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Effects of cyclooxygenase-1/cyclooxygenase-2 inhibition on leukocyte/endothelial cell interactions in the rat mesentery

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAID) inhibit cyclooxygenase activity and cause gastrointestinal damage in part by promoting leukocyte accumulation in the mucosa. Our aim was to evaluate the effects of selective blockade of the isoenzymes cyclooxygenase-1 and cyclooxygenase-2 on leukocyte adhesion in vivo. Leukocyte/endothelial cell interactions were examined in rat mesenteric venules before and after treatment with indomethacin, SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole, cyclooxygenase-1 inhibitor), DFP (5,5-dimethyl-3-(2-propoxy)-4-(4-methanesulfonyl)-2(5*H*)-furanone, cyclooxygenase-2 inhibitor), or SC-560 plus DFP (20 mg/kg, i.v. each). Indomethacin increased leukocyte-rolling flux, reduced rolling velocity and elicited significant leukocyte adhesion. Neither SC-560 nor DFP induced these effects although their co-administration reproduced the indomethacin response. Indomethacin and SC-560, but not DFP, abolished cyclooxygenase-1 activity in blood. Plasma from indomethacin- or DFP-, but not from SC-560-treated rats abolished cyclooxygenase-2 activity in activated A549 cells. Specific blockade of cyclooxygenase-1 or cyclooxygenase-2 does not induce any inflammatory event in the rat mesentery and the inflammatory response observed with non-selective NSAIDs seems to be due to the inhibition of both isoenzymes.

Keywords: Cyclooxygenase-1; Cyclooxygenase-2; NSAID (nonsteroidal anti-inflammatory drug); Leukocyte/endothelium interaction

1. Introduction

Nonsteroid anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs, even though they may induce serious gastrointestinal complications (Hawkey, 1999; Mitchell and Warner, 1999). Inhibition of cyclooxygenase, and therefore prostaglandin production, is the common mechanism of action of the NSAIDs (Vane, 1971). Two isoforms of cyclooxygenase, named cyclooxygenase-1 and cyclooxygenase-2, have been described. In general terms, cyclooxygenase-1 is constitutive and present in, for example, the endothelium, stomach and kidney whereas cyclooxygenase-2 is induced by pro-inflammatory cytokines and endotoxin in cells in vitro and at inflammatory sites in vivo (Vane et al., 1998). This pattern of expression has given rise to the hypothesis that the therapeutic, anti-inflammatory effect of these agents is attributable to their ability to inhibit cyclooxygenase-2 whereas the side effects of NSAIDs would be due to cyclooxygenase-1 blockade (Mitchell et al., 1993). As a consequence, great efforts have been made to synthesise cyclooxygenase-2 selective agents in the belief that they will have similar anti-inflammatory actions to traditional NSAIDs while being devoid of the gastrotoxic side-effects (Hawkey, 1999; Mitchell and Warner, 1999). The availability of these new selective drugs has allowed more precise examination of the relative roles of each isoenzyme in the responses mediated by prostanoids and the effects of NSAIDs. Interestingly, some of these examinations have suggested that the initial hypothesis regarding the relative roles of cyclooxygenase-1 and cyclooxygenase-2, i.e. that the anti-inflammatory effects of NSAIDs were attributable to their ability

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to inhibit cyclooxygenase-2 and their side-effects of NSAIDs were attributable to blockade of cyclooxygenase-1 blockade, could be too simplistic. For example, cyclooxygenase-1 seems to be involved in some inflammatory responses (Wallace et al., 1998, 1999) whereas, in certain settings, cyclooxygenase-2 activity plays a key role in preserving gastric mucosal integrity (Maricic et al., 1999).

Wallace et al. (1990, 1991) first demonstrated that NSAID-induced gastric ulcerations were attenuated in neutropaenic rats and were greatly reduced by inhibition of leukocyte/endothelium interactions. Furthermore, concomitant administration of an inhibitor of leukocyte adherence, e.g. nitric oxide, prevents both the accumulation of leukocytes and the ulcerogenic action of these drugs (Wallace et al., 1994; Calatayud et al., 1999). These experimental studies indicate that NSAIDs induce gastrointestinal damage at least in part by promoting the accumulation of neutrophils in the mucosa. This effect may well be due to suppression of the tonic production of anti-adhesive prostaglandins (such as prostacyclin) by the vascular endothelium. Here we have analysed the relative contribution of cyclooxygenase-1 and cyclooxygenase-2 to the regulation of leukocyte/endothelial cell interactions in the mesentery.

2. Materials and methods

2.1. Intravital microscopy

The rat mesenteric preparation was set up as described recently (Tailor et al., 1997). Sprague–Dawley rats (200–250 g) were anaesthetised with Inactin (sodium thiopentobarbital 120 mg kg^{-1} i.p.); the neck and abdominal areas were gently shaved, and a tracheotomy was performed to facilitate breathing during experimentation. A midline abdominal incision was made and a loop of the ileal mesentery was exteriorised and superfused at a rate of 2 ml/min with warmed (37 °C) bicarbonate-buffered saline (pH 7.4). The exposed tissue was kept moist by covering with saline-soaked gauze to minimise heat loss and fluid evaporation.

The animals were placed in a supine position on a Plexiglas viewing stage in preparation for microscopic observation; the mesentery was mounted onto a Zeiss Axioskop FS (Zeiss, Welwyn Garden City, UK) microscope stage. A long distance objective lens (magnification × 20; Zeiss) and an eyepiece (magnification × 40; Zeiss) were used to observe the microcirculation. The preparation was trans-illuminated with a 12-V, 100-W halogen light source. A Hitachi CCD colour camera (model KPC571 Tokyo, Japan) acquired images that were displayed onto a Sony Trinitron colour video monitor (model PVM 144OQM) and recorded onto a Sony super-VHS videocassette recorder (model SVO-9500 MDP) for subsequent off-line analysis. A video time–date generator (FOR-A video timer, model VTG-33, Tokyo, Japan) projected the time, date, and stopwatch functions onto the monitor. Single unbranched mesenteric venules (20–40 μm in diameter) were selected for study and the diameter was measured on-line with a video calliper (Microcirculation Research Institute, Texas A&M University, College Station, TX, USA). The number of rolling, adherent and emigrated leukocytes was determined off-line during playback of videotaped images. A leukocyte was defined as adherent to venular endothelium if it remained stationary for a period equal to or greater than 30 s. Adherent cells were expressed as the number per 100 μm length of venule. Rolling leukocytes were defined as those white blood cells that move at a velocity less than that of erythrocytes in the same vessel. Leukocyte rolling velocity was determined by the time required for a leukocyte to transverse a given distance along the length of the venule and is expressed as μm/s. Flux of rolling leukocytes was measured as the number of cells that could be seen moving past a defined reference point in the vessel per minute. The same reference point was used throughout the experiment because leukocytes may roll for only a section of the vessel before rejoining the flow of the blood or firmly adhering. Emigration was measured as the number of emigrated leukocytes in the area surrounding the vessel (100 μm length of venule × 50 μm at each side). Centreline red blood cell velocity was also determined on-line by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow was calculated from the product of mean red blood cell velocity (V_{mean} = centreline velocity/1.6) and cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8(V_{mean}/D_v)$, in which D_v is venular diameter.

A 30-min equilibration time was allowed and the last 5-min period was recorded for assessing the baseline values (time 0). Then indomethacin, the cyclooxygenase-1 selective inhibitor SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole) (Smith et al., 1998), the cyclooxygenase-2 specific agent DFP (5,5-dimethyl-3-(2-propoxy)-4-(4-methanesulfonyl)-2(5*H*)-furanone) (Leblanc et al., 1999) or SC-560 plus DFP (20 mg/kg each) were administered through the jugular vein and the images were recorded for 5 min beginning 15, 30, and 60 min after drug injection. All protocols comply with the European Community guidelines for the use of experimental animals and were approved by the ethics committee of St. Bartholomew's and the Royal London School of Medicine and Dentistry.

2.2. Cell culture

Human airway epithelial cells, A549 cells (European Collection of Animal Cell Cultures, ref. No. 86012804) were cultured in 96-well plates with DMEM supplemented with 10% foetal calf serum and L-glutamine (4 mM). To induce the expression of cyclooxygenase-2, A549 cells were exposed to interleukin-1β (10 ng/ml) for 24 h.

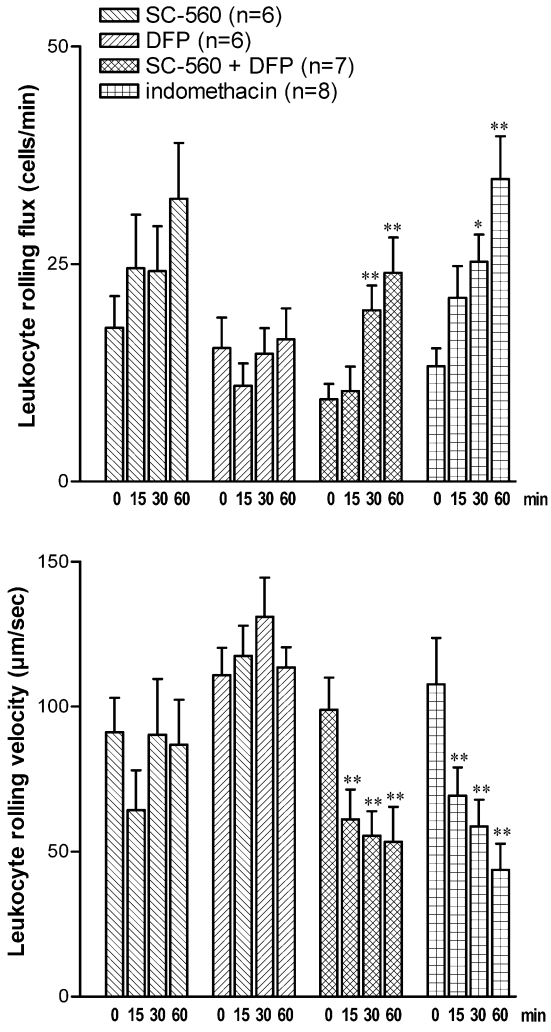

Fig. 1. Effects of SC-560, DFP and indomethacin (20 mg/kg, i.v. each) on leukocyte rolling flux and leukocyte rolling velocity. These parameters were evaluated in Inactin-anaesthetised rats using intravital microscopy of unbranched mesenteric venules (20–40 μm in diameter) superfused with bicarbonate-buffered saline (37 °C, pH 7.4). Measurements were made before and 15, 30 and 60 min after drug administration. Results are expressed as mean ± S.E.M. * $P<0.05$ and ** $P<0.01$ vs. respective basal value (repeated measures ANOVA + Dunnett's test).

2.3. Cyclooxygenase-1 and cyclooxygenase-2 activities

Blood was collected into heparin (19 units/ml) from the carotid artery of pentobarbital (60 mg/kg i.p.) anaesthetised rats before and 60 min after treatment with indomethacin, SC-560, DFP or SC-560 plus DFP (20 mg/kg each). For assay of cyclooxygenase-1, blood was aliquoted in 100-μl volumes into the individual wells of 96-well plates and treated for 30 min with the calcium ionophore, A23137 (50 μM) to stimulate platelets. The plates were then centrifuged (1500 × *g*, 4 °C, 5 min), and the plasma was collected and immediately frozen. Concentrations of thromboxane B_2 (as a measure of thromboxane A_2 formation and so cyclooxygenase-1 activity in platelets) in these samples were determined by radioimmunoassay. This indicates the level of cyclooxygenase-1-inhibitory drug present in vivo at the time of blood sampling. For cyclooxygenase-2 assay, blood was centrifuged (12 000 × *g*, 2 min) and the plasma collected. The medium from A549 cells, which had been exposed to interleukin-1β for the preceding 24 h to induce the expression of cyclooxygenase-2, was replaced with these plasma samples (100 μl) and the cells incubated in the presence of A23137 (50 μM) for 30 min to stimulate the formation of prostaglandin E_2. Plasma was then collected and the concentration of prostaglandin E_2 in these samples determined by radioimmunoassay as a measure of the activity of cyclooxygenase-2 in A549 cells (Giuliano and Warner, 1999). This reports the level of cyclooxygenase-2-inhibitory drug present in vivo at the time of blood sampling.

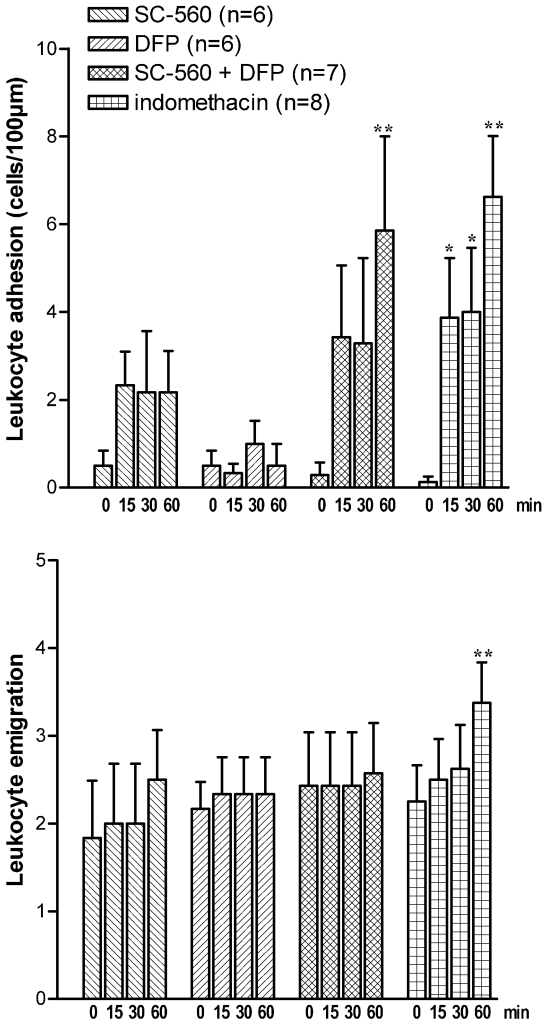

Fig. 2. Effects of SC-560, DFP and indomethacin (20 mg/kg, i.v. each) on leukocyte adhesion and leukocyte emigration. These parameters were evaluated in Inactin-anaesthetised rats using intravital microscopy of unbranched mesenteric venules (20–40 μm in diameter) superfused with bicarbonate-buffered saline (37 °C, pH 7.4). Measurements were performed before and 15, 30 and 60 min after drug administration. Results are expressed as mean ± S.E.M. * $P<0.05$ and ** $P<0.01$ vs. respective basal value (repeated measures ANOVA + Dunnett's test).

2.4. Drugs

DFP (5,5-dimethyl-3-(2-propoxy)-4-(4-methanesulfonyl)-2(5*H*)-furanone) was a gift from Dr. Robert Young, Merck-Frosst, Canada. SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole) was a gift from Dr. Günter Trummlitz, Boehringer Ingelheim Pharma, Germany. Interleukin-1β was purchased from Roche. Other compounds were from Sigma.

2.5. Statistical analysis

Repeated measures ANOVA was used to analyse data obtained in the same animals before and after drug administration. Dunnet's test was used as a post test to compare values after treatment with the corresponding basal value. ANOVA plus Newman–Keuls test was used for comparisons between groups of animals receiving different treatments.

3. Results

Indomethacin (20 mg/kg, i.v.) induced a significant time-dependent increase in leukocyte rolling flux accompanied by a progressive reduction in the velocity of rolling in mesenteric venules (Fig. 1). Neither SC-560 nor DFP (both 20 mg/kg, i.v.) induced any significant change in leukocyte rolling, though there was a trend to increased rolling flux in the former group. Rats receiving both drugs (SC-560 plus DFP, both 20 mg/kg, i.v.) experienced an increase in leukocyte rolling flux and a reduction in rolling velocity similar to those induced by indomethacin (Fig. 1). Adhesion of leukocytes to the venular endothelium was significantly increased in rats administered with indomethacin all along the experimental period. No significant changes in this parameter were observed after SC-560 or DFP administration ($p > 0.05$ both, repeated measures ANOVA), while rats receiving both drugs showed a response analogous to that observed with indomethacin (Fig. 2). Leukocyte emigration was only slightly increased in indomethacin treated rats (Fig. 2). None of these treatments modified venular shear rate (Table 1).

Table 1
Venular shear rate before and 15, 30 and 60 min after administration of indomethacin, SC-560, DFP or SC-560 plus DFP (all 20 mg/kg, i.v.)

	Shear rate (γ)			
	0 min	15 min	30 min	60 min
Indomethacin	501 ± 39	479 ± 77	416 ± 42	375 ± 49
SC-560	449 ± 32	396 ± 41	413 ± 45	402 ± 37
DFP	506 ± 54	489 ± 65	470 ± 54	505 ± 69
SC-560 + DFP	505 ± 54	461 ± 65	460 ± 69	471 ± 78

Venular wall shear rate was calculated based on the Newtonian definition $\gamma = 8(V_{mean}/D_v)$, in which V_{mean} corresponds to "centreline velocity/1.6" and D_v is venular diameter. Centreline red blood cell velocity was measured using an optical Doppler velocimeter. Results are expressed as mean ± S.E.M. $P > 0.05$ for all treatments (repeated measures ANOVA).

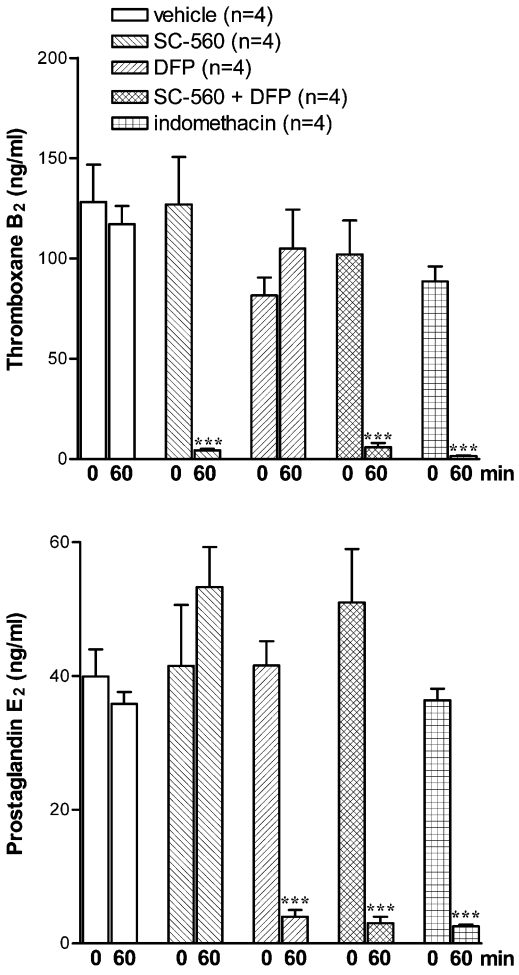

Fig. 3. Effects of SC-560, DFP and indomethacin (20 mg/kg, i.v. each) or their vehicle (sodium bicarbonate 5%, glucose 2.5% in distilled water, 1 ml/kg, i.v.) on cyclooxygenase-1 and cyclooxygenase-2 activity. Rat blood samples were collected 60 min after drug administration. Cyclooxygenase-1 activity was determined as thromboxane B_2 synthesis in whole blood treated for 30 min with the calcium ionophore A23137 (50 μM). Cyclooxygenase-2 activity was evaluated in human airway epithelial A549 cells that had been incubated for the previous 24 h with interleukin-1β (10 ng/ml). Plasma taken from rats 60 min after treatment with the test compounds was then incubated with A549 cells in the presence of A23137 (50 μM) for 30 min after which time the production of prostaglandin E_2 was determined. Results are expressed as mean ± S.E.M. *** $P < 0.01$ vs. respective basal value (ANOVA + Newman–Keuls test).

Cyclooxygenase-1 activity was almost completely inhibited in blood from rats treated 60 min before with indomethacin, SC-560 or SC-560 plus DFP (20 mg/kg, i.v. each). In comparison, treatment with DFP (20 mg/kg, i.v.) did not modify the activity of this isoenzyme (Fig. 3). The activity of cyclooxygenase-2 in activated (interleukin-1β, 10 ng/ml, − 24 h) A549 cells was nearly abolished by plasma samples from rats treated (− 60 min) with indomethacin, DFP or SC-560 plus DFP while it was unaffected by plasma from rats receiving only SC-560 (Fig. 3). Prostaglandin E_2 levels in the plasma removed from vehicle treated animals was 0.8 ± 0.1 ng/ml ($n = 6$).

4. Discussion

In the gastrointestinal tract, the only isoform of cyclooxygenase expressed under physiological circumstances is cyclooxygenase-1 (Kargman et al., 1996). This observation led to the generally accepted concept that cyclooxygenase-1 is the isoenzyme supporting the prostanoid mediated "housekeeping" functions in this system and that inhibition of cyclooxygenase-1 by NSAIDs explains their gastrotoxic effect. In contrast, cyclooxygenase-2 was found to be the predominant isoform present at inflammatory sites and appeared to be responsible for the pro-inflammatory effects of prostanoids in this setting (Vane et al., 1998). This scheme led to the development of cyclooxygenase-2 selective inhibitors able to exert the anti-inflammatory action of the classic non-selective NSAIDs without compromising mucosal integrity in the gastrointestinal tract. The availability of these drugs has allowed the distinction of the actions of both isoenzymes and the results obtained, including those reported here, challenge this too simplistic initial view. Firstly, it has been shown that the anti-inflammatory effects of NSAIDs are dependent upon inhibition of both cyclooxygenase-1 and cyclooxygenase-2, suggesting a contributory role of cyclooxygenase-1 to the inflammatory response (Wallace et al., 1998, 1999). Experiments carried out in cyclooxygenase-1 deficient mice support this notion as these animals showed a lower sensitivity to certain inflammatory stimuli (Langenbach et al., 1995). Secondly, cyclooxygenase-2 activity seems to be essential to preserve mucosal integrity when the gastrointestinal mucosa is under challenge (e.g. ischemia–reperfusion) (Maricic et al., 1999). Thirdly, cyclooxygenase-2 plays a central role in gastrointestinal ulcer healing as its blockade significantly delays this process (Brzozowski et al., 2001). More recently, the selective inhibitor of cyclooxygenase-1, SC-560, has been synthesised and its use has extended the above notions and demonstrated that inhibition of both cyclooxygenase-1 and cyclooxygenase-2 is required to induce gastric lesions (Wallace et al., 2000).

Accumulation of leukocytes in the gastrointestinal mucosa plays a causative role in lesion development after NSAID intake (Wallace et al., 1990). We have therefore analysed the role of cyclooxygenase-1 and cyclooxygenase-2 inhibition in the pro-inflammatory actions of NSAIDs in the gastrointestinal tract. Our results show that intravenous administration of a gastrolesive dose of indomethacin increased leukocyte rolling flux, which indicates that indomethacin activates leukocyte and/or endothelial cells and, as a consequence, more leukocytes abandon the blood stream to roll over the endothelial layer. Furthermore, a progressive increase in the strength of the leukocyte/endothelial cell interactions together with resultant reductions in leukocyte rolling velocity and increases in leukocyte adherence were observed. Analysis of the activities of cyclooxygenase-1 and cyclooxygenase-2 revealed that the dose of indomethacin used caused an almost complete inhibition of both isoenzymes. Most importantly, when we used the cyclooxygenase-1 selective inhibitor SC-560 we found an equivalent inhibition of cyclooxygenase-1 to that caused by indomethacin, without changes in cyclooxygenase-2 activity and without effects on leukocyte/endothelium interactions in the mesenteric venules. The cyclooxygenase-2 selective agent DFP also failed to produce any pro-inflammatory effects, despite inducing a substantial inhibition of cyclooxygenase-2 activity equivalent to that observed with indomethacin. These results indicate that inhibition of cyclooxygenase-1 or cyclooxygenase-2 alone is not enough to induce an inflammatory response similar to that induced by indomethacin. The effects of indomethacin were, however, mimicked by co-treatment with the two selective inhibitors, which suggests that the pro-inflammatory action of non-selective NSAIDs is indeed cyclooxygenase-dependent and that inhibition of the two isoforms of this enzyme is required to increase leukocyte/endothelium interactions. These findings are in line with those reporting the requirement of inhibition of both, cyclooxygenase-1 and cyclooxygenase-2, to cause gastric lesions (Wallace et al., 2000) and add to the increasing number of reports demonstrating that the roles of cyclooxygenase-1 and cyclooxygenase-2 cannot be easily separated but seem to be highly coordinated (Brzozowski et al., 2000, 2001; Langenbach et al., 1995; Maricic et al., 1999; Tanaka et al., 2001; Wallace et al., 1998, 1999, 2000).

We do not know at the moment the cellular location of the enzymes whose inhibition induces the increase in leukocyte/endothelial cell interactions. It was generally assumed that the release of the inhibitory factors prostacyclin and nitric oxide (NO) from endothelial cells reduced leukocyte/endothelial interactions and that, in physiological circumstances, the synthesis of these substances relied upon the constitutive isoforms of cyclooxygenase and NO-synthase, i.e. cyclooxygenase-1 and endothelial NO-synthase. However, recent studies in humans support the possibility of cyclooxygenase-2 being a major source of prostacyclin synthesis in healthy volunteers, as its production appeared to be strongly inhibited by the selective cyclooxygenase-2 inhibitors celecoxib and rofecoxib (Catella-Lawson et al., 1999; McAdam et al., 1999). Cyclooxygenase-2 has not been found in endothelial cells in vitro under resting conditions although a rapid increase in its expression was detected when cells were subjected to physiological levels of shear stress (Topper et al., 1996). Cyclooxygenase-2 is also a rapid response gene in inflammatory cells (Pouliot et al., 1998). Whatever the cellular location of cyclooxygenase-1 and cyclooxygenase-2, it seems clear that both activities are involved in the homeostatic control that protects the organism against the damaging effects of leukocyte accumulation and activation. However, their function appears to be redundant as the activity of one of them is enough to preserve homeostasis. This finding adds further support to the idea that there are numerous mechanisms that work to preserve the integrity of the gastric

mucosa (Whittle et al., 1990). They also suggest that the greater gastrointestinal tolerability associated with cyclooxygenase-2-selective agents is explained by substantial inhibition of both cyclooxygenase-1 and cyclooxygenase-2 being required for gastrotoxicity to be displayed; i.e. it is not inhibition of cyclooxygenase-1 alone that underlies the gastrotoxic effects of the NSAIDs.

Our results corroborate those of Wallace et al. (2000) who showed that superfusion with SC-560 did not induce leukocyte adhesion in rat mesenteric venules. However, in contrast to the results obtained with DFP in the present study, they observed increased adherence of leukocytes in venules superfused with a different cyclooxygenase-2 selective drug (celecoxib). The reasons for this discrepancy are not clear at the moment, but may be explained by the greater selectivity towards cyclooxygenase-2 of DFP compared to celecoxib (Warner et al., 1999). Alternatively, it may be that, as reported for other actions induced by NSAIDs (Ahnen, 1998), the pro-inflammatory effect of celecoxib was unrelated to cyclooxygenase inhibition.

In summary, our results demonstrate that, in contrast to non-selective NSAIDs, cyclooxygenase-1 or cyclooxygenase-2 selective inhibitors do not exert a pro-inflammatory action in rat mesenteric venules and this is consistent with the reported gastric sparing action of these drugs. We also demonstrate that pro-inflammatory events develop when both cyclooxygenase-1 and cyclooxygenase-2 are inhibited, which indicates that the two isoenzymes act co-ordinately to prevent tissue damage resulting from the excessive accumulation of inflammatory cells.

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