

Selective Cyclooxygenase-2 Inhibition by Nimesulide in Man¹

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ABSTRACT

Prostaglandins are generated through two isoforms of the enzyme cyclooxygenase, the constitutively expressed cyclooxygenase (Cox)-1 and Cox-2, which is induced at sites of inflammation. Selective inhibition of Cox-2 is desirable as this may avoid the gastropathy and platelet inhibition seen with nonselective agents. Moreover, these agents will allow us to examine the relative contribution of the two isoforms to prostaglandin formation in man. We examined the activity of nimesulide, a Cox-2 selective nonsteroidal antiinflammatory drug, *in vitro* against purified enzymes and *in vivo* in man. Nimesulide 100 mg twice daily or aspirin 300 mg three times daily were administered randomly for 14 days to 20 subjects complaining of musculoskeletal pain. Serum thromboxane B₂ was determined as an index of Cox-1 activity and endotoxin-induced prostaglandin E₂ formation in whole blood as an index of Cox-2

activity. Urinary excretion of prostaglandin metabolites was determined by GC/MS. Nimesulide was highly selective against ovine Cox-2, so that at concentrations attained *in vivo*, it had no effect on Cox-1 but completely suppressed Cox-2. Aspirin markedly inhibited serum thromboxane B₂ (181.92 ± 19.77 to 2.83 ± 0.96 ng/ml, *P* < .002), whereas nimesulide had very little effect (207.53 ± 47.30 to 181.15 ± 54.59 ng/ml). In contrast, nimesulide suppresses endotoxin-induced prostaglandin E₂ formation (35.03 ± 8.73 to 2.62 ± 0.95 ng/ml, *P* = .002). As expected, aspirin reduced TX metabolite excretion, whereas nimesulide had no significant effect. In contrast, both compounds suppressed PGI₂ formation to the same extent. The findings suggest that TX is largely Cox-1 derived. Moreover, Cox-2 is expressed in man and generates prostaglandin I₂.

PG play an important role in many biological systems, including hemostasis, integrity of the gastric mucosa, renal function and the inflammatory response. The first step in the formation of prostaglandins is oxidation of arachidonic acid by the enzyme Cox, the target for NSAIDs (Vane, 1971; DeWitt *et al.*, 1991). Two isoforms of the enzyme are known to exist, Cox-1 and Cox-2 (Hla and Neilson, 1992). Cox-1 is constitutively expressed in most tissues including the kidney, and the epithelial cells lining the gastrointestinal tract and is the only isoform of the enzyme expressed in platelets (O'Neill and Ford-Hutchinson., 1993; Funk *et al.*, 1991). Cox-2 is absent from most normal tissues, but is inducible by cytokines, growth factors and hormones (Kujubu *et al.*, 1991; Jones *et al.*, 1993; Fu *et al.*, 1990; Rimarachin *et al.*, 1994). In experimental models, Cox-2 is induced and is responsible for the increase in prostaglandin formation at sites of inflammation. Cox-2 expression has been demonstrated also in the synovial tissues from patients with rheumatoid arthritis (Crofford *et al.*, 1994).

Most NSAIDs discriminate poorly between the two isoforms and indeed inhibit Cox-1 to a greater extent than Cox-2. Inhibition of Cox-1, the isoform expressed in the stomach, may be responsible for the gastrointestinal injury and peptic ulceration seen in patients taking NSAIDs (Allison *et al.*, 1992). Furthermore, inhibition of Cox-1 in platelets by NSAIDs suppresses TXA₂ formation and platelet aggregation (Patrono *et al.*, 1985; FitzGerald, 1991; Patrono, 1994). The combination of mucosal injury and a hemostatic defect probably contributes to the most serious complication of these drugs, gastrointestinal bleeding. It is possible, however, to discriminate pharmacologically between the two isoforms and several Cox-2 selective compounds have been described (Meade *et al.*, 1993; Gans *et al.*, 1990; Panara *et al.*, 1995). These compounds would have a number of advantages, including greater potency against the generation of prostaglandins at sites of inflammation and preservation of gastrointestinal prostaglandin formation and platelet function.

In this study, we explored the effects of nimesulide, a Cox-2 selective NSAID, on prostaglandin and thromboxane formation in man using doses shown to be effective in inflammatory disorders. Serum TXB₂ was used as an index of Cox-1 activity, whereas Cox-2 activity was measured as lipopo-

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ABBREVIATIONS: Cox, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; TMPP, N N N'N'-tetramethyl-*p*-phenylenediamine dihydrochloride; GC/MS, gas chromatography, mass spectrometry; EIA, enzyme immunoassay; PG, prostaglandin; TX, thromboxane; DMSO, dimethyl sulfoxide.

lysaccharide-induced PGE₂ formation in whole blood. In addition, we determined urinary TXB₂, 11-dehydro TXB₂, 6-keto PGF_{1α} and 2,3-dinor-b-keto-PGF_{1α} as a markers of prostaglandin generation *in vivo*.

Methods

Cox-1 and Cox-2 assays. Assays were performed spectrophotometrically at 37°C using a Pharmacia LKB Ultraspec 111 (Pharmacia Biotech, Herts., UK) measuring the oxidation of TMPD (BDH/Merck Ltd, Poole, Dorset, England) at 611 nm (Kulmacz., 1987). Cyclooxygenase assay mixtures contained 1 ml of 0.1 M Tris-HCl, pH 8.0, 200 μM TMPD, 1 μM hematin (Sigma Chemical Co., St. Louis, MO), arachidonic acid (Cayman Chemical Co., Ann Arbor, MI) and 1.1 μg Cox-1 or 2.2 μg Cox-2 (Cayman Chemical Co.). Nimesulide (Helsinn Birex, Dublin, Ireland) in DMSO was incubated with enzyme for 1 min before initiating the reaction by the addition of 20 μM arachidonic acid (K_m 2–10 μM) (Marshall *et al.*, 1987).

Platelet aggregation. Platelet aggregation was performed as previously described (Fitzgerald *et al.*, 1988) in human platelet-rich plasma by light transmission using a four channel platelet aggregometer (Biodata, model PAP-4 Horsham, PA). Platelet aliquots (450 μl) were preincubated for 1 min in the presence of 1, 3 or 10 μM nimesulide in DMSO or with DMSO alone before the addition of arachidonic acid at a final concentration of 0.66 mM. Platelet aggregation was determined at 4 min after the addition of agonist.

Subjects. The study was approved by the Ethics Committee of Beaumont Hospital, Dublin and all patients gave informed, written consent. Twenty patients complaining of nonspecific musculoskeletal pains were recruited into the study. None of the patients were on NSAIDs, corticosteroids or any other drugs interfering with PG formation over the 14 days before study. There were 10 males and 10 females with a mean age of 59.8 yr. Peripheral venous blood samples were taken from each patient at 48 and 24 hr before starting the study to obtain baseline PG levels. The patients were randomly assigned to either 100 mg of nimesulide per orum twice daily or 300 mg of aspirin per orum three times daily for a 2-wk period in an open label fashion, followed by a washout period of 10 days. Blood samples were taken on days 2, 5, 10 and 14 during administration of the drug and on days 2, 5 and 10 after drug withdrawal. Urine was obtained as a spot sample before any treatment and 2 hr after drug administration on day 14.

Cox-1 activity in whole blood. Serum TXB₂ was assayed as previously described (Patrignani *et al.*, 1994). Briefly, nonanticoagulated whole blood was allowed to clot in nonsiliconised glass tubes at 37°C for 1 hr. Serum was separated by centrifugation at 1000 × *g* for 10 min and stored at -20°C. TXB₂ levels were determined using specific colorimetric EIA (Assay Designs, Inc., Ann Arbor, MI).

Induction of Cox-2 in whole blood. Cox-2 activity was determined as described by Patrono *et al.* (1994). Briefly, 1-ml aliquots of

whole blood containing 10 IU of sodium heparin were incubated both in the presence and absence of LPS, derived from *Escherichia coli* 026:B6 (Sigma Chemical Co.) 10 μg/ml at 37°C for 24 hr. The contribution of platelet Cox-1 was suppressed by the addition of 200 μM aspirin. As aspirin is rapidly inactivated by hydrolysis, induced Cox-2 activity is unaffected. Preliminary experiments demonstrated that the PGE₂ formed in this assay was Cox-2 dependent. Plasma was separated by the centrifugation at 1000 × *g* for 10 min and stored at -20°C until assayed for PGE₂ by EIA (Assay Designs, Inc.).

Determination of urinary eicosanoids by GC/MS. Urinary PG metabolites of prostacyclin (6-keto-PGF_{1α}, 2,3-dinor-6-keto-PGF_{1α}) and thromboxane A₂ (11-dehydro-TXB₂) were determined by negative ion, chemical ionization-GC/MS using deuterated internal standards for 11-dehydro TXB₂, 6-keto PGF_{1α} and 2,3-dinor-b-keto-PGF_{1α} (Cayman Chemical Co.) (Pratico *et al.*, 1995). The sample was derivatized as the pentafluorobenzyl ester, trimethylsilyl ether and GC/MS analysis was performed using a Varian 3400 gas chromatograph linked to a Finnigan Incos XL mass spectrometer operated in the negative ion, chemical ionization mode. Analytes were monitored by selected monitoring of the mass ion. Urinary TXB₂ was determined by EIA (Assay Designs, Inc.).

Statistical analysis. The data are expressed as mean ± S.E.M. The data were analysed by Friedman's nonparametric, two-way analysis of variance, followed by the Wilcoxon paired nonparametric test where appropriate. This makes no assumptions as to the distribution of the data.

Results

Cyclooxygenase Cox-1 and Cox-2 enzyme assay. Purified enzyme assays were used to examine the effect of nimesulide on both Cox-1 and Cox-2 in a cell free system using arachidonic acid as the substrate, and TMPD as the cosubstrate (fig. 1). In the presence of 20 μM arachidonic acid, nimesulide markedly suppressed Cox-2 with an IC₅₀ of 0.01 μM. Maximum plasma concentrations after repeated oral administration of nimesulide (100 mg twice daily for 7 days) have been reported to be less than 10 μM (Davis and Brogden., 1994), at which there was no detectable effect on Cox-1.

Platelet aggregation. To examine the effect of nimesulide on platelet function at plasma concentrations achieved *in vivo*, platelet aggregations in response to 0.66 mM arachidonic acid were performed. The vehicle, DMSO at 1%, had no effect on platelet aggregation. When compared to control, nimesulide at concentrations of 1, 3 and 10 μM had no effect on the extent of platelet aggregation (fig. 2). An increase in the lag time to platelet aggregation was seen, however, with nimesulide. Nimesulide has been reported to scavenge the hydroxyl radical (Maffei-Fracino *et al.*, 1993), which in turn may delay Cox activation and subsequent platelet aggregation (Violi *et al.*, 1988).

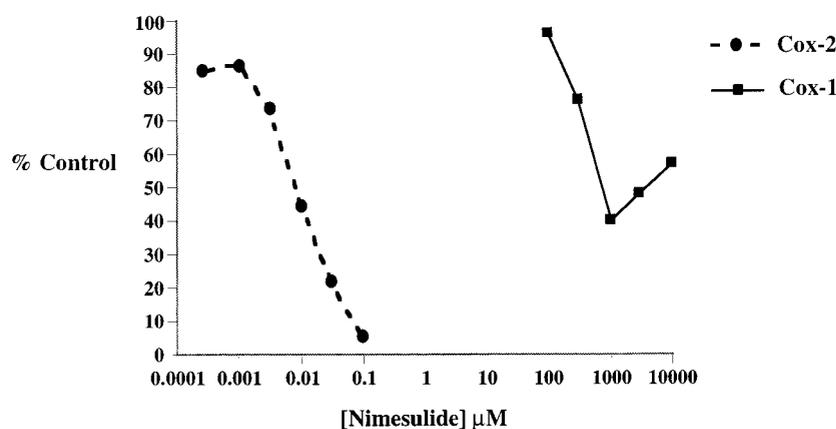


Fig. 1. Inhibition of the ovine Cox-1 and Cox-2 isozymes by nimesulide. Cox-1 1.1 μg or Cox-2 2.2 μg was incubated with TMPD 200 μM and hematin 1 μM and the reaction initiated with 20 μM arachidonic acid.

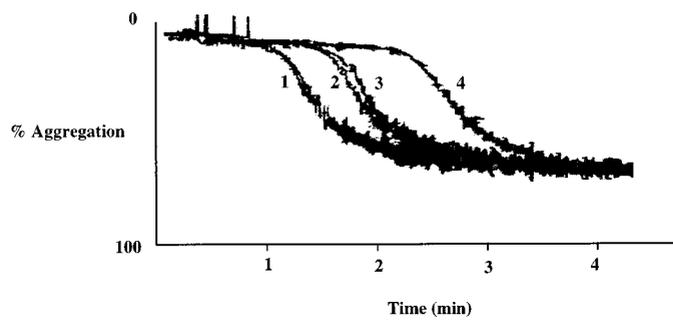


Fig. 2. Platelet aggregations in the presence of DMSO alone (tracing 1), 1 μ M nimesulide (tracing 2), 3 μ M nimesulide (tracing 3) and 10 μ M nimesulide (tracing 4).

Clinical study. One patient was withdrawn from the study 3 days after starting aspirin due to indigestion. The remaining patients completed the protocol and there were no symptoms reported.

Inhibition of Cox-2. Incubation of the whole blood with LPS *ex vivo* resulted in a marked induction of PGE₂ formation in both the nimesulide and aspirin groups before drug administration. Aspirin had no effect on LPS-induced PGE₂ formation (fig. 3, upper panel). In contrast, LPS-induced PGE₂ generation was markedly suppressed by nimesulide throughout the administration of drug (fig. 3, lower panel). LPS-induced PGE₂ formation had returned to 51.4 \pm 30.8% of

baseline 48 hr after withdrawal of nimesulide and was similar to predrug levels by day 5. Neither aspirin nor nimesulide had an effect on control samples incubated for 24 hr without the addition of LPS. Levels of PGE₂ in aspirin treated patients were 96.1 \pm 28.9% of control, while levels in nimesulide treated patients were 95.9 \pm 22.3% of control.

Inhibition of Cox-1. Serum TXB₂ was markedly suppressed by aspirin (fig. 4, upper panel) and, as expected, recovered slowly after drug withdrawal. In contrast, serum TXB₂ was little affected by nimesulide, with active serum TXB₂ levels at day 14 of 86.6 \pm 14.5% of predose levels (fig. 4, lower panel).

PG metabolite excretion. Urinary 6-keto-PGF_{1 α} and TXB₂ are hydrolysis products of the parent compounds and may reflect either renal or systemic production of the prostacyclin and TXA₂, respectively. In contrast, 2,3-dinor-6-keto-PGF_{1 α} and 11-dehydro-TXB₂ are enzymatic metabolites reflecting systemic generation of the parent compounds. Aspirin inhibited excretion of TXB₂ (to 32.9 \pm 12.6% of control), and 11-dehydro-TXB₂ (to 44.4 \pm 24.6% of control; P = .03) whereas nimesulide had no significant effect (urinary TXB₂, 83.1 \pm 20% of control and urinary 11-dehydro-TXB₂, 79.3 \pm 10.1% of control) (fig. 5, upper panel). In contrast, both drugs suppressed urinary 6-keto-PGF_{1 α} and 2,3-dinor-6-keto-PGF_{1 α} , and to a similar extent. Urinary 6-keto-PGF_{1 α} fell to 64.5 \pm 15.8% of control, and 2,3-dinor-6-keto-PGF_{1 α} to 36.5 \pm 10.1% of control P = .01, in aspirin-treated patients. In patients being administered nimesulide, 6-keto-PGF_{1 α}

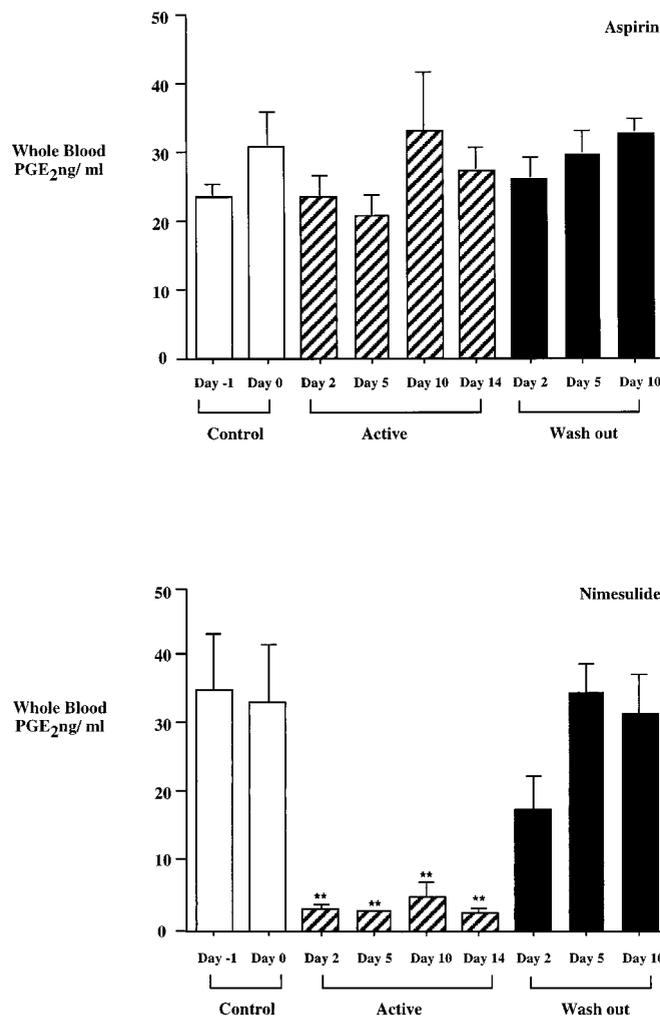


Fig. 3. PGE₂ after LPS induction of whole blood before (control), during (active) and after treatment of patients with aspirin (upper panel) and nimesulide (lower panel). ** P < .002.

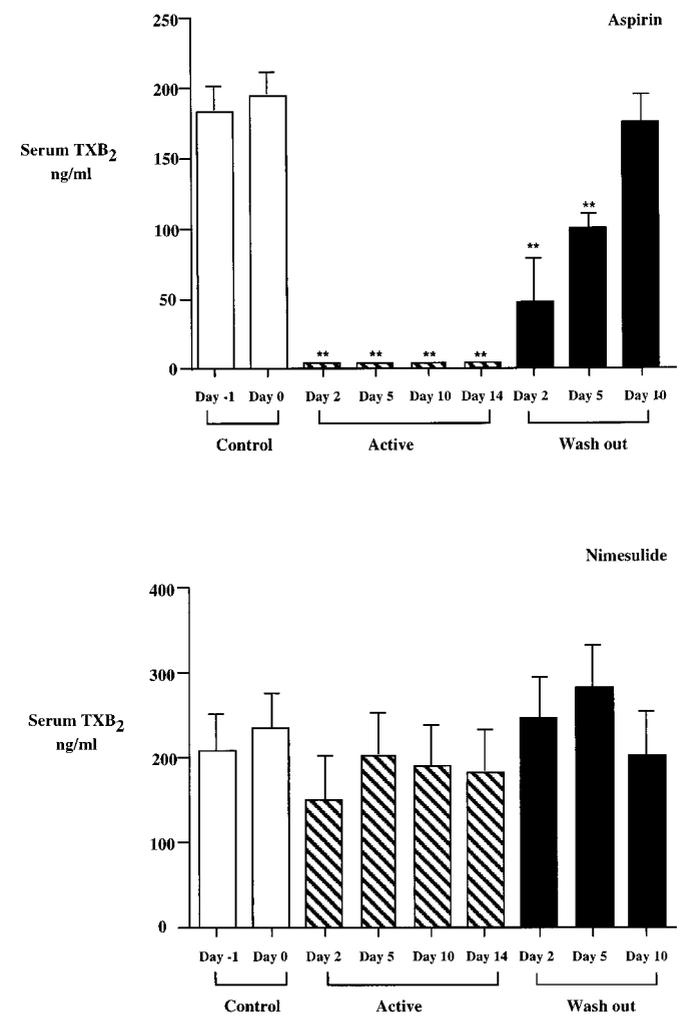


Fig. 4. Serum TXB₂ before (control), during (active) and after treatment of patients with aspirin (upper panel) and nimesulide (lower panel). ** P < .002.

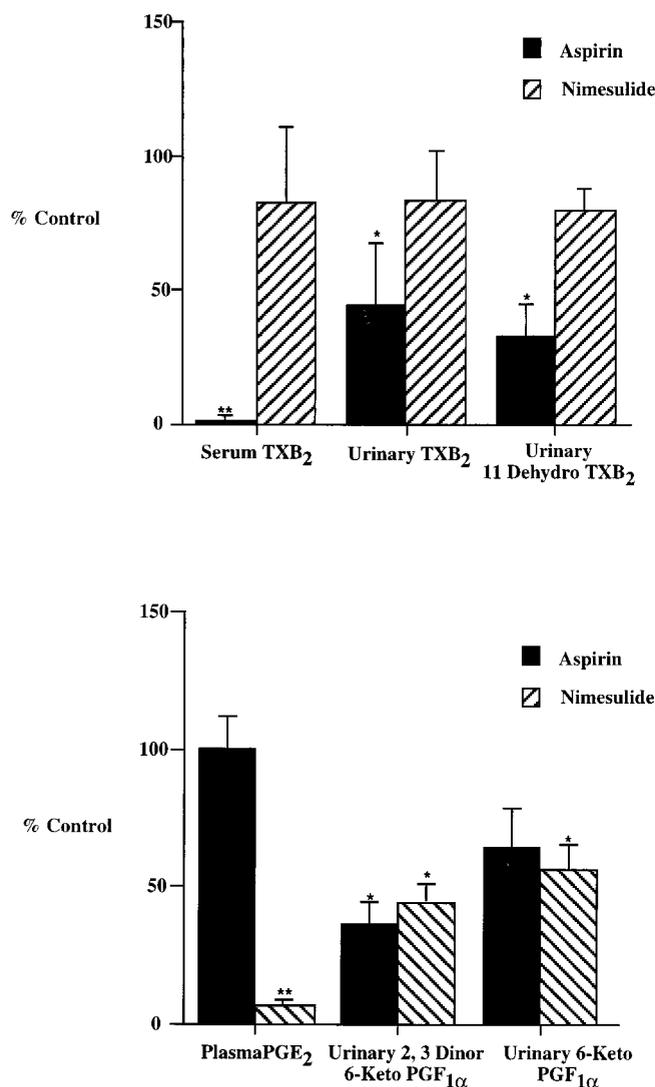


Fig. 5. Thromboxane (upper panel) and prostacyclin metabolite excretion after treatment with aspirin or nimesulide. Also shown are serum TXB₂ and LPS-induced PGE₂ for day 14. Results are expressed as % of control \pm S.E.M. ** $P < .002$; * $P < .05$.

fell to $55.9 \pm 11.1\%$ of control ($P = .03$) and 2,3-dinor-6-keto-PGF_{1 α} to $44.0 \pm 8.2\%$ of control ($P = .03$) (fig. 5, lower panel).

Discussion

Although the Cox-1 and Cox-2 exhibit a high degree of homology, particularly at the active sites, there are important structural differences. Substitution of an isoleucine and histidine within the Cox-1 substrate pocket for a valine and arginine in Cox-2 creates a side channel that is responsible for the selectivity of some compounds (Picot *et al.*, 1994; Loll *et al.*, 1995; Kurumbail *et al.*, 1996; Wong *et al.*, 1997). The selectivity of compounds varies widely depending on whether they are tested on purified proteins, cell systems or *in vivo*. *In vitro*, we found that nimesulide was a potent inhibitor of purified ovine Cox-2, but was five orders of magnitude less potent against Cox-1. As there may be species differences, we also examined the effect on human enzymes in platelets (Cox-1) where it had no effect, and in human umbilical endothelial cells induced by PMA to express Cox-2, where there

was marked suppression of prostacyclin formation (data not shown).

These studies, however, do not address the selectivity of drugs *in vivo*. For example, several NSAIDs interact in a noncompetitive fashion with cyclooxygenase over time so that during chronic administration a cumulative effect on Cox-1 may be seen. We used serum TXB₂ formation as a marker of platelet Cox-1 activity. As platelets express Cox-1 only and cannot generate new enzyme, serum TXB₂ is a highly sensitive index of any cumulative effect on Cox-1 *in vivo*. Moreover, as cumulative effects on platelet Cox-1 may be delayed, as shown with ultra low-dose aspirin regimens (McAdam *et al.*, 1996), we administered the drug for 14 days. In this study, we used aspirin as it has easily detectable effects on platelet Cox-1, resulting in a marked suppression of serum TXB₂. Samples were obtained at 2 hr after drug administration, corresponding to peak plasma levels. Although serum TXB₂ was markedly suppressed by aspirin, nimesulide had no significant effect.

To explore the Cox-2 effects of the two treatments, we examined LPS-induced PGE₂ formation, which is due to the induction of Cox-2, in anticoagulated whole blood (Patrignani *et al.*, 1994). The samples were pretreated with aspirin that irreversibly destroys all Cox activity in the samples. Nimesulide markedly suppressed LPS-induced PGE₂ formation over the period of administration. Interestingly, nimesulide had no effect in samples incubated for 24 hr without the addition of LPS, demonstrating the absence of Cox-2 in noninduced cells.

To determine the effect on *in vivo* prostaglandin formation, we examined the generation of two cyclooxygenase products, prostacyclin and thromboxane, by determining the excretion of their metabolites in urine. This approach avoids the many artifacts encountered measuring prostaglandins in blood samples, where the products detected largely reflect cell activation during the sampling procedure. Moreover, GC/MS has the sensitivity to detect the low concentrations of products that are present in urine and the specificity to distinguish metabolites with a high structural homology.

Aspirin, as expected, reduced urinary TXB₂ and 11-dehydro-TXB₂, much of which is platelet in origin. In contrast, nimesulide had only a minor effect, in close agreement with the serum TXB₂ findings. These data suggest that TXA₂ generated *in vivo* is largely Cox-1 derived, presumably from platelets. In contrast, nimesulide reduced the urinary excretion of both 6-keto-PGF_{1 α} and 2,3-dinor-6-keto-PGF_{1 α} , suggesting that these products are generated in part through Cox-2. Aspirin also inhibited prostacyclin biosynthesis, which is consistent with previous findings (Pedersen and FitzGerald, 1984). This could be due to inhibition of Cox-1 or Cox-2 or both because at the plasma concentration achieved with this dose, aspirin would be expected to inhibit the two isoforms (Meade *et al.*, 1993; Ali *et al.*, 1980).

The tissue source of the Cox-2-mediated PG formation in our patients is unknown. The study was performed in a group of patients that might be expected to receive NSAIDs rather than young, healthy subjects, and not surprisingly the mean age was 56 yr. It is possible that although there were no overt signs of inflammatory conditions, some degree of inflammation was present. Moreover, there are reports of Cox-2 expression in human tissues, including the brain (Lukiw and Bazan, 1997), stomach (Ristimaki *et al.*, 1997; Soydan *et al.*, 1997), kidney (Schneider and Stahl, 1998) and at sites of

atherosclerosis (Belton O, Leahy A and Fitzgerald DJ, unpublished observations).

NSAID-induced gastropathy is a major cause of hospital admission and death, accounting for half as many deaths as occur from road traffic accidents. Experimental data show that inhibition of Cox-1 plays a major role in NSAID-induced gastropathy and this is avoided using a Cox-2 inhibitor despite equivalent suppression of inflammation (Futaki *et al.*, 1993; Boyce *et al.*, 1994). Another important complication of NSAIDs, particularly in the elderly, is nephropathy (Henrich *et al.*, 1996; Bennett *et al.*, 1996). NSAID-induced nephropathy is a class effect and so is likely to reflect the primary pharmacological activity of NSAIDs. There are reports that both isozymes are expressed in the normal human kidney (Schneider and Stahl, 1998) with Cox-2 present in endothelial and smooth muscle cells of arteries and veins and in podocytes (Komhoff *et al.*, 1997). Our study did not distinguish between renal and systemic PG generation. Although urinary 6-keto-PGF_{1 α} was inhibited by nimesulide, this may have reflected a fall in systemic formation of the parent compound as urinary 2,3-dinor-6-keto-PGF_{1 α} , which is formed in the liver, was also reduced. It remains to be seen to what extent each isoform contributes to renal PG formation and if sparing of renal PG formed by Cox-1 will limit the appearance of NSAID-induced nephropathy. Whether nimesulide or similar Cox-2 selective inhibitors will reduce the risks associated with NSAID use is as yet unclear. Although nimesulide has a good safety record, randomized, double-blind studies specifically addressing gastric and renal toxicity are awaited.

In summary, nimesulide is a highly selective Cox-2 inhibitor. Chronic administration of nimesulide in man suppressed prostacyclin but not TX formation. The findings suggest that TX formation is largely Cox-1 derived. Moreover, Cox-2 is expressed in man and generates prostacyclin.

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