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Role of tumour necrosis factor-α and inducible nitric oxide synthase in the prevention of nitro-flurbiprofen small intestine toxicity

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Abstract

The present study compares the intestinal toxicity of nitro-flurbiprofen and flurbiprofen in order to determine their differential properties on tumour necrosis factor-α production and inducible nitric oxide synthase induction. Rats received one s.c. injection of flurbiprofen, nitro-flurbiprofen at equimolar dose of solvent. Twenty-four hours later, the rats were sacrificed and small intestine tissue was taken up for macroscopical quantification of ulceration, ex vivo production of tumour necrosis factor-α and nitrites, and determination of tissue inducible nitric oxide synthase and myeloperoxidase activities. Anti-inflammatory activity was examined in the carrageenan-induced paw edema model. We demonstrated that flurbiprofen induced dose-dependently small intestine production of tumour necrosis factor-α, nitrites, myeloperoxidase and inducible nitric oxide synthase activities. On the other hand, nitro-flurbiprofen did neither induce tumour necrosis factor-α nor nitrite production. Concurrently, no small intestine ulceration was observed with nitro-flurbiprofen whereas flurbiprofen induced dose-dependent ulceration. Nitro-flurbiprofen is devoid of intestinal toxicity despite inhibiting cyclooxygenase activity. This is associated with the absence of tumour necrosis factor-α and inducible nitric oxide synthase induction in normal rats. Nitro-flurbiprofen is an anti-inflammatory drug with a much more favorable gastro-intestinal toxicity profile than flurbiprofen.

Keywords: NSAID (non steroidal anti-inflammatory drug); Nitro-flurbiprofen; Intestinal toxicity; TMF-α (tumour necrosis factor-α); Nitric oxide (NO) synthase, inducible

1. Introduction

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with numerous side effects, the most frequent being at the gastrointestinal level (Rainsford, 1985). The potentially serious gastrointestinal pathology of NSAIDs may manifest itself as ulceration, bleeding and perforation resulting in more than 70,000 hospitalizations and 7000 deaths annually in the USA (Fries, 1991). This toxicity has been well documented both in humans and animals (Levi et al., 1990; Schmassmann et al., 1989).

In contrast with gastric toxicity of NSAIDs, intestinal toxicity caused by these drugs received close attention only recently. In humans, intestinal toxicity induced by NSAIDs is probably underestimated but with an 111 In-leucocytes technique, an inflammation of the small intestine and/or the colon has been reported in 65% of patients receiving long-term treatment with NSAIDs for osteoarthritis or rheumatoid arthritis (Bjarnasson et al., 1993; Davies, 1995). In rats, administration of NSAIDs can induce acute and chronic small intestinal ulcers (Elson et al., 1995). Initial steps of intestinal ulcers involve increased mucosal permeability which can be restored by exogenous prostaglandin administration (Davies et al., 1994). The mechanisms by which NSAIDs cause intestinal damage are explained mainly on the basis of inhibition of cyclooxygenase by NSAIDs and consequently by decreased synthesis of prostaglandins (Whittle, 1981). However, in addition to prostaglandins, nitric oxide is involved in inflammatory responses, including those in rheumatic or arthritic disease (McCartney-Francis et al., 1993; Stefanovic-Racic et al., 1994; Weinberg et al., 1994). Nitric oxide synthases and cyclooxygenases have constitutive and

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cytokine-inducible forms, and a cross-talk between nitric oxide synthases and cyclooxygenases pathway has become evident (Tetsuka et al., 1994). Actually, one approach to reduce gastro-intestinal toxicity is to develop NSAID-nitroxybutylesters (nitro-NSAIDs) that are capable of generating nitric oxide (Wallace et al., 1995a; Wallace et al., 1994a; Wallace, 1997) and suppress cyclooxygenases (type 1 and 2) as efficiently as the parent NSAID (Wallace et al., 1994b; Wallace et al., 1994c). There is growing evidence that endogenous nitric oxide regulates mucosal barrier integrity under physiological conditions and counters the increase in mucosal permeability associated with acute pathophysiological states (Alican and Kubes, 1996). The protective effects of nitric oxide include maintenance of blood flow, inhibition of platelet and leukocyte adhesion and/or aggregation within the vasculature, modulation of mast cell reactivity and scavenging of reactive oxygen metabolites such as superoxide (McCall et al., 1989). In support of this, it has been shown that the severity of NSAID-induced gastric damage can be reduced by nitric oxide donors (Wallace et al., 1994b) while an inhibitor of nitric oxide synthase increased it (Wallace et al., 1994c). Tumour necrosis factor-α has recently been proposed to be involved in NSAID-induced gastric injury since treatment with pentoxifylline prevents gastric injury (Santucci et al., 1995; Appleyard et al., 1996). Moreover, we have recently shown that tumour necrosis factor-α and the subsequent induction of inducible nitric oxide synthase play a pivotal role in indometacin-induced small intestine ulceration (Bertrand et al., 1997).

The aims of this study have been: first—to compare flurbiprofen and nitro-flurbiprofen in terms of their anti-inflammatory effect and their ulcerogenic properties at intestinal level both in normal and carrageenan-induced paw edema, and second—to determine whether nitro-flurbiprofen was capable of regulating tumour necrosis factor-α production in the small intestine tract and at the site of peripheral inflammation (hindpaw).

2. Methods

2.1. Animals

Nine-week-old male Wistar rats (Janvier, Le Genest St Isle, France) were fed standard laboratory chow and tap water ad libitum.

2.2. Treatment protocol in normal rats

Rats were deprived of food, but not water, for 18 h and were then given either flurbiprofen (10 and 30 mg/kg) (Sigma, St Quentin Fallavier, France), nitro-flurbiprofen (15 and 45 mg/kg; equimolar doses) (kindly provided by P del Soldato, NicOx, Paris, France) or the vehicle subcutaneously (s.c.) in the intrascapular region, in a volume of 5 ml/kg. Higher doses of nitro-flurbiprofen were used because the nitric oxide-releasing moiety of the compound accounts for 33% of the molecular weight. The rats were refed 5 h after s.c. injection. The compounds were initially dissolved in dimethylsulfoxide and diluted in 0.5% methylcellulose to a final concentration of the former of 5%. The rats were decapitated 24 h later. The small intestine was opened along the antimesenteric border and gently rinsed with phosphate-buffer saline pH 7.4 (Gibco, Life Technologies, France). The tissue was photographed to quantify ulceration. Samples of 1 cm-length pieces of small intestine were taken (10, 20 and 30 cm above the ileo-caecal valve) and pooled for the tissular determination of myeloperoxidase and nitric oxide synthase activities, and ex vivo measurement of tumour necrosis factor-α and nitrite production.

2.3. Treatment protocol in carrageenan induced paw edema

The experiments performed in normal rats (described above in paragraph 2.2) has confirmed the results we previously obtained with indometacin (Bertrand et al., 1997), in that tumour necrosis factor-α was not significantly increased in gastric tissue. Consequently, in the second part of the experiments, we decided to optimize the induction conditions for the exploration of intestinal damage: the animals were refed just after NSAID injection instead of 5 h after it. This protocol minimizes gastric ulceration but increases small intestine lesions (Weissenborn et al., 1985). Rats deprived of food, but not water for 18 h, were treated with flurbiprofen (30 mg/kg) or equimolar dose of nitro-flurbiprofen (45 mg/kg) or the vehicle. Thirty min later, a 0.1% solution of lambda carrageenan (kindly provided by Dr. D. Willoughby, London, UK) (0.1 ml) was injected into the right hind foot pad. The rats were refed immediately after the s.c. injection. The volume of the edema was recorded by means of plethysmography after carrageenan injection and every hour thereafter for 5 h. Changes in paw volume relative to the measurement taken just after carrageenan administration were calculated. At necropsy, inflammatory hindpaws were taken and kept at −80°C and dissected as previously described (Chen et al., 1994). For tumour necrosis factor-α, nitrites and prostaglandin-E_2, small intestine tissues were treated as described in 'Treatment Protocol in Normal Rats' except that the rats were sacrificed 5 h after carrageenan injection to be close to the peak of tumour necrosis factor-α concentrations in edematous tissue (Chen et al., 1994).

2.4. Intestinal damage scores

Small intestine ulcerations were long-shaped, segmental and sometimes confluent. Damage was scored by tracing the outline of ulcerated areas from twofold magnified photographic images onto paper, and weighting the cut-

outs. The small intestine damage score (expressed in cm^2) corresponds to the sum of ulcerated areas (Allison et al., 1992).

2.5. Myeloperoxidase activity in tissue samples

Myeloperoxidase activity was measured as described by Maehly and Chance (1954). Myeloperoxidase was extracted from tissue, frozen −80°C, by suspending the material in lysis buffer containing 20 mM KH_2PO_4 and 1.4 mM hexadecyltrimethyl ammonium bromide (HTAB) (pH = 6.0), before homogenization on ice with a Polytron homogenizer. Then, the homogenate was sonicated 10 s on ice, frozen at −80°C, sonicated 10 min on ice and centrifuged. The suspension was assayed spectrophotometrically for myeloperoxidase activity: 500 μl of suspension was combined with 2500 μl of buffer (pH = 6.0) containing 0.16 mM Na_2HPO_4, 18.4 mM KH_2PO_4, 44.8 μM guaiacol and 70 μmol/l hydrogen peroxide. The kinetics of absorbance at 470 nm were recorded with a spectrophotometer thermostated at 40°C. One unit of myeloperoxidase activity is defined as that degrading 1 μmol of peroxide per minute (Freehold, 1972). The change in absorbance for each sample was expressed in international units (IU) by using a standard curve of horseradish peroxidase established in the same experimental conditions. Myeloperoxidase activity is expressed as units (U) per gram of total protein, measured according to Lowry et al. (1951).

2.6. Nitric oxide synthase activities in tissue samples

Tissue inducible nitric oxide synthase activity was estimated by measuring the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline as described by Bush et al. (1992). Tissue samples (approximately 250 mg) were homogenized in buffer (pH = 7.4) containing 50 mM Tris–HCl, 1 mM dithiothreitol, 23.4 μM leupeptin, 14.6 μM pepstatin and 1 mM phenylmethylsulfonylfluoride. After sonication on ice and centrifugation at 250 g for 30 min at 4°C, 100 μl of the supernatant were added to a reaction mixture containing 50 mM Tris–HCl (pH = 7.4), 1.58 μM [^{14}C]L-arginine, 200 μM NADP, 10 μM flavine mononucleotide, 10 μM flavine adenine dinucleotide, 1 mM dithiothreitol, 50 μM tetrahydrobiopterine and 50 mM valine, with 2 mM $CaCl_2$ (total nitric oxide synthase activity) or 1 mM EDTA and 1 mM EGTA (inducible nitric oxide synthase activity). After 45 min of incubation at 37°C, the enzymatic reaction was terminated by adding cold phosphate buffer (pH = 5.5) containing 3 mM EDTA. [^{14}C]L-citrulline was separated by applying the samples to columns containing pre-equilibrated Dowex AG50W-X8, eluting them with water and measuring the amount of radioactivity by means of scintillation counting. Protein content was measured according to Lowry et al. (1951). Enzyme activity is expressed as picomol of citrulline formed per milligram of protein per hour. Specific nitric oxide synthase activity was defined as citrulline formation that was abolished by incubation of the supernatants with *N*ω-nitro-L-arginine methyl (300 μM) and was characterized by the effects of incubation with EDTA/EGTA (1 mM).

2.7. Culture ex vivo

After the sacrifice of rats, intestinal tissue was carefully washed three times in sterile and pyrogen-free normal saline, then cultured into 24-well plates (Nuncleon, France) with 1.6 ml of RPMI 1640 medium (Sigma, St Quentin Fallavier, France) without phenol red and supplemented with penicillin (100 IU/ml)/streptomycin (100 μg/ml). The tissue was cultured for 5 h in a humid atmosphere at 37°C with 5% CO_2. After that time, supernatants were collected and kept at −80°C before the determination of tumour necrosis factor-α, nitrites and prostaglandin-E_2.

2.8. Tumour necrosis factor-α assays in supernatants

The cytotoxic activity of tumour necrosis factor-α was tested on serial dilutions of supernatants obtained from either small intestine tissue cultured ex vivo or homogenized plantar tissue as previously described (Chen et al., 1994). Tumour necrosis factor-α activity was quantified on actinomycin D-treated murine L929 fibroblasts as previously described (Chen et al., 1994). The tumour necrosis factor-α titer (U/ml) was defined as the reciprocal of sample dilution that caused half-maximal cell destruction. Recombinant human tumour necrosis factor-α (kindly provided by Knoll/BASF, Ludwigshafen, Germany) was used for the standard curve. The tumour necrosis factor activity of samples was completely neutralized by preincubation of samples with anti-tumour necrosis factor antibody, at 37°C for 2 h. Less than 10 ng of anti-tumour necrosis factor antibody was necessary to abolish 1 U of tumour necrosis factor activity. The control IgG had no effect (data not shown). Final concentrations were expressed as U/g of cultured tissue or as U/mg of protein for the assay in carrageenan-induced paw edema.

2.9. Nitrites and prostaglandin-E_2 in cultured supernatants

Nitrite concentrations were determined in duplicate by a spectrofluorimetric method using diaminonaphthalene as substrate (Misko et al., 1993). Prostaglandin-E_2 was measured at two serial dilution with a specific EIA kit (Stallergènes, Fresnes, France). Its detection limit is 7.8 pg/ml.

2.10. Statistical analysis

The results are expressed as mean ± S.E.M. Comparison was performed by non parametric tests or ANOVA as appropriate. P values less than 0.05 were considered significant.

3. Results

3.1. Effects of NSAIDs in normal rats

3.1.1. Small intestine damage scores

Administration of flurbiprofen by s.c. route induced penetrating ulcers along the mesenteric border of small intestine tissue. The average small intestine damage scores were 0.11 ± 0.08 and 0.65 ± 0.11 cm^2 for 10 and 30 mg/kg doses, respectively (Fig. 1). In the rats treated with nitro-flurbiprofen, intestinal ulcers were not observed at each of the doses tested, except one out of eight rats that developed small ulceration as assessed by a damage score of 0.099 cm^2 for the 15 mg/kg dose. The average intestinal damage scores were not significantly different from that observed in solvent-treated rats and nitro-flurbiprofen 45 mg/kg was significantly different ($P = 0.001$) from flurbiprofen 30 mg/kg and 10 mg/kg (Fig. 1).

3.1.2. Tumour necrosis factor-α production by small intestine tissue

At the small intestine level, flurbiprofen 30 mg/kg significantly ($P = 0.0008$) induced tumour necrosis factor-α concentrations, with 235.14 ± 32.26 U/g of tissue compared to solvent-treated rats in which no tumour necrosis factor-α was detected. By contrast, nitro-flurbiprofen at the two doses tested did not induce tumour necrosis factor-α production (Fig. 2).

3.1.3. Myeloperoxidase and inducible nitric oxide synthase activities, and nitrite production in intestine

In an attempt to quantify the infiltration of small intestine mucosa by neutrophils, we assessed intestine myeloperoxidase activity. In small intestine tissue, myeloperoxidase activity was significantly ($P = 0.0011$) increased to 5.58 ± 0.52 U/g of protein in rats treated with 30 mg/kg flurbiprofen (Fig. 3A). The levels of myeloperoxidase activity found in the small intestine tissue of rats treated with nitro-flurbiprofen were not significantly different from the value in solvent-treated rats: 3.32 ± 0.46 and 2.75 ± 0.31 U/g of protein for 15 and 45 mg/kg nitro-flurbiprofen, respectively, vs. 2.81 ± 0.24 U/g of protein for solvent-treated rats. Moreover, myeloperoxidase activity with the highest dose of nitro-flurbiprofen was significantly different ($P = 0.0015$) from the value in the rats receiving 30 mg/kg flurbiprofen (Fig. 3A).

Inducible nitric oxide synthase activity in small intestine tissue was dose-dependently increased in flurbiprofen-treated rats with 1.70 ± 0.28 and 3.83 ± 0.96 pmol/mg of protein/h for 10 and 30 mg/kg doses respectively and was significantly ($P = 0.021$ and 0.001, respectively) different from solvent-treated rats (0.78 ± 0.25 pmol/mg of protein/h) (Fig. 3B). In the same way, nitrite production by small intestine tissue was significantly ($P = 0.001$) increased in flurbiprofen treated rats with 342.63 ± 122.0 nmol/g of tissue for 30 mg/kg dose (Fig. 3C). By contrast, inducible nitric oxide synthase activity and nitrite production at small intestine level in nitro-flurbiprofen treated rats at each doses tested, returned near to control values and were not significantly different from solvent-treated rats (Fig. 3B,C). Small intestine inducible nitric oxide synthase activity induced by nitro-flurbiprofen 45 mg/kg (1.37 ± 0.48 pmol/mg protein/h) was significantly different ($P = 0.012$) from flurbiprofen 30 mg/kg (3.83 ± 0.96 pmol/mg protein/h). In parallel, small intestine nitrite production induced by nitro-flurbiprofen 45 mg/kg (13.99 ± 5.03 nmol/g of tissue) was significantly different ($P = 0.0011$) from flurbiprofen 30 mg/kg (342.63 ± 122.0 nmol/g of tissue) (Fig. 3B,C).

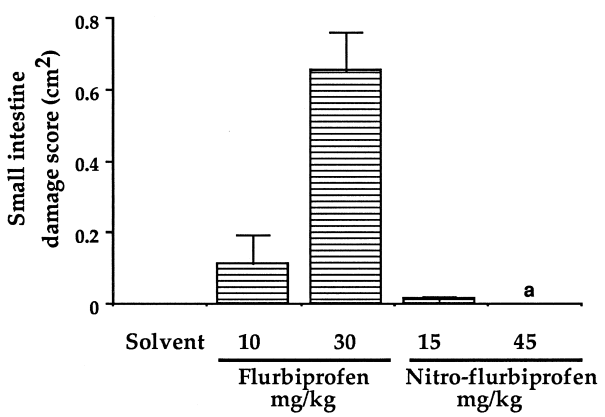

Fig. 1. Effects of flurbiprofen and nitro-flurbiprofen on small intestine damage score. Eight rats per group received solvent, flurbiprofen (10, 30 mg/kg) or nitro-flurbiprofen (15, 45 mg/kg) by s.c. route. Twenty-four hours later the rats were sacrificed. Intestinal damage score corresponded to the sum of ulceration areas (cm^2). Results are the mean ± S.E.M. ($n = 8$). [a] $P < 0.01$ compared with equimolar dose of flurbiprofen.

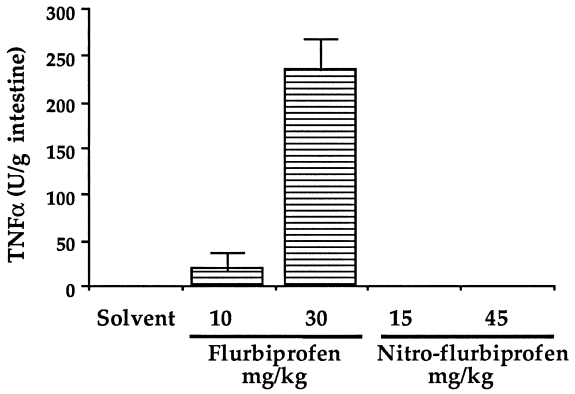

Fig. 2. Effects of flurbiprofen and nitro-flurbiprofen on small intestine tumour necrosis factor-α concentration. Tumour necrosis factor-α was determined in supernatants obtained from intestine cultured for 5 h at 37°C. Data were obtained from the same rats than in Fig. 1. Results are the mean ± S.E.M. ($n = 8$).

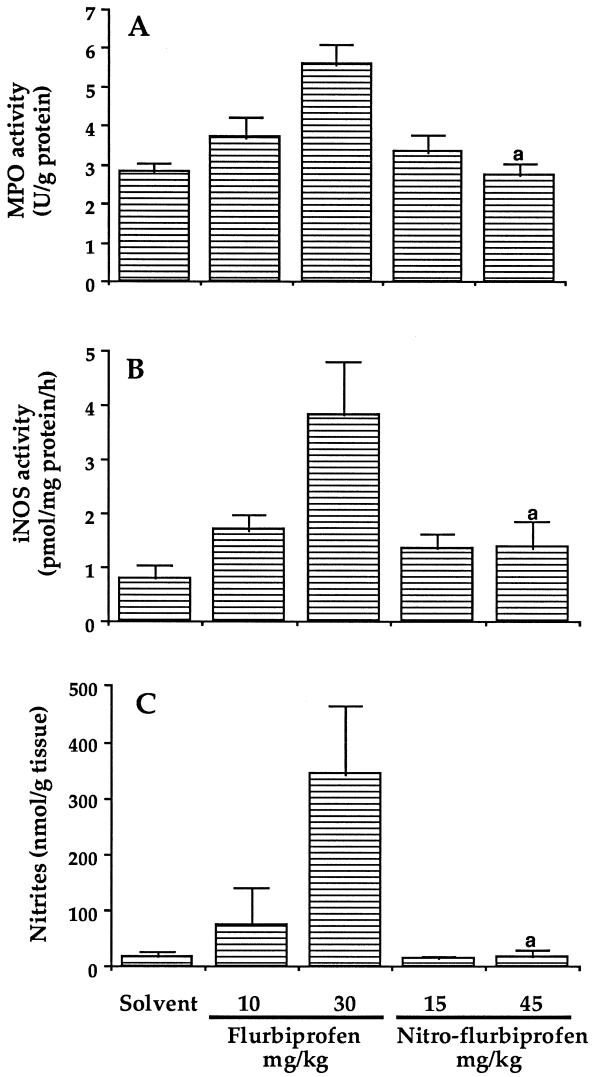

Fig. 3. Intestinal effects of flurbiprofen and nitro-flurbiprofen on myeloperoxidase (A) and inducible nitric oxide synthase (B) activities, and nitrite production (C). Enzyme activities were determined from homogenized small intestine as described in Section 2. Nitrites were quantified in supernatants of small intestine cultured ex vivo for 5 h at 37°C. Data were obtained from the same rats than in Fig. 1. Results are the mean ± S.E.M. ($n = 8$). [a]$P < 0.01$ compared with equimolar dose of flurbiprofen.

3.2. Effects of NSAIDs in rats with carrageenan-induced paw edema

3.2.1. Anti-inflammatory activity

As shown in Fig. 4, both flurbiprofen (30 mg/kg) and nitro-flurbiprofen (45 mg/kg) significantly ($P = 0.001$) reduced the paw edema induced by carrageenan compared to the solvent-treated group. There were no significant differences between the two drugs at any of the time points examined. Similar effects of the two compounds were also obtained using a lower dose (10 and 15 mg/kg flurbiprofen or nitro-flurbiprofen, respectively). At the fifth hour, flurbiprofen (10 mg/kg) reduced edema volume by 65.2 ± 12.4% and nitro-flurbiprofen (15 mg/kg) by 49.5 ± 7.1%, the differences between the two drugs being non-significant (ANOVA).

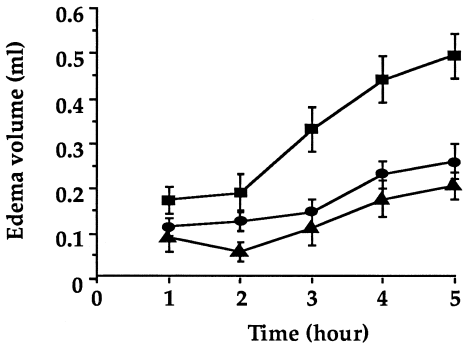

Fig. 4. Time-course effects of flurbiprofen and nitro-flurbiprofen on carrageenan-induced paw edema. Five rats per group received either solvent, flurbiprofen (30 mg/kg) or nitro-flurbiprofen (45 mg/kg) by s.c. route, 30 min before carrageenan injection. At various times after carrageenan injection, paw edema was recorded for (solvent – ■ –; flurbiprofen – ▲ – and NO-flurbiprofen – ● –) after carrageenan injection were determined. Results are the mean ± S.E.M. ($n = 5$). [a]$P < 0.03$ compared with equimolar dose of flurbiprofen.

3.2.2. Tumour necrosis factor-α concentrations in plantar edema

Rats receiving solvent in the paw did not produce tumour necrosis factor-α. Rats receiving carrageenan in the paw produced tumour necrosis factor-α with a level of 37.19 ± 5.49 U/mg of protein 5 h after carrageenan injection. Administration of flurbiprofen (30 mg/kg) and nitro-flurbiprofen (45 mg/kg) by s.c. route increased tumour necrosis factor-α concentrations in tissue of carrageenan-treated rats significantly ($P = 0.004$ and 0.002, respectively) (Fig. 5). There were no significant differences in paw-edema tumour necrosis factor-α concentrations between flurbiprofen and nitro-flurbiprofen groups. The smaller dose of NSAIDs also increased plantar tumour

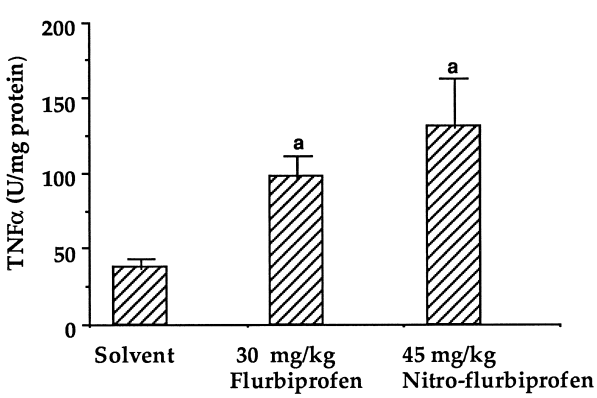

Fig. 5. Effects of flurbiprofen (30 mg/kg) and nitro-flurbiprofen (45 mg/kg) on tumour necrosis factor-α production in edematous plantar tissue. Tumour necrosis factor-α 5 h after carrageenan injection were determined. Results are the mean ± S.E.M. ($n = 5$).

Table 1
Effects of flurbiprofen and nitro-flurbiprofen on small intestine damage score, tumour necrosis factor-α and prostaglandin-E_2 production in rats with carrageenan-induced paw edema (same rats as in Fig. 5)

	Small intestine damage score (cm^2)	TNFα (U/g tissue)	PGE_2 (ng/g tissue)
Solvent	0	105.51 ± 51.07	5.30 ± 1.51
Flurbiprofen 30 mg/kg	3.78 ± 0.51[a]	846.48 ± 165.16[a]	2.02 ± 0.68[a]
Nitro-flurbiprofen 45 mg/kg	0.03 ± 0.03	107.41 ± 42.79[b]	0.84 ± 0.17[a]

Small intestine damage score corresponded to the sum of ulceration areas (cm^2).
Tumour necrosis factor-α (TNF-α) and prostaglandin-E_2 (PGE2) were determined in supernatants obtained from intestine cultured for 5 h at 37°C.
Results are mean ± S.E.M. ($n = 5$).
[a] $P < 0.01$ compared with solvent-treated group.
[b] $P < 0.01$ compared with flurbiprofen-treated group.

necrosis factor-α levels by comparison with rats receiving only carrageenan (data not shown).

3.2.3. Damage scores, tumour necrosis factor-α and prostaglandin-E_2 concentrations in small intestine tissue

Administration of flurbiprofen (30 mg/kg) and nitro-flurbiprofen (45 mg/kg) by s.c. route in rats with plantar paw edema produced small intestine ulceration as assessed by a significant ($P = 0.001$) damage score of 3.78 ± 0.51 cm^2. Only one out of ten rats treated with nitro-flurbiprofen 45 mg/kg developed very mild ulceration (damage score 0.03 cm^2) (Table 1). The rats treated with flurbiprofen had a significant ($P = 0.003$) increase in small intestine tumour necrosis factor-α concentrations while the small intestine tumour necrosis factor-α concentrations in nitro-flurbiprofen-treated rats returned near to control values. The prostaglandin-E_2 level produced by the intestine of control rats was 5.30 ± 1.51 ng/g of tissue. Flurbiprofen 30 mg/kg and nitro-flurbiprofen 45 mg/kg significantly reduced ($P = 0.008$) the production of prostaglandin-E_2 by 62% and 84%, respectively (Table 1). This indicates that s.c. administration of nitro-flurbiprofen was at least as potent as flurbiprofen to inhibit cyclooxygenase activity at small intestine level. The lowest dose of flurbiprofen was also associated with significant intestinal damage score (1.74 ± 0.75 cm^2 vs. 0 cm^2 in carrageenan group, $n = 6$) and small intestine tumour necrosis factor-α production (289.8 ± 100.2 U/g of tissue vs. 10.7 ± 6.8 U/g of tissue in carrageenan group). Again, nitro-flurbiprofen (15 mg/kg) did not induce significant changes at small intestine level by comparison with the carrageenan group, in terms of damage score and tumour necrosis factor-α production (data not shown).

4. Discussion

In this study, small intestine toxicity and anti-inflammatory potency of nitro-flurbiprofen were evaluated in comparison to the parent drug flurbiprofen. Nitro-flurbiprofen and flurbiprofen retained the anti-inflammatory activity in the rat carrageenan-induced paw edema, and simultaneously exerted no small intestine toxicity while the two compounds reduced intestinal prostaglandin-E_2 production to the same extent. The effect of flurbiprofen on the small intestine was characterized by the induction of tumour necrosis factor-α production and the activation of inflammation pathways relative to the primary cytokine. By contrast, nitro-flurbiprofen did not induce inflammation nor tumour necrosis factor-α release, even at a dose three times higher than that of flurbiprofen eliciting frank small intestine ulcerations. On the other hand, both flurbiprofen and nitro-flurbiprofen slightly increased, the levels of tumour necrosis factor-α, locally in carrageenan-induced paw edema.

The ability of NSAIDs to induce small intestine injury has been well documented both clinically (Bjarnasson et al., 1993) and experimentally (Yamada et al., 1993). The mechanisms through which these agents produce the injury remain unclear but all NSAIDs inhibit prostaglandin synthesis and it has been widely proposed that this represents a common and necessary mechanism of action (Vane, 1971). Attempts to develop NSAIDs with reduced irritant effects on the gastrointestinal tract have primarily involved changes in the formulation of these drugs (enteric coating, prodrugs requiring metabolism in the liver) and more recently, attempts have been made to develop NSAIDs which selectively inhibit the inducible form of cyclooxygenase, in the hope that these drugs will not produce gastrointestinal injury.

The mechanisms of the reduction of the toxicity of nitro-flurbiprofen are complex. By contrast with gastric lesions, the induction of intestinal damage by NSAIDs does not primarily depend on neutrophil activation. We have recently reported that small intestine ulcerations induced by indometacin are associated with early mucosal production of tumour necrosis factor-α and are prevented by drugs that inhibit tumour necrosis factor-α synthesis and by glucocorticoids (Bertrand et al., 1997; Mauprivez et al., 1997; Appleyard et al., 1996). In the present study, our results confirmed that flurbiprofen also possessed the properties to damage the small intestine and to induce the inflammatory cascade locally. The reduced small intestine

lesions observed with nitro-flurbiprofen correlates with the absence of local tumour necrosis factor-α and nitrite production and inducible nitric oxide synthase induction, 24 h after drug administration. This suggests that the mechanism through which nitro-flurbiprofen has reduced intestinal 'ulcerogenicity' depends, at least in part, on its effects on tumour necrosis factor-α release at small intestine level.

On the other hand, flurbiprofen and nitro-flurbiprofen increased the production of tumour necrosis factor-α concentration locally in carrageenan-treated rats. Wallace and coworkers have shown that nitro-naproxen and naproxen induced similar small increase in serum tumour necrosis factor-α levels (up to 30 U/ml) 3 h after their administration (Davies et al., 1997). We found that plantar tissue levels of tumour necrosis factor-α, measured 5 h after carrageenan injection, were increased in the same extent by flurbiprofen and nitro-flurbiprofen. So it appears that the effects of nitro-flurbiprofen on tumour necrosis factor-α release differ in the small intestine by comparison with other sites such as plasma and plantar edema. As flurbiprofen and nitro-flurbiprofen decreased similarly prostaglandins synthesis in the small intestine, the discrepancy could be due to a differential local release of nitric oxide in the gastrointestinal tract and in other sites (e.g., plantar edema). With glyceryl trinitrate, nitro-flurbiprofen is an organic nitrate characterized by the radical $C-O-NO_2$. It is interesting to notice that glyceryl trinitrate can be metabolized to nitric oxide by cytochrome *P*-450 (CYP3A) (Delaforge et al., 1993) which is the main isoenzyme present in the digestive tract. Moreover, metabolism of glyceryl nitrate to nitric oxide in smooth muscle cells is potentiated by lipopolysaccharide (Salvemini et al., 1992). Thus, the digestive tract could be a privileged site for enzyme conversion of nitro-NSAIDs to nitric oxide.

A relationship between the enterohepatic recirculation of the NSAIDs and their ability to cause intestinal damage was recently suggested (Reuter et al., 1997). However, the absence of small intestine damage after nitro-flurbiprofen cannot be explained by a lower enterohepatic recirculation for the following reasons. First, prostaglandin-E_2 production in the small intestine was reduced as potently by nitro-flurbiprofen than by flurbiprofen following their s.c. administration. Second, a previous study had shown that the administration of nitro-flurbiprofen by the same route (s.c.) protect the small intestine from the injury induced by endotoxin (Wallace et al., 1995b) demonstrating that the nitric oxide releasing moiety of this compound has a protective effect on the small intestine. Third, the bioavailability of nitro-NSAID is decreased (approximately 35%) after oral administration in rats and nitro-flurbiprofen is transformed in flurbiprofen in less than 24 h after oral administration (unpublished data from NicOx). Our results show that the s.c. administration of 45 mg/kg of nitro-flurbiprofen (corresponding to an equimolecular dose of 30 mg/kg flurbiprofen) did not induced any intestinal lesion while the s.c. administration of a dose as low as 10 mg/kg flurbiprofen induced intestinal lesions in all rats. Although we did not measure bile concentration of flurbiprofen in our study, we can assume (according to the unpublished data from NicOx reported below) that 24 h after s.c. administration of 45 mg/kg of nitro-flurbiprofen, the concentration of flurbiprofen in bile was higher than after the administration of 10 mg/kg flurbiprofen. That is the reason why the protective effect of nitro-flurbiprofen cannot only be explained by a decrease in the enterohepatic recirculation.

The mechanisms of tumour necrosis factor-α release by NSAIDs in the small intestine remain to be determined. Tumour necrosis factor-α production is strongly regulated by soluble mediators. In particular prostaglandins, such as prostaglandin-E_2, inhibits tumour necrosis factor-α synthesis in a feed back loop, via increased intracellular concentrations of cAMP (Spengler et al., 1989). Prostaglandin-E_2 and nitric oxide act synergistically in many physiological processes, particularly in the maintenance of tissue integrity at cellular and extracellular levels (Radomski et al., 1988). Prostaglandin-E_2 and nitric oxide are synthesized by constitutive and inducible isoenzymes (Kujubu et al., 1991; Moncada and Higgs, 1993). The inducible forms of cyclooxygenase (COX 2) and inducible nitric oxide synthase are induced, with a similar time course, by lipopolysaccharide, interleukin-1β, tumour necrosis factor-α and interferon-γ. Further evidence of the cooperative interactions between nitric oxide and prostaglandin-E_2 in modulating mucosal defense is that these mediators can regulate the activity of the enzymes responsible for the synthesis of one another (Salvemini et al., 1995). Tumour necrosis factor-α and interleukin-1β are known to induce inducible nitric oxide synthase but the mechanism still remains incompletely understood. Previous data indicated that exogenous nitric oxide derived from nitro-flurbiprofen (Mariotto et al., 1995), like endogenous nitric oxide (Park et al., 1994), exerted an inhibitory action on inducible nitric oxide activity. Our results reinforce this hypothesis. There is growing evidence that cytokine signalling is more complex than originally thought, involving kinases, phosphatases, small GTP-binding proteins (Taniguchi, 1995; Heller and Krönke, 1994; Kolesnick and Golde, 1994). In this regard, acetylsalicylic acid and sodium salicylate, devoid of small intestine toxicity, inhibit the activation of nuclear factor-κB (NF-κB) (Kopp and Ghosh, 1994) and inhibit the induction of inducible nitric oxide synthase (Farivar et al., 1996). Similarly, glucocorticoids could inhibit, first, the activation of NF-κB induced by tumour necrosis factor-α, and second, the induction of inducible nitric oxide synthase induced by NF-κB (Finco and Baldwin, 1995; Auphan et al., 1995; Scheinman et al., 1995; Kleinert et al., 1996). Moreover, the authors demonstrated in cultured cells that the inhibition of constitutive production of nitric oxide induces the activation of NF-κB while nitric oxide

donors inhibit the activation of NF-κB induced by tumour necrosis factor-α (Peng et al., 1995). Whether nitro-flurbiprofen exerts its protective effects by inhibiting NF-κB, therefore after the induction of inducible nitric oxide synthase, remains to be demonstrated.

In conclusion, these results suggest that nitro-flurbiprofen may offer a useful alternative to existing flurbiprofen. Nitro-flurbiprofen is an anti-inflammatory drug with a more favorable gastro-intestinal toxicity profile than flurbiprofen. Nitro-NSAIDs did not induce injury and inflammation in the small intestine and might be very useful in patients with inflammatory bowel disease needing NSAIDs for arthritis. The results obtained with nitro-NSAIDs, if translated into human terms, suggest that nitro-NSAIDs could have advantages over both new NSAIDs and no NSAIDs (Hawkey, 1995). However, further studies are required to better understand the mechanism(s) through which nitro-NSAIDs preserve gastro-intestinal integrity despite keeping their anti-inflammatory properties.

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