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Intestinal tolerability of nitroxybutyl-flurbiprofen in rats

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Abstract

***Background*—Nitric oxide derivatives of non-steroidal anti-inflammatory drugs (NSAIDs) are thought to be much less ulcerogenic than their parent compounds.**

***Aim*—To compare the effect and potency of flurbiprofen and nitroxybutyl-flurbiprofen to uncouple mitochondrial oxidative phosphorylation (an early pathogenic event in NSAID enteropathy), increase intestinal permeability (transitional stage), and cause macroscopic small intestinal damage.**

***Methods*—In vitro uncoupling potency was assessed using isolated coupled rat liver mitochondria and in vivo by electron microscopy of rat small intestinal mucosa (two hours after the drugs). A dose-response study with flurbiprofen (single doses of 5, 10, 20, and 40 mg/kg) and equimolar doses of nitroxybutyl-flurbiprofen was performed; assessing their effect on intestinal permeability (at 18–20 hours), with ^{51}Cr EDTA, and the number of pointed (<5 mm) and longitudinal (>5 mm) small intestinal ulcers at 24 hours.**

***Results*—Flurbiprofen, but not nitroxybutyl-flurbiprofen, stimulated coupled respiration in vitro. Both drugs, however, uncoupled in vivo; in the case of nitroxybutyl-flurbiprofen possibly because hydrolysis of its ester bond released free flurbiprofen. Intestinal permeability was uniformly and equally increased with both drugs compared with controls. The number of small intestinal ulcers, pointed and longitudinal, was significantly reduced with nitroxybutyl-flurbiprofen apart from the number of longitudinal ulcers with the highest dose.**

***Conclusions*—These studies show that nitroxybutyl-flurbiprofen is associated with significantly less macroscopic damage in the small intestine than flurbiprofen but was associated with mitochondrial damage in vivo and caused similar increases in permeability of the small intestine, suggesting that its beneficial effect is on the later pathogenic stages of the damage.**

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The main concern with the use of non-steroidal anti-inflammatory drugs (NSAIDs) is the frequency and severity of their gastrointestinal side effects,[1] which affect both the stomach and small intestine.[2 3] The development of safer NSAIDs depends on insights into the mechanism by which these drugs cause gastrointestinal toxicity. We and others have suggested that there may be two stages to the damage, a topical (biochemical) and a systemic phase (tissue reaction)[4–6] linked by increased intestinal permeability. The topical phase may be due to NSAID induced uncoupling of mitochondrial oxidative phosphorylation (Somasundaram *et al*, manuscript submitted),[6 7] rather than by inhibition of cyclooxygenase[8] and leads to increased intestinal permeability.[9] Subsequently the tissue reaction is the result of mucosal exposure of luminal aggressive factors; neutrophil recruitment and changes in the microcirculation being important for the development of inflammation and ulcers.[6] An alternative suggestion is that NSAIDs damage the intestinal vascular epithelium directly during drug absorption, with expression of adhesion molecules and activation of neutrophils,[10–13] which cause damage by lysosomal release, generation of reactive oxygen species,[14] and by impairing microcirculatory blood flow. There are data to support both the main hypotheses of damage. Based on the this an attempt has been made to limit the gastrointestinal toxicity of NSAIDs by attaching a nitric oxide moiety to conventional NSAIDs in the hope that the nitric oxide released during drug absorption would counteract some of the above detrimental vascular effects. Initial reports of nitric oxide NSAIDs in animals have been encouraging, showing minimal damage compared with the parent NSAID.[15–17]

The attachment of the nitric oxide group to NSAIDs is achieved by linkage of a nitroxybutyric group.[15 16] This connection modifies the NSAID carboxylic group, which is essential for effective inhibition of cyclooxygenase.[18] It is therefore possible that some of the beneficial effect of nitric oxide-NSAIDs (NO-NSAIDs) might be due to rendering them non-acidic and hence ineffective as proton translocators (Somasundaram *et al*, manuscript submitted).[19] Here we report aspects of the topical phase of damage (in vitro and in vivo effects on mitochondria) and changes in intestinal permeability in relation to the macroscopic damage caused by flurbiprofen compared with nitroxybutyl-flurbiprofen (NO-flurbiprofen).

Methods

ANIMALS

Male pathogen free Sprague-Dawley rats (Charles River), 6–8 weeks old and weighing 200–250 g, were used throughout these studies.

A dose response study was done by administration (without sedation) of flurbiprofen (5, 10, 20, and 40 mg/kg), NO-flurbiprofen (7·5, 15, 30, and 60 mg/kg; which are roughly equimolar to the doses of flurbiprofen, respectively), or vehicle by gastric gavage to six (fasting) rats in each group followed by 1 ml water. However, this comparison may be biased against NO-flurbiprofen as previous studies have shown that flurbiprofen and NO-flurbiprofen have comparable efficacy on a weight to weight basis.[15] The highest dose of flurbiprofen was selected to be a roughly equivalent dose (extrapolated from maximum recommended doses in humans) of 20 mg/kg indomethacin in rats, which is the dose of indomethacin most commonly used to induce intestinal damage.

The drugs were initially dissolved in 10% dimethyl sulphoxide and then diluted so that the final concentration of dimethyl sulphoxide was less than 5%. The dissolved drugs were shielded from exposure to light and used within one hour. Control animals received 1 ml vehicle and 1 ml water. After the gavage animals remained fasting for a further two hours after which they had free access to standard rat food and water. They were again fasted the next night and placed in metabolic cages for the intestinal permeability test after which they were anaesthetised (with Hypnovel-Hypnorm) and underwent laparotomy with removal of the whole of the small intestine. Animals were then killed by terminal anaesthesia and cervical dislocation.

PURITY AND STABILITY OF FLURBIPROFEN AND NO-FLURBIPROFEN

The purity of the solutions of flurbiprofen and NO-flurbiprofen was checked using reversed phase high pressure liquid chromatography (HPLC) with ultraviolet detection (276 nm). A Spectrasystem HPLC system was used (Thermo Separation Products, Stone, Cheshire, UK) with a gradient pump (P4000), autosampler (AS3000), and ultraviolet detector (UV2000). Separation was by reversed gradient elution, using a gradient of acetic acid (0·1 mM, pH 4·27) and acetonitrile (0–20 minutes 40% acetonitrile changing from 20–25 minutes to 60% acetonitrile which is maintained until 35 minutes). A C18 column was used (RP-18 LiChrosorb 5 mm, 250×4·6 mm, Phenomenex, Macclesfield, Cheshire, UK) at room temperature. Samples were dissolved in methanol and 25 µl was injected on to the column. The retention times were: flurbiprofen 23·5 minutes, indomethacin (internal standard) 26·4 minutes, and NO-flurbiprofen 39 minutes.

The stability of the two NSAIDs within the intestine was assessed by incubating (shielded from light) flurbiprofen and NO-flurbiprofen for one hour at 37°C with intestinal mucosal scrapings from male Sprague-Dawley rats that had been fasted overnight.

MITOCHONDRIAL FUNCTION

In vitro uncoupling experiments were done on isolated coupled liver mitochondria.[20] The liver was cut into 1 cm^3 pieces and homogenised in a 74 mM sucrose, 225 mM mannitol, 1 mM EDTA, 5 mg/ml BSA solution in 10 mM MOPS-NaOH buffer, pH 7·4, by six strokes of a tight fitting teflon pestle. The homogenate was centrifuged at 500 *g* for 10 minutes and the resulting supernatant recentrifuged at 12000 *g* for 10 minutes. The pellet was resuspended in the isolation medium and centrifuged for 10 minutes at 12000 *g*, to give the resulting mitochondrial enriched pellet used for these experiments. All procedures were carried out at 4°C. Protein concentration was determined using a BCA protein assay kit (Pierce, Illinois, USA).

Oxygen consumption, P/O values, and respiratory control ratios were measured with a Clarke type oxygen electrode (Rank Brothers, Cambridge, UK).[21] The reaction mixture consisted of 150 mM sucrose, 10 mM potassium chloride, 5 mM magnesium chloride, and 1 mM potassium phosphate in 10 mM HEPES-NaOH buffer, pH 7·4. Mitochondrial protein (1–5 mg/ml final concentration), substrates, and drugs were introduced into the chamber (1·0 ml capacity) through a small hole in the lid by syringe. Reactions were carried out at 30°C under magnetic stirring. P/O ratios and respiratory control (using carbonyl cyanide-(trifluoromethoxy) phenylhydrazone (FCCP) as uncoupler) were measured on each of the mitochondrial preparations at the start and end of each series of experiments. P/O ratios with succinate as substrate were 4·0 (SEM 1·6). Respiratory control values ranged from 2 to 5.

The effects of drug addition on coupled mitochondrial respiration over a concentration range of 0·01–5·0 µmol/mg mitochondrial protein was monitored by oxygen uptake for two minutes after drug addition. Results are expressed as a mean (SEM) percentage of control (absence of drug) of the duplicates of 4–10 experiments performed on different days.

All substrates, FCCP, and flurbiprofen were obtained from Sigma (Sigma Chemical Company Ltd, Dorset, UK) and NO-flurbiprofen was provided by Dr P Del Soldato (Nicox, Dublin, Ireland).

For the in vitro studies flurbiprofen and NO-flurbiprofen were solubilised in dimethyl sulphoxide before dilution and the pH adjusted to 7·4. Control experiments used the solvent alone. The final concentrations of dimethyl sulphoxide never exceeded 2%.

The in vivo effects of flurbiprofen and NO-flurbiprofen on mitochondria were assessed by electron microscopy. Groups of four rats each received flurbiprofen (10 mg/kg), NO-flurbiprofen (15 mg/kg), or vehicle after an overnight fast, by gastric gavage. Two hours later an abdominal incision was made under anaesthesia, the stomach opened, and a catheter placed in the second part of the duodenum. The whole of the small intestine was then flushed, avoiding distension, with a 3·0% gluteraldehyde solution in 0·1 M sodium phosphate phosphoric acid buffer, pH 7·3–7·4. One cm length of jejunum (30 cm distal to the ligament of Trietz) was then routinely

processed for electron microscopy and placed in gluteraldehyde for three days. After fixation, samples were washed in 1% osmium tetroxide in 0·1 M sodium phosphate buffer (pH 7·3–7·4) followed by a phosphate buffer wash. Dehydration was achieved by increasing concentrations of acetone (25%, 50%, 70%, 90%, 100%) and infiltration in araldite and acetone mixture (50:50) overnight. Samples were washed (two hours) in neat resin, embedded in araldite, and cut ultrathin with an ultratome (Richart Ultracut-E). The samples were then examined with a Joel 1200 cm electron microscope in transmission mode. All samples had been coded and morphology was assessed by one pathologist unaware of the treatments.

INTESTINAL PERMEABILITY

Gastric intubation (without anaesthesia) was carried out after an overnight fast 18–20 hours after receiving the NSAIDs or vehicle. 51-Chromium labelled ethylenediaminetetraacetate (^{51}CrEDTA; Amersham International, Amersham, UK; 0·5 ml; 10 mCi) was administered (containing approximately 50 nmol ^{51}CrEDTA), followed by 1 ml water.[22] The animals were placed in individual metabolic cages for five hours for collection of urine. Laparotomy was then performed under anaesthesia and the bladder emptied by puncture. The urine obtained was mixed with that excreted during the five hour test period. Urine was counted along with standards in a Wallac 1284 gamma counter for five minutes, which allowed minimal detectable activity of <0·001% of the administered dose.

MACROSCOPIC DAMAGE

The intestinal mucosa was exposed by a cut through the mesenteric side and laid out on a piece of cork for assessment of macroscopic damage 24 hours after giving the drugs. The assessments were carried out by one person who was unaware of the treatments. An ulcer count was made distinguishing between pointed (<5 mm) and longitudinal (>5 mm) ulcers.

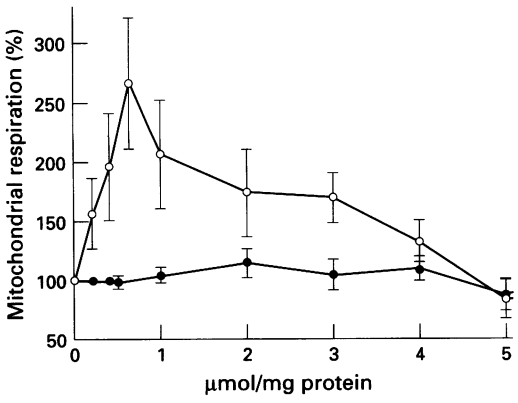

Figure 1: Effect of flurbiprofen (○) and NO-flurbiprofen (●) on coupled rat liver mitochondrial respiration in vitro. The rate of oxygen use is expressed as a percentage relative to the baseline respiration. Each data point represents the mean (SEM) of four to eight experiments.

STATISTICAL ANALYSES

The SYSTAT statistic package was used for calculations. Results are expressed as means (SEM). Statistical differences between groups were assessed by the non-parametric Mann-Whitney U test.

Results

MITOCHONDRIAL FUNCTION

Dimethyl sulphoxide alone, at the concentrations and volumes used in the experiments, had no effect on purified mitochondrial oxygen consumption. Figure 1 shows that flurbiprofen stimulates respiration in coupled mitochondria over a concentration range of 0·05 to 4 μmol/mg protein. At concentrations of flurbiprofen above 5 μmol/mg protein respiration is greatly inhibited. NO-flurbiprofen by comparison had no significant effect on mitochondrial respiration over the concentrations tested.

Figure 2 shows representative electron microscopy from animals two hours after receiving vehicle only and the NSAIDs. All four control rats were normal, with mitochondria of uniform size and clearly visible cristae. However, there were identical and equally prominent mitochondrial changes in all the rats receiving flurbiprofen and NO-flurbiprofen. The changes ranged from subtle elongation of mitochondria and swelling to condensation of the matrix due to loss of clarity of the cristae. In some cases there was mitochondrial vacuolisation with disruption of the cristae. The range of abnormalities is compatible and highly suggestive of mild to severe uncoupling of oxidative phosphorylation or inhibition of electron transport,[23] the only other common cause for such changes being exposure to very hyperosmolar solutes.

The endoplasmic reticulum was slightly vacuolated and distended in both groups of animals receiving NSAIDs. The brush border and other subcellular organelles were unaffected by the drugs.

PURITY AND STABILITY OF FLURBIPROFEN AND NO-FLURBIPROFEN

Flurbiprofen and NO-flurbiprofen had distinctly different retention times. Single peaks were obtained with the neat drugs dissolved in dimethyl sulphoxide. However, after incubation with intestinal homogenate for 60 minutes the amount of NO-flurbiprofen was reduced by 50% with a corresponding increase in flurbiprofen, suggesting that the ester linkage of NO-flurbiprofen can be cleaved by intestinal esterases.

INTESTINAL PERMEABILITY

Figure 3 shows that intestinal permeability to ^{51}CrEDTA was significantly increased after each dose of flurbiprofen and NO-flurbiprofen. There was no significant ($p>0·1$) difference between excretion values between groups receiving 5, 10, 20, and 40 mg/kg flurbiprofen or equimolar doses of NO-flurbiprofen.

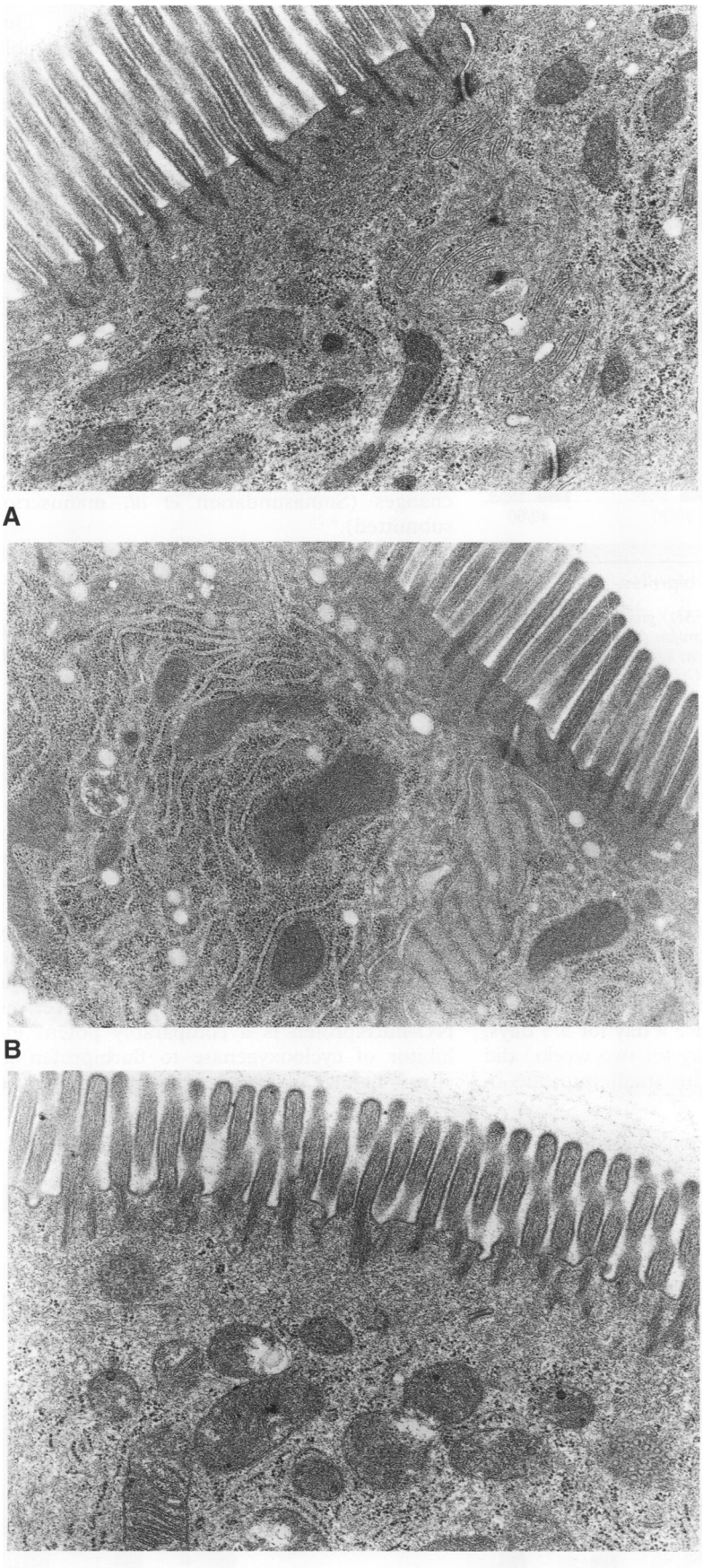

Figure 2: Representative electron microscopic appearances of mucosa of the small intestine two hours after gastric gavage of vehicle, flurbiprofen (10 mg/kg), or NO-flurbiprofen (15 mg/kg). (A) normal appearances of mitochondria after vehicle. The mitochondrial changes after flurbiprofen and NO-flurbiprofen were identical. These ranged from subtle swelling and elongation of mitochondria with loss of clarity of the cristae (B: flurbiprofen) to the occasional vacuolisation of mitochondria and disruption of cristae (C: NO-flurbiprofen). These changes mirror the range of changes in mitochondrial morphology when exposed to increasing concentrations of uncouplers of oxidative phosphorylation. Similar changes are seen with inhibitors of the respiratory chain and very hyperosmotic solutes.[23] Original magnification×14 000.

Furthermore, there was no significant difference ($p>0{\cdot}1$) in the urinary excretion of ^{51}CrEDTA between groups of rats receiving different doses of the drugs.

MACROSCOPIC DAMAGE

Figure 4 shows that the number of pointed ulcers (<5 mm) and longitudinal ulcers (>5 mm) were significantly fewer after NO-flurbiprofen than after each equimolar dose of flurbiprofen except for the highest dose, with which there was no significant difference between the number of longitudinal ulcers between the two drugs.

Discussion

The multistep pathogenic framework for NSAID induced gastrointestinal toxicity proposes that there are distinct and sequential phases of damage. The first is due to the "topical" action of NSAIDs to uncouple oxidative phosphorylation, which leads to increased intestinal permeability (Somasundaram *et al*, manuscript submitted). This may then transform the biochemical phase of damage to an inflammatory reaction, by allowing mucosal exposure of luminal substances, and the development of ulcers mediated either by altered microcirculatory blood flow or by neutrophils.[10–13]

Here we assessed three important pathophysiological aspects of NSAID induced damage to the small intestine at different time points after single doses of flurbiprofen and NO-flurbiprofen. Firstly, we showed that chemical modification of flurbiprofen to NO-flurbiprofen rendered it ineffective as a mitochondrial uncoupler in vitro. Secondly, in vivo the two drugs were equally associated with mitochondrial damage at the electron microscopy level. Thirdly, there was no significant difference between the increased intestinal permeability after the two drugs. Collectively this suggests that the nitric oxide moiety of NO-flurbiprofen does not have a role in altering the early pathogenic events in NSAID enteropathy. A possible reason for the apparent discrepancy between the in vitro and in vivo mitochondrial results is that the ester bond of NO-flurbiprofen may be cleaved by intestinal esterases[24] (present also in abundance in pancreatic secretions) to yield the parent NSAID, which is then able to exert its biochemical effect during drug absorption.

By contrast with the similarities in the early pathophysiological changes seen with flurbiprofen and NO-flurbiprofen, the latter is associated with a significantly improved tolerability of the small intestine as shown in the reduction in number of ulcers, as has been found in the stomach.[15 16] These findings conform to the idea that NO-flurbiprofen releases nitric oxide and thereby causes vasodilatation, increases microcirculatory mucosal blood flow, and prevents neutrophil recruitment,[15 16] all of which are important components in the later stages of NSAID induced gastrointestinal damage.[4 6 11 25] Previous gastrointestinal tolerability studies with NO-flurbiprofen and

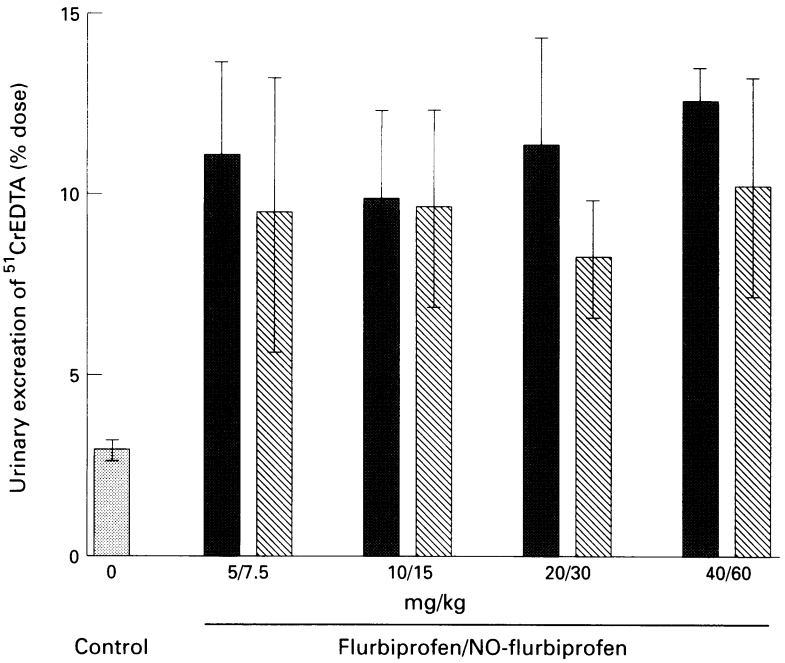

Figure 3: Five hour urinary excretion of ^{51}CrEDTA (mean (SEM)) given 18–20 hours after equimolar doses of flurbiprofen (grey bars) and NO-flurbiprofen (striped bars). Intestinal permeability was significantly increased from baseline at each dose of the NSAID. There was no significant difference in the urinary excretion of ^{51}CrEDTA between the two drugs at any of the doses.

nitrofenac (nitroxybutyl-diclofenac) have shown minimal gastric damage compared with the parent compound when given at doses comparable with those used in the present study.[15 16] Furthermore NO-flurbiprofen given orally to Wister rats (15 mg/kg twice a day) for a week was not associated with any small intestinal pathology whereas all five rats given equimolar doses of flurbiprofen had damage to the small intestine.[15] Similarly, subcutaneous nitrofenac (30 mg/kg twice a day for 3·5 days, and 15 mg/kg twice a day for two weeks) did not cause damage to the small intestine in Wister rats or rabbits, respectively.[16 17] Our results, although not directly comparable because of the different strain of animals used and mode of drug administration, extend these findings and show that at equimolar doses NO-flurbiprofen is associated with significantly less small intestinal ulceration than flurbiprofen. Reduced ulceration along with the findings that it does not seem to affect the early pathogenic events (namely, uncoupling of mitochondrial oxidative phosphorylation leading to the permeability changes) in the damage supports the contention that nitric oxide-NSAID may counteract some of the later vascular effects of NSAIDs. Indirectly this provides some evidence for the importance of the microcirculation in the formation of ulcers as opposed to inflammation which may be predominantly driven by the permeability changes (Somasundaram *et al*, manuscript submitted).[6 26]

Although not directly studied here, it seems possible that the esterification of flurbiprofen which occurs when butyric nitric oxide is attached provides additional protection for the stomach mucosa as this renders the drugs incapable of acting as proton translocators; circumventing the topical phase of damage (Somasundaram *et al*, manuscript submitted). Esterification of other NSAIDs, without introduction of the nitric oxide group, provides compounds with less gastric ulcerogenic action while maintaining in vivo anti-inflammatory activity.[27 28] Because most NSAIDs are relatively ineffective as cyclooxygenase inhibitors without the carboxyl group,[18] it was suggested that in vivo anti-inflammatory activity of esterified NSAIDs was due to generation of the parent compound by intestinal or plasma esterases,[4 27] However, as NO-flurbiprofen is a comparably potent inhibitor of cyclooxygenase to flurbiprofen in vitro[29] neither a difference in cyclooxygenase

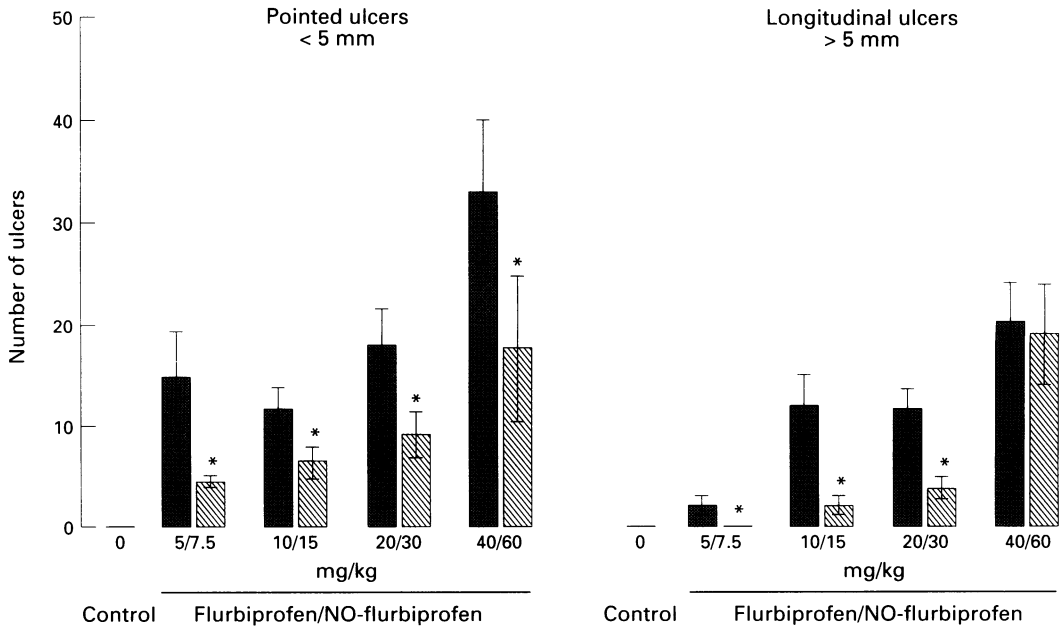

Figure 4: Number of pointed and longitudinal ulcers after flurbiprofen (grey bars) and NO-flurbiprofen (striped bars) 23–25 hours after administration of vehicle or the drugs. The number of ulcers was significantly ($p<0·01$) less with NO-flurbiprofen at each equimolar dose to flurbiprofen except in the number of longitudinal ulcers with the highest dose.*

inhibition or esterification of the NSAID would seem to explain our results. On the contrary there is a differential effect of NO-flurbiprofen and flurbiprofen on the later pathophysiological stages of the damage, as opposed to the early stages, implying an additional beneficial effect of nitric oxide as detailed above. The importance of the nitric oxide group is also evident from other studies, in particular by the finding of Elliott *et al*, who showed that nitrofenac accelerated healing of experimental gastric ulcers in the rat.[30]

In conclusion, orally administered flurbiprofen and NO-flurbiprofen in the rat are equally associated with changes in mitochondrial morphology, that are indicative of uncoupling of oxidative phosphorylation, and increased intestinal permeability. However, the development of ulcers is significantly decreased with NO-flurbiprofen, which is consistent with the suggestion that alterations in the microcirculation may be the driving force in converting the biochemical damage of NSAIDs into ulcers.

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