. ($n = 3$).
IC_{50} vs. control (1 h at 37°C). [a] $P < 0.05$.

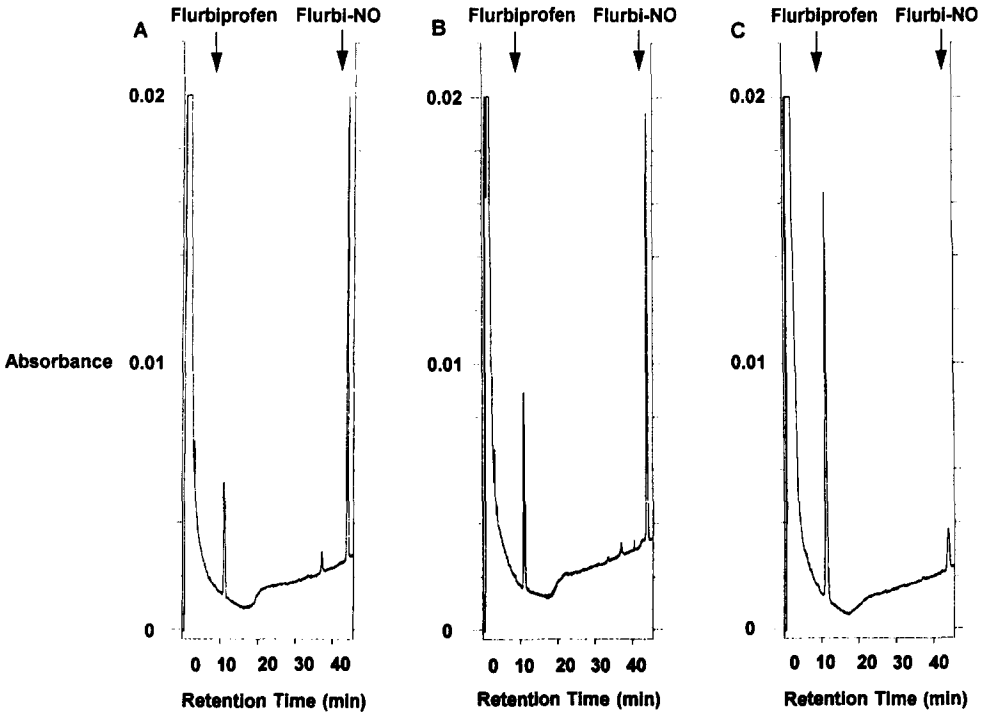

Fig. 2. Time-dependent formation of flurbiprofen from flurbinitroxybutylester in human whole blood. Flurbinitroxybutylester (Flurbi-NO, 28 μM) was incubated in whole-blood samples for 1 h at 37°C (panel A) and for 3 h at room temperature + 1 h at 37°C (panel B). Aliquots of heparinized whole blood were incubated with flurbinitroxybutylester (28 μM) for 24 h at 37°C (panel C). Blood samples were centrifuged at 1600 × *g* for 10 min and 10 μl of serum or plasma were directly injected into a RP-HPLC system. Flurbiprofen and flurbinitroxybutylester were separated using a mobile phase consisting of a gradient prepared from water:acetic acid (100:0.1) (solvent A) and acetonitrile:acetic acid (100:0.1) (solvent B) as follows: 0–15 min 40% solvent B, 60 min 70% solvent B. The flow rate was 1 ml/min. Absorbance was assessed at 250 nm. The retention time of the standards is indicated by the arrows.

was added to whole blood and the concentration of flurbiprofen and flurbinitroxybutylester in serum and in plasma was assessed by RP-HPLC. At $t = 0$, only flurbinitroxybutylester was present in whole blood (not shown); after 1 h of incubation at 37°C, 20% of flurbinitroxybutylester was converted to flurbiprofen, while after 3 h of incubation at room temperature followed by 1 h at 37°C, the peak representing flurbiprofen increased to 35% of flurbinitroxybutylester (Fig. 2). When flurbinitroxybutylester was incubated in heparinized whole blood for 24 h at 37°C, the compound was almost completely converted to flurbiprofen (Fig. 2).

To evaluate whether the inhibition of thromboxane B_2 production by flurbinitroxybutylester in whole blood allowed to clot for 1 h at 37°C might be influenced by its transformation to flurbiprofen, we measured the time course of thromboxane B_2 production in the absence and in the presence of flurbinitroxybutylester (28 μM). As shown in Fig. 3, the inhibition of serum thromboxane B_2 production by flurbinitroxybutylester was significantly ($P < 0.05$) lower at 5 min (71 ± 4.4%, mean ± S.D., $n = 3$) than at 1 h (87 ± 4.4%) of incubation. When the same samples were analysed by RP-HPLC for their content of flurbinitroxybutylester and flurbiprofen, we found that the time-dependent inhibition of thromboxane B_2 production was associated with conversion of flurbinitroxybutylester to flurbiprofen (Fig. 3). However, the concentration of flurbiprofen (approximately 1 μM) detected in whole blood incubated for 5 min with 28 μM of flurbinitroxybutylester could only partially account for the extent of inhibition of the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 measured at this time point, i.e. 71%. In fact, this concentration of flurbiprofen can only cause an approximately 50% inhibition of platelet prostaglandin endoperoxide synthase-1 activity (Table 1).

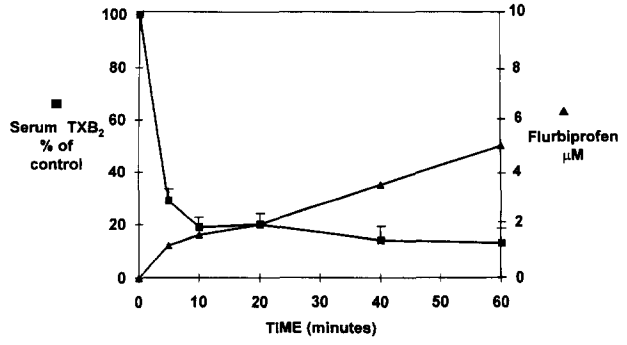

Fig. 3. Time-course of inhibition of whole-blood thromboxane B_2 production by flurbinitroxybutylester and its conversion to flurbiprofen. Flurbinitroxybutylester (28 μM) was incubated with 1-ml aliquots of whole blood at 37°C for 0–60 min and the concentrations of thromboxane B_2, flurbiprofen and flurbinitroxybutylester were measured at various time points.

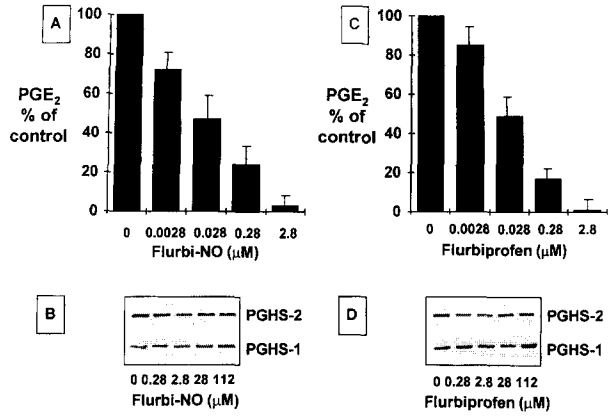

Fig. 4. Effects of flurbinitroxybutylester and flurbiprofen on prostaglandin E_2 production and prostaglandin endoperoxide synthase (PGHS) isozyme expression by lypopolysaccharide-stimulated human monocytes. Increasing concentrations of flurbinitroxybutylester (Flurbi-NO, panels A and B) or flurbiprofen (panels C and D) were incubated for 24 h at 37°C with human monocyte suspensions in the presence of lypopolysaccharide (10 μg/ml). Supernatants were assayed for immunoreactive prostaglandin E_2 while monocytes were lysed and proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques. Equal amounts of proteins (10 μg) were loaded onto all lanes. Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG and streptavidin-peroxidase. This figure is representative of 4 experiments.

Due to the instability of flurbinitroxybutylester in whole blood on prolonged incubation at 37°C, we compared the inhibitory effects of the 2 compounds on the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 in isolated monocytes. Isolated monocytes respond to lypopolysaccharide (10 μg/ml) with a time-dependent increase in prostaglandin E_2 production that correlates with the expression of prostaglandin endoperoxide synthase-2 (Patrignani et al., 1994). After 24 h of incubation, prostaglandin E_2 production averaged 4.3 ± 2.5 ng/10^6 cells (mean ± S.D., $n = 4$). Flurbinitroxybutylester and flurbiprofen incubated with human monocytes in the presence of lypopolysaccharide for 24 h, inhibited prostaglandin E_2 production with similar IC_{50} (0.03 ± 0.02 and 0.03 ± 0.02 μM, respectively, mean ± S.D., $n = 4$). Fig. 4 depicts the dose-response curves for inhibition of lypopolysaccharide-induced monocyte prostaglandin endoperoxide synthase-2 cyclooxygenase activity by flurbiprofen and flurbinitroxybutylester. Flurbiprofen and flurbinitroxybutylester, at concentrations that completely suppressed the production of prostaglandin E_2, did not affect lypopolysaccharide-induced expression of monocyte prostaglandin endoperoxide synthase-2 as analysed by Western blot using specific antibodies directed against a unique amino-acid sequence present in human prostaglandin endoperoxide synthase-2 (Habib et al., 1993). Similarly, the constitutive expression of prostaglandin endoperoxide synthase-1 was not affected by either drug to any detectable extent (Fig. 4).

We evaluated the effects of flurbiprofen and flurbinitroxybutylester on the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 induced in cultured human monocytes by the treatment with lypopolysaccharide (10 μg/ml) for 24 h and stimulated with the calcium ionophore, A23187 (1 μg/ml), for an additional 1 h. A23187 caused a 7-fold increase in the production of prostaglandin E_2 by monocytes incubated for 24 h with saline expressing only prostaglandin endoperoxide synthase-1: 0.7 ± 0.06 vs. 0.1 ± 0.04 ng/ml (mean ± S.D., $n = 3$). In contrast, A23187 stimulated more than 100-fold the production of prostaglandin E_2 by monocytes incubated for 24 h with lypopolysaccharide expressing both prostaglandin endoperoxide synthase-2 and prostaglandin endoperoxide synthase-1: 11.6 ± 2 vs. 0.1 ± 0.04 ng/ml (mean ± S.D., $n = 3$). Under these experimental conditions, flurbiprofen and flurbinitroxybutylester inhibited prostaglandin E_2 production in response to A23187 with similar IC_{50} : 0.06 ± 0.05 vs. 0.14 ± 0.12 μM, respectively (mean ± S.D., $n = 3$).

4. Discussion

In the present study, we have investigated whether the addition of a nitroxybutyl moiety to flurbiprofen could affect its inhibitory effects on the cyclooxygenase activity of prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2 in vitro. Since flurbiprofen is a time-dependent, irreversible inhibitor of the 2 prostaglandin endoperoxide synthase isozymes (Laneuville et al., 1994), we studied the effects of flurbiprofen and flurbinitroxybutylester on the cyclooxygenase activity of lypopolysaccharide-induced monocyte prostaglandin endoperoxide synthase-2 and thrombin-stimulated platelet prostaglandin endoperoxide synthase-1 in a recently described whole-blood model (Patrignani et al., 1994). Under these experimental conditions, the effects of inhibitors are evaluated on biologically relevant target cells, selectively expressing the isozymes that use arachidonate released from endogenous lipid pools and in the presence of plasma proteins. The main finding of the present study was that flurbiprofen inhibited prostaglandin endoperoxide synthase-1 and prostaglandin endoperoxide synthase-2 with similar IC_{50} values while flurbinitroxybutylester showed a 13-fold preference for prostaglandin endoperoxide synthase-2 due to an apparent 10-fold reduction in its capacity to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase-1.

In whole blood, flurbinitroxybutylester was slowly converted to flurbiprofen and this, in turn, may have contributed to the extent of inhibition of the cyclooxygenase activity of prostaglandin endoperoxide synthase-1. In contrast, flurbiprofen and flurbinitroxybutylester inhibited the cyclooxygenase activity of monocyte prostaglandin endoperoxide synthase-2 with comparable potency. This occurred under experimental conditions where flurbinitroxy-

butylester was stable (i.e. in isolated monocytes incubated for 1 h in the absence of plasma proteins).

Based on the crystal structure of sheep prostaglandin endoperoxide synthase-1, it has been proposed that the carboxylic acid group of flurbiprofen is located in a favourable position for interacting with the guanidinium group of Arg^{120} present in the hydrophobic cyclooxygenase channel (Picot et al., 1994). Thus, the presence of the nitroxybutyl moiety on the carboxyl group of flurbiprofen might decrease its affinity for prostaglandin endoperoxide synthase-1. In contrast, the prostaglandin endoperoxide synthase-2-selective inhibitors that have been developed thus far lack a carboxylic acid group (Vane and Botting, 1995), thus suggesting that this group is not important for their interaction with the cyclooxygenase binding site of prostaglandin endoperoxide synthase-2. This might explain the similar potency shown by flurbiprofen and flurbinitroxybutylester towards the cyclooxygenase activity of prostaglandin endoperoxide synthase-2. At variance with our results, Mitchell et al. (1994) demonstrated that flurbinitroxybutylester and flurbiprofen were equipotent to inhibit the cyclooxygenase activity of purified prostaglandin endoperoxide synthase-1 and of the isozyme constitutively expressed in bovine aortic endothelial cells in the presence of exogenous substrate. It is likely that different sensitivities of the bovine and human prostaglandin endoperoxide synthase-1 to flurbinitroxybutylester might account for the apparent discrepancy.

The administration of flurbinitroxybutylester but not flurbiprofen to rats has been demonstrated to increase plasma nitrate/nitrite levels consistently with the release of NO in vivo (Wallace et al., 1994a). However, this was not associated with any change in systemic blood pressure. The addition of a nitroxybutyl group to flurbiprofen, ketoprofen and diclofenac has been shown to reduce their unwanted ulcerogenic activity without altering their anti-inflammatory activity (Wallace and Cirino, 1994; Wallace et al., 1994a,b). It has been suggested that released NO can protect the gastrointestinal mucosa from injury. In fact, NO and NO donors have been shown to protect the gastric mucosa in experimental ulcer models (MacNaughton et al., 1989; Kitagawa et al., 1990).

It has been recently demonstrated that NO can stimulate the cyclooxygenase activity of prostaglandin endoperoxide synthase isozymes (Salvemini et al., 1993). Thus, the reduced capacity of flurbinitroxybutylester to suppress the cyclooxygenase activity of platelet prostaglandin endoperoxide synthase-1 demonstrated in the present study might be due to activation of the enzyme by released NO. However, this mechanism seems unlikely to explain our findings because: (1) NO should also activate the inducible prostaglandin endoperoxide synthase-2 enzyme; and (2) NO has also been reported to inhibit prostaglandin endoperoxide synthase activity (Vane et al., 1994; Stadler et al., 1993). Mitchell et al. (1994) demonstrated that NO is not liberated from flurbinitroxybutylester in cultured bovine aortic endothelial cells and J774.2 macrophages. No information is available on the release of NO from flurbinitroxybutylester in whole blood in vitro.

Wallace et al. (1994a) compared the severity of gastric mucosal injury and inhibition of prostaglandin E_2 production following the administration of flurbinitroxybutylester and flurbiprofen to rats. 5 h following oral administration, flurbinitroxybutylester caused significantly less gastric mucosal injury than flurbiprofen despite producing comparable suppression of prostaglandin E_2 biosynthesis. It is likely that conversion of flurbinitroxybutylester to flurbiprofen by esterases contributed to inhibition of the cyclooxygenase activity of gastric prostaglandin endoperoxide synthase-1 by flurbinitroxybutylester.

In conclusion, the addition of a nitroxybutyl moiety to flurbiprofen seems to reduce its capacity to inhibit the cyclooxygenase activity of human platelet prostaglandin endoperoxide synthase-1. Whether this effect will result in a reduced risk of gastrointestinal toxicity and bleeding remains to be studied in humans.

Acknowledgements

We thank Prof. Carlo Patrono for his review of the manuscript. 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

Barnett, J., J. Chow, D. Ives, M. Chiou, R. Mackenzie, E. Osen, B. Nguien, S. Tsing, C. Bach, J. Freire, H. Chan, E. Sigal and C. Ramesha, 1994, Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system, Biochim. Biophys. Acta 1209, 130.

Bateman, D.N., 1996, Re-evaluation of gut toxicity of NSAIDs, in: Improved Non-Steroid Anti-Inflammatory Drugs, COX-2 Enzyme Inhibitors, eds. J. Vane, J. Botting and R. Botting (Kluwer and William Harvey) p. 189.

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.

Chan, C.-C., S. Boyce, C. Brideau, A.W. Ford-Hutchinson, R. Gordon, D. Guay, R.G. Hill, C.-S. Li, J. Mancini, M. Penneton, P. Prasit, R. Rasori, D. Riendeau, P. Roy, P. Tagari, P. Vickers, E. Wong and I.W. Rodger, 1995, Pharmacology of a selective cyclooxygenase-2 inhibitor, L-745,337: a novel nonsteroidal anti-inflammatory agent with an ulcerogenic sparing effect in rat and nonhuman primate stomach, J. Pharmacol. Exp. Ther. 274, 1531.

Ciabattoni, G., F. Pugliese, M. Spaldi, G.A. Cinotti and C. Patrono, 1979, Radioimmunoassay measurement of prostaglandin E_2 and $F_{2\alpha}$ in human urine, J. Endocrinol. Invest. 2, 173.

De Lean, A., P.J. Munson and D. Rodbard, 1978, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves, Am. J. Physiol. 235, E97.

Grossman, C.J., J. Wiseman, F.S. Lucas, M.A. Trevethick and P.J. Birch, 1995, Inhibition of constitutive and inducible cylooxygenase activity in human platelets and mononuclear cells by NSAIDS and COX 2 inhibitors. Inflamm. Res. 44, 253.

Habib, A., C. Créminon, Y. Frobert, J. Grassi, P. Pradelles and J. Maclouf, 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.

Hla, T. and K. Neilson, 1992, Human cyclooxygenase-2 cDNA, Proc. Natl. Acad. Sci. USA 89, 7384.

Jones, D.A., D.P. Carlton, T.M. McIntyre, G.A. Zimmerman and S.M. Prescott, 1993, Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines, J. Biol. Chem. 268, 9049.

Kitagawa, H., F. Takeda and H. Kohei, 1990, Effects of endotelium-derived relaxing factor on the gastric lesion induced by HCl in rats, J. Pharmacol. Exp. Ther. 253, 1133.

Kraemer, S.A., E.A. Meade and D.L. DeWitt, 1992, Prostaglandin endoperoxide synthase gene structure: identification of the trascriptional start site and 5'-flanking regulatory sequences, Arch. Biochem. Biophys. 293, 391.

Kujubu, D.A. and H.R. Herschman, 1992, Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase gene, J. Biol. Chem. 267, 7991.

Laneuville, O., D.K. Breuer, D.L. DeWitt, T. Hla, C.D. Funk and W.L. Smith, 1994, Differential inhibition of human prostaglandin endoperoxide H synthase-1 and -2 by nonsteroidal anti-inflammatory drugs, J. Pharmacol. Exp. Ther. 271, 927.

Lopez-Belmonte, J., B.J. Whittle and S. Moncada, 1993, The action of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa, Br. J. Pharmacol. 108, 73.

MacNaughton, W.K., G. Cirino and J.L. Wallace, 1989, Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach, Life Sci. 45, 1869.

Masferrer, J.L., B.S. Zweifel, P.T. Manning, S.D. Hauser, K.M. Leahy, W.G. Smith, P.C. Isakson and K. Seibert, 1994, Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic, Proc. Natl. Acad. Sci. USA 91, 3228.

Mitchell, J.A., P. Akarasereenont, C. Thiemermann, R.J. Flower and J.R. Vane, 1993, Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase, Proc. Natl. Acad. Sci. USA 90, 11693.

Mitchell, J.A., G. Cirino, P. Akarasereenont, J.L. Wallace, R.J. Flower and J.R. Vane, 1994, Flurbinitroxybutylester, a novel anti-inflammatory drug devoid of ulcerogenic activity, inhibits cyclo-oxygenase-1 and cyclo-oxygenase-2, Can. J. Physiol. Pharmacol. 72 (Suppl. 1), 270 (Abstract).

O'Banion, M.K., H.B. Sadowski, V. Winn and D.A. Young, 1991, A serum and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase related protein, J. Biol. Chem. 266, 23261.

Panara, M.R., A. Greco, G. Santini, M.G. Sciulli, M.T. Rotondo, R. Padovano, M. di Giamberardino, F. Cipollone, F. Cuccurullo, C. Patrono and P. Patrignani, 1995, Effects of the novel anti-inflammatory compounds, *N*-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulphonamide (NS-398) and 5-methanesulphonamido-6-(2,4-difluorothiphenyl)-1-indanone (L-745,337), on the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases, Br. J. Pharmacol. 116, 2429.

Patrignani, P., M.R. Panara, A. Greco, O. Fusco, C. Natoli, S. Iacobelli, F. Cipollone, A. Ganci, C. Crèminon, J. Maclouf and C. Patrono, 1994, Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases, J. Pharmacol. Exp. Ther. 271, 1705.

Patrono, C., G. Ciabattoni, E. Pinca, F. Pugliese, G. Castrucci, A. De Salvo, M.A. Satta and B.A. Peskar, 1980, Low dose aspirin and inhibition of thromboxane B_2 production in healthy subjects, Thromb. Res. 17, 317.

Picot, D., P.J. Loll and R.M. Garavito, 1994, The x-ray crystal structure of the membrane protein prostaglandin H_2 synthase-1, Nature 367, 243.

Salvemini, D., T.P. Misko, J.L. Masferrer, K. Seibert, M.G. Currie and P. Needleman, 1993, Nitric oxide activates cyclooxygenase enzymes, Proc. Natl. Acad. Sci. USA 90, 7240.

Seibert, K., Y. Zhang, K. Leahy, S. Hauser, J. Masferrer, W. Perkins, L. Lee and P. Isakson, 1994, Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain, Proc. Natl. Acad. Sci. USA 91, 12013.

Smith, W.L. and D.L. DeWitt, 1995, Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal antiinflammatory drugs, Semin. Nephrol. 15, 179.

Soll, A.H., W.M. Weinstein, J. Kurata and D. McCarthy, 1991, Nonsteroidal anti-inflammatory drugs and peptic ulcer disease, Ann. Intern. Med. 114, 307.

Stadler, J., B.G. Harbrecht, M. Di Silvio, R.D. Curran, R.D. Jordan, M.L. Simmons and T.R. Billiar, 1993, Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells, J. Leukocyte Biol. 53, 165.

Vane, J.R. and R.M. Botting, 1995, New insights into the mode of action of anti-inflammatory drugs, Inflamm. Res. 44, 1.

Vane, J.R., J.A. Mitchell, I. Appleton, A. Tomlinson, D. Bishop-Bailey, J. Croxtall and D.A. Willoughby, 1994, Inducible isoforms of cyclooxygenase and nitric oxide synthase in inflammation, Proc. Natl. Acad. Sci. USA 91, 2046.

Wallace, J.L. and G. Cirino, 1994, The development of gastrointestinal-sparing nonsteroidal anti-inflammatory drugs, Trends Pharmacol. Sci. 15, 405.

Wallace, J.L., B. Reuter, C. Cicala, W. Mcknight, M.B. Grisham and G. Cirino, 1994a, Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat, Gastroenterology 107, 173.

Wallace, J.L., B. Reuter, C. Cicala, W. McKnight, M. Grisham and G.L. Cirino, 1994b, A diclofenac derivative without ulcerogenic properties, Eur. J. Pharmacol. 257, 249.