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Role of Vascular Mechanisms Involved in the Acute Gastric Mucosal Injury Induced by Droxicam and Piroxicam in Rats

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Abstract

We describe the formation of severe gastric erosions produced in fasted rats by intragastric administration of droxicam and its active species piroxicam, non-steroidal anti-inflammatory drugs of the oxicam group. The time course of gastric damage and the possible role of mucus secretion, changes of gastric vascular permeability, and neutrophil activation in the development of droxicam- and piroxicam-induced gastric lesions, were also investigated.

Both drugs dose-dependently (1·25–20 mg kg^{-1}) caused acute gastric haemorrhagic erosions in the rat. These lesions were significantly greater with piroxicam treatment 6 h after dosing. Only the lower doses of droxicam and piroxicam (1·25 mg kg^{-1}) induced a significant increase of mucus gel production at different times (3 and 6 h). However, there was no increase in the concentration of its components. Oral pretreatment of the animals with either agent did not induce any changes on the values of mucosal vascular permeability. In contrast, myeloperoxidase activity as an index of neutrophil infiltration was significantly increased. A marked relationship was found between the lesion index and myeloperoxidase activity.

These results suggest that neutrophil infiltration could play an important role in the pathogenesis of gastric mucosal injury induced by these oxicam agents.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been a mainstay in the treatment of inflammatory diseases such as rheumatoid arthritis. However, the long-term use of these drugs is associated with significant adverse effects, most notably gastric ulceration, bleeding and perforation. A number of different strategies have been used to obtain NSAIDs with reduced gastric toxicity. These include formulating the agents with an enteric coating to prevent absorption in the stomach, and the development of prodrugs, which are not active until they have undergone metabolism in the liver, and so prevent the irritant properties of these drugs on the gastric mucosa.

Members of the oxicam family are not carboxylic acids, but they are acidic by virtue of their enolic 4-hydroxy substituent. Droxicam is a prodrug that after oral administration is hydrolysed to piroxicam, the active species, in the gastrointestinal tract before absorption (Martinez & Sanchez 1991). Piroxicam, like droxicam, has shown anti-inflammatory, analgesic and antipyretic activity, but after its administration the haemorrhagic lesions of the gastric mucosa occurred in about 20% of osteoarthritis patients. Both the beneficial and detrimental effects of oxicams are likely to be attributable to the ability of these drugs to suppress prostaglandin synthesis (Bohl et al 1990).

Recently, a vascular aetiology for NSAID gastropathy has been proposed, based on the demonstrable ability of these agents to cause vascular endothelial damage, local decrease in mucosal blood flow and activation of polymorphonuclear leucocytes (Wallace et al 1990). The aim of the present work was to study the possible role of vascular mechanisms involved in the formation of gastric erosions produced by oral administration of droxicam compared with piroxicam, such as modifications of vascular permeability and increase in neutrophil infiltration. Thus, we can determine whether its formulation as a prodrug induces a protective effect on the gastric mucosa of the rat.

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Materials and Methods

Animals, groups, and drug preparation

Male and female Wistar rats, 180–250 g were placed in single cages in a controlled room (temperature 22–24°C and humidity 70–75%). The animals were deprived of food for 24 h before the experiments, but had free access to water. They were randomly assigned to groups and were treated with droxicam (1·25, 5·0, 10·0, 20·0 and 40·0 mg kg^{-1} body weight, Esteve SA, Barcelona, Spain) and piroxicam (1·25, 5·0, 10·0 and 20·0 mg kg^{-1} body weight, Pfizer SA, Madrid, Spain), respectively. The agents were suspended in 1% Tween 20 and were administered by the intragastric route (1 mL/100 g body weight). Control groups received vehicle in a comparable volume.

Gastric mucosal injury. Biochemical study of gastric mucus

Groups of 8–10 rats each were treated with droxicam and piroxicam, and were killed at 3 and 6 h, respectively, using an overdose of anaesthetic, and their stomachs removed and opened along the greater curvature. Briefly, the length and width of each lesion was measured and the product was expressed in terms of the ulcer index (UI, mm^2). The gastric mucus gel was obtained by scraping the surface of the mucosa with a glass slide and was immediately homogenized in 4 mL of distilled water. The weight of mucus (g) was the difference between the weight of homogenate and that of the original 4 mL of water. Total proteins (mg mL^{-1}) were determined from one portion of the homogenate (1 mL), following the colorimetric technique of Lowry et al (1951). The hexosamine content (μg mL^{-1}) was determined as described by Escolar et al (1987). The doses that produced 50 and 80% of the gastric damage were calculated by the graphic method (UI plotted against log dose), and were chosen for the main experiments (droxicam: 12·5 and 20·4 mg kg^{-1}, piroxicam: 3·7 and 8·5 mg kg^{-1}, respectively). The minimum dose required to cause gastric damage (1·25 mg kg^{-1}) was also used. The duration of the ulcerogenic effect of both drugs was examined after intragastric administration of the dose giving 80% of the gastric lesions at various intervals (3, 6 and 9 h).

Determination of microvascular permeability

The microvascular permeability was evaluated 6 h after treatment, by measuring the extravasated amount of the dye Evan's blue in the mucosa (Takeuchi et al 1987). The rats were anaesthetized with sodium pentothal (Abbott SA, 60 mg kg^{-1}). Then, 1 mL 1% Evan's blue was injected intravenously 30 min before the animal was killed. The stomach was removed, opened along the greater curvature and placed in pre-weighed tubes with 9 mL formamide. The dye was extracted in formamide at 65°C for 12 h and quantified by light absorbance at 620 nm (Perkin Elmer Lambda 3). The amount in the tissue (μg (mg tissue)$^{-1}$) was calculated from a standard curve.

Assessment of leucocyte involvement

Neutrophil infiltration in-vivo has previously been assessed by measuring granulocyte-specific enzymes such as myeloperoxidase in tissue (Grisham et al 1990). Myeloperoxidase activity in this experimental model was measured in new groups of animals 6 h after treatment with droxicam (1·25, 12·5 and 20·4 mg kg^{-1}) and piroxicam (1·25, 3·7 and 8·5 mg kg^{-1}).

Briefly, one sample from corpus gastric was obtained from each animal. Samples were excised and rapidly rinsed with ice-cold saline. The tissue was thawed, weighed and homogenized in 10 volumes 50 mM phosphate buffered saline pH 7·4. The homogenate was centrifuged at 20 000 *g*, 20 min, 4°C. The pellet was again homogenized in 10 volumes 50 mM phosphate buffered saline pH 6·0 containing 0·5% hexadecyltrimethylammonium bromide (HETAB) and 10 mM EDTA. The HETAB-containing homogenate was subjected to one cycle of freezing and thawing and a brief period of sonication. A sample of homogenate (0·5 μL) was added to a 0·5-mL reaction volume containing 80 mM phosphate buffered saline pH 5·4, 0·5% HETAB and 1·6 mM 3,3′,5,5′-tetramethylbenzidine. The mixture was incubated at 37°C for 5 min and the reaction started by the addition of 0·3 mM H_2O_2.

Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37°C. The reaction was terminated by the sequential addition of catalase (20 μg mL^{-1}) and 2 mL 0·2 M sodium acetate pH 3·0. The changes in absorbance at 655 nm were measured spectrophotometrically. One unit of myeloperoxidase activity was defined as the amount of enzyme present that produced a change in absorbance of 1·0 min^{-1} at 37°C in the final reaction volume containing the acetate.

Blood leucocyte counts

New groups of animals were used for leucocyte counts. Animals were anaesthetized by inhalation of diethyl ether 3–6 h after dosing with droxicam, piroxicam or vehicle. The thorax was opened and approximately 2 mL of blood was withdrawn by cardiac puncture (using a 21-gauge butterfly needle) and added to vials containing EDTA as an anticoagulant. Blood smears were prepared and

examined microscopically for leucocyte counts using a Neubauer camera. Blood counts were assessed by an observer unaware of the treatment.

Statistical analysis

Values are given as arithmetic means ± s.e.m. Differences between groups were analysed using the Mann–Whitney *U*-test (changes in ulcer index, UI) and the Student's *t*-test for unpaired data.

Results

All animals tested showed gastric erosions 3 and 6 h after drug administration. Piroxicam (1·25–10·0 mg kg^{-1}) produced a progressive and significant increase in the area of the lesions from 0·20 ± 0·14 to 1·99 ± 0·92 mm^2 at 3 h, and 0·21 ± 0·12 to 3·92 ± 1·12 mm^2 at 6 h. However, no changes were observed on UI with the higher dose, 20 mg kg^{-1} (2·01 ± 0·87 mm^2 at 3 h and 3·96 ± 0·63 mm^2 at 6 h, respectively). The gastric damage induced by droxicam was significantly lower at all doses tested (Table 1). The doses that produced 80% of damage in gastric mucosa (droxicam 20·5 mg kg^{-1}, piroxicam 8·5 mg kg^{-1}) were selected for the study of the duration of the gastrolesive effect (3, 6 and 9 h). The surface of gastric mucosa affected increased up to 6 h, with no significant decrease up to 9 h. The groups treated with 1·25 mg kg^{-1} droxicam and piroxicam at 3 and 6 h showed a significant increase in the amount of gastric mucus compared with control ($P < 0·01$ and $P < 0·001$, respectively) (Table 2); however, there was no enhancement of quality (proteins and hexosamine content, data not shown). Mucus secretion did not change significantly with respect to the control group with the rest of treatment. Table 3 shows the Evan's blue concentration in the stomach tissue, as the criterion of permeability of the gastric blood vessels, after 6 h of droxicam and piroxicam administration. We observed no increase in its concentration compared with control animals. Table 3 also compares myeloperoxidase activity as an index of neutrophil infiltration in control mucosal samples with those obtained after administration of droxicam (1·25, 12·5 and 20·4 mg kg^{-1}) and piroxicam (1·25, 3·7 and 8·5 mg kg^{-1}) at 6 h of treatment. Our data show that myeloperoxidase activity was significantly increased in a dose-dependent manner compared with control. These results indicate that there was substantial neutrophil influx into the mucosa in response to injury in rats. However, in vehicle-treated animals the leucocytes present in blood-samples were $4·08 \times 10^3 \pm 1·45 \times 10^3$ mm^{-3} and their numbers remain essentially constant throughout the experimental period. Droxicam and piroxicam treatment had no significant effects on the number of circulating leucocytes after 3–6 h.

Table 1. Gastric ulcer index (mm^2) after oral administration of droxicam and piroxicam at 3 and 6 h of treatment.

Treatment	Dose (mg kg^{-1})	Ulcer index (mm^2)	
		3 h	6 h
Droxicam	1·25	0·13 ± 0·04	0·17 ± 0·04
	5	0·36 ± 0·05	0·51 ± 0·15
	10	0·84 ± 0·19	0·53 ± 0·24
	20	0·98 ± 0·17	1·35 ± 0·47
	40	1·00 ± 0·24	1·57 ± 0·21
Piroxicam	1·25	0·20 ± 0·14	0·21 ± 0·12
	5	1·67 ± 0·43*	2·32 ± 0·36***
	10	1·99 ± 0·92	3·92 ± 1·12***
	20	2·01 ± 0·87	3·96 ± 0·63**

*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with the same dose of droxicam.

Table 2. Gastric mucus secretion in rats after oral administration of droxicam and piroxicam at 3 and 6 h of treatment.

Treatment	Dose (mg kg^{-1})	Gastric mucus (g)	
		3 h	6 h
Control	–	0·085 ± 0·010	0·079 ± 0·014
Droxicam	1·25	0·172 ± 0·020**	0·160 ± 0·012**
	5	0·141 ± 0·021	0·162 ± 0·022
	10	0·123 ± 0·013	0·132 ± 0·020
	20	0·124 ± 0·010	0·112 ± 0·014
	40	0·112 ± 0·016	0·110 ± 0·010
Piroxicam	1·25	0·180 ± 0·041***	0·210 ± 0·040***
	5	0·092 ± 0·023	0·142 ± 0·031
	10	0·080 ± 0·013	0·111 ± 0·014
	20	0·083 ± 0·011	0·113 ± 0·010

**$P < 0·01$, ***$P < 0·001$ compared with control.

Table 3. Changes in myeloperoxidase activity and mucosal microvascular permeability after oral administration of droxicam and piroxicam at 6 h of treatment.

Treatment	Dose (mg kg^{-1})	Myeloperoxidase (%)	Permeability (%)
Control	–	99·90 ± 7·17	99·90 ± 11·54
Piroxicam	1·25	137·60 ± 10·54**	69·19 ± 8·93
	3·70	141·32 ± 12·16**	92·87 ± 8·08
	8·50	156·29 ± 24·08*	116·14 ± 23·63
Droxicam	1·25	126·25 ± 5·34**	71·10 ± 7·14
	12·50	133·07 ± 10·68**	78·68 ± 12·55
	20·24	165·81 ± 9·34***	93·24 ± 11·45

*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with control.

Discussion

The present data show that droxicam provokes haemorrhagic lesions in the corpus mucosa of rats during a 6-h test period (1·25–40 mg kg^{-1}) although the damage was already observed in the stomach 3 h after administration. The doses which produce 50 and 80% of ulceration were 12·5 and 20·4 mg kg^{-1}, respectively and these doses were tenfold those for piroxicam (3·7 and 8·5 mg kg^{-1}). These findings are in agreement with those of Jane & Rodriguez de la Serna (1991). It has been hypothesized that this fact is due to delayed gastric absorption; droxicam must undergo dissolution and hydrolysis prior to absorption into the gastric mucosa as piroxicam (Jane & Rodriguez de la Serna 1991). The pathogenesis of NSAID's gastropathy is poorly understood but has been causally linked to the topical irritation of the mucosa. Droxicam, at least theoretically, does not produce this effect on the stomach walls. However, the systemic effect of droxicam could be involved. The beneficial (anti-inflammatory, analgesic, antipyretic, antithrombotic) and detrimental (ulcerogenic) effects of NSAIDs are likely to be attributable to the ability of these drugs to suppress prostaglandin synthesis. Reduced prostaglandin production in the gastrointestinal tract is thought to result in decreased cytoprotective mechanisms, such as mucus–bicarbonate barrier stimulation or the restitution of microcirculation damage (Robert et al 1983). It has been recently shown that mammalian cells contain two related, but unique cyclooxygenase isozymes, referred to as COX-1 and COX-2. These studies have demonstrated that the two isozymes are pharmacologically distinct. COX-1 is a constitutively expressed enzyme (Simmons et al 1991) and early observations indicate that it is involved in producing prostaglandins which regulate cellular housekeeping functions, such as gastric cytoprotection, vascular homeostasis and kidney functions. This enzyme is present in most tissues, such as stomach and vascular smooth muscle (De Witt et al 1993). In contrast, COX-2 appears only to be expressed in inflamed tissue following exposure to growth factors, lymphokines, and other mediators of inflammation. Piroxicam, as well as other NSAIDs such as indomethacin and sulindac, preferentially inhibit murine COX-1 (De Witt et al 1993). Thus it is possible that mucosal prostaglandin inhibition could also be the most important mechanism of droxicam-induced damage, since this drug is hydrolysed to piroxicam in the gastrointestinal tract. Other mechanisms involved in the pathogenesis of the NSAIDs, such as the depletion of mucus gel or changes on the vascular permeability in the gastric mucosa, seem not to be implicated in droxicam or piroxicam gastropathy. Our results show that these agents provoke gastric lesions but this seems not to be associated with a decrease of the mucus secretion. Indeed, the lowest gastrolesive doses, 1·25 and 5 mg kg^{-1}, produce a significant increase in the amount of gel layer. This fact could be interpreted as a self-defence mechanism of the gastric mucosa. Robert et al (1983) have shown that mild irritants such as 10–20% ethanol or HCl and NaOH at low concentrations are cytoprotective, and suggest the existence of a prostaglandin-mediated self-defence mechanism in the stomach. It is possible that these drugs, at lower doses, produce an enhancement of mucus gel by a similar process, but higher doses do not induce any changes in this parameter.

On the other hand, a number of previous studies have provided evidence for a critical role of circulating neutrophils in the pathogenesis of NSAID gastric erosions (Wallace et al 1990). In particular, neutrophil adherence to the vascular endothelium appears to be one of the earliest detectable events after NSAID administration (Asako et al 1992), and may account for a number of other events identified as important in the development of mucosal injury. At least three mechanisms are involved in damage induced by polymorphonuclear leucocytes. First, by production of reactive oxygen metabolites as neutrophil-derived free radicals are known to contribute significantly to the gastric ulceration induced by ethanol (Kvietys et al 1990) and they can influence vascular tone by accelerating the inactivation of endothelium-derived relaxing factor; second, by release of proteinases that can contribute to gastrointestinal ulceration; and third, by clogging of the microvasculature and, therefore, exacerbating tissue ischaemia. Thus, it is possible to establish an association between the appearance of ulceration and the presence of infiltrating neutrophils at the damage site. Six hours after droxicam and piroxicam administration, there was a progressive increase in the area and severity of ulceration parallel to that of neutrophil infiltration; however, there was no significant change in the number of circulating leucocytes.

In conclusion, our findings suggest that neutrophil infiltration could play an important role in the pathogenesis of gastric mucosal injury induced by these oxicam agents, and that the ulcerogenicity produced by droxicam, under our experimental conditions, is less than that found for piroxicam. This assumption could be related, at least in part, to the absence of topical irritation induced by this prodrug. Other mechanisms such as reduction of

mucus secretion, or changes on mucosal microvascular permeability, seem not to be implicated in droxicam- or piroxicam-induced gastric damage.

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