A comparison of the anti-inflammatory and anti-nociceptive activity of nitroaspirin and aspirin

[1]O.A. al-Swayeh, [2]R.H. Clifford, [3]P. del Soldato & *,[2]P.K. Moore

[1]Department of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia; [2]Messengers & Signalling Research Group, School of Biomedical Sciences, King's College London, Hodgkin Building, Guy's Campus, London SE1 9RT and [3]NiCOX SA, Sophia-Antipolis, France

1 Nitroaspirin (2.5–50 mg kg^{-1}, i.p. or 2.5–100 mg kg^{-1}, p.o.) and aspirin (2.5–100 mg kg^{-1}, i.p. or p.o.) exhibit anti-inflammatory activity in the carrageenan-induced hindpaw oedema model in the rat. When administered i.p., nitroaspirin was a more effective anti-oedema agent than aspirin particularly in the 'early' phase (i.e. up to 60 min) of the response. The ED$_{50}$ values for nitroaspirin and aspirin as inhibitors of the 'late' phase response (measured at 180 min) were 64.3 μmol kg^{-1} and >555 μmol kg^{-1}, respectively. When administered p.o., neither nitroaspirin nor aspirin exhibited significant anti-inflammatory activity in the 'early' phase and were of similar potency in the 'late' phase. Thus, at the highest dose used (100 mg kg^{-1}, 360 min) orally administered nitroaspirin (aspirin in parenthesis) inhibited oedema formation by 46.9$\pm$1.6% (47.2$\pm$3.8%, both $n=6$, $P<0.05$).

2 Nitroaspirin and aspirin (25–200 mg kg^{-1}, p.o.) caused dose-related inhibition of the hyperalgesia to mechanical stimulation following intraplantar injection of carrageenan in the rat. ED$_{50}$ values were 365 μmol kg^{-1} and 784 μmol kg^{-1}, respectively. Neither drug influenced the threshold for mechanical stimulation in the contralateral (i.e. untreated) hindpaw.

3 Nitroaspirin and aspirin (2.5–100 mg kg^{-1}, p.o.) caused dose-related inhibition of acetic acid induced abdominal constrictions in the mouse (ED$_{50}$ values of 154.7 μmol kg^{-1} and 242.8 μmol kg^{-1}, respectively).

4 Nitroaspirin and aspirin (>200 mg kg^{-1}, p.o.) reduced the 'late' phase (but not the 'early' phase) of the formalin-induced hindpaw licking assay in the mouse. Similarly, nitroaspirin and aspirin (>50 mg kg^{-1}, p.o.) prolonged tail withdrawal latency following application of a noxious heat stimulus in the mouse.

British Journal of Pharmacology (2000) **129**, 343–350

Introduction

Aspirin and like non-steroidal anti-inflammatory drugs (NSAID) have been extensively employed in the clinic for their anti-inflammatory and analgesic activity. The mechanism of action is widely believed to involve inhibition of cyclo-oxygenase (COX) mediated biosynthesis of prostacyclin (PGI$_2$) and prostaglandin E$_2$ (PGE$_2$, Moncada *et al.*, 1973). At the site of inflammation, these prostanoids exhibit both pro-inflammatory and pro-nociceptive activity and potentiate the effect of other pro-inflammatory mediators such as histamine and 5-hydroxytryptamine. Unfortunately, the side effects of aspirin in the gastrointestinal tract (e.g. haemorrhage, ulceration), resulting from reduced formation of vasodilator and cytoprotective prostanoids in the gastric mucosa, limit the usefulness of these compounds in the clinic (Wallace, 1997).

In recent years, a number of strategies have been adopted in an attempt to reduce the gastrointestinal side effects of aspirin and like NSAID. For example, the realization that COX exists as two separate isoforms *viz.* COX-1 and COX-2 (reviewed by Vane *et al.*, 1998) has led to the development of a number of selective COX-2 inhibitors, which exhibit anti-inflammatory activity (COX-2 inhibition) but with reduced gastrointestinal toxicity (COX-1 inhibition) (for review, see Fung & Kirschenbaum, 1999).

An alternative approach to the development of less toxic NSAID is provided by the nitro-NSAID (NO-NSAID) class of compounds including nitroaspirin. To date, experiments in animals have revealed that, compared with aspirin, oral administration of nitroaspirin is associated with a marked reduction in gastrointestinal damage (Wallace *et al.*, 1994a). Furthermore, nitroaspirin protects the stomach against injury following haemorrhagic shock (Wallace *et al.*, 1997) and HCl/ethanol (Takeuchi *et al.*, 1998) and additionally promotes the healing of pre-existing ulcers (Ukawa *et al.*, 1997). Other NO-NSAID including nitrodiclofenac (Wallace *et al.*, 1994b) and nitronaproxen (Davies *et al.*, 1997) also cause reduced gastrointestinal damage when compared with the corresponding parent NSAID. In each case, nitro-NSAID most probably 'spare' the gastrointestinal tract by the local release of nitric oxide (NO) resulting in increased stomach mucosal blood flow.

The improved gastrointestinal tolerability of nitro-NSAID (*c.f.* NSAID) suggest that these compounds may prove of value in the clinic. However, whilst the effect of nitroaspirin on gastrointestinal function has been extensively investigated (see above), its ability to reduce oedema formation and to modify algesia/hyperalgesia in experimental animals has, perhaps surprisingly, received relatively little attention. Indeed, to the best of our knowledge, there has been no systematic assessment of the ability of nitroaspirin *vis-à-vis* aspirin to exert anti-

*Author for correspondence; E-mail: phillip.moore@kcl.ac.uk

oedema and anti-nociceptive activity in a range of animal models and using a range of doses and by different routes of administration. To this end, we have now compared the ability of nitroaspirin and aspirin to influence, (i) carrageenan-induced hindpaw oedema and mechanical hyperalgesia in the rat and (ii) formalin-induced hindpaw licking, acetic acid induced abdominal constriction and tail flick latency to thermal stimulation in the mouse. Some of these results have been presented previously in preliminary form (al-Swayeh *et al*., 1999).

Methods

Rats (male, Wistar, 80 – 240 g, Tucks Ltd., U.K.) and mice (male, LACA, 22 – 35 g, Tucks Ltd., U.K.) were purchased from accredited suppliers and maintained in the Biological Services Unit of this College until required. All experiments were carried out 'blind' in that the observer was not aware of the identity or dose of drugs administered to individual animals and were conducted under the conditions of the Animals Scientific Procedures Act, U.K. (1986).

Measurement of carrageenan-induced hindpaw oedema formation in the rat

Rats (male, Wistar, 160 – 240 g) were used in this study. Experiments were conducted essentially as described previously (Handy & Moore, 1998a). Animals were housed for 1 week prior to use and thereafter acclimatized in the laboratory for 1 h prior to commencement of the experiment. Hindpaw volume (ml) was determined as a measure of oedema formation, before, and at timed intervals (30 min – 6 h), after intraplantar carrageenan injection. For this purpose, animals were held firmly and the volume of the injected hindpaw determined by plethysmography using a commercially available device (Ugo Basile Ltd.). Nitroaspirin (2.5 – 50 mg kg^{-1}), aspirin (2.5 – 100 mg kg^{-1}) or vehicle (0.5% w v^{-1} carboxymethylcellulose, CMC, 1 ml kg^{-1}) were administered i.p. or p.o. 15 min prior to intraplantar injection of a standard volume (100 μl) of carrageenan (2% w v^{-1}). At the end of the experiment, animals were killed by a blow to the head and exsanguination. Results are mean $\pm$ s.e.mean, $n = 6$.

Measurement of carrageenan-induced hindpaw hyperalgesia

Rats (male, Wistar, 80 – 120 g) were used in this study. Experiments were conducted essentially as described previously (Handy & Moore, 1998b). Animals were housed for 1 week prior to the experiment. Animals were weighed, marked for identification purposes and allowed to habituate to the thermostatically controlled test room (ambient temperature, 22°C) for at least 1 h prior to commencement of the experiment. 'Baseline' measurements of hindpaw mechanical nociceptive threshold were obtained as described below. Thereafter, hindpaw inflammation was produced by intraplantar injection of carrageenan (2% w v^{-1}, 150 μl) into either hindpaw chosen at random. Nitroaspirin or aspirin (25 – 200 mg kg^{-1}, p.o.) or an appropriate volume (5 ml kg^{-1}, p.o.) of vehicle (0.5% w v^{-1} CMC) were administered 2.5 h after carrageenan injection and the mechanical nociceptive threshold determined 30 min thereafter.

The threshold response to a noxious mechanical stimulus was determined using an Ugo Basile algesiometer as described previously (Laird *et al*., 1996; Handy & Moore, 1998b). Briefly, each hindpaw was positioned in turn under a conical probe

surface (tip radius approx. 1 mm) and gradually increasing pressure applied to the hindpaw surface until the animal vocalized at which point the measurement was terminated. Mechanical nociceptive threshold for both the injected and contralateral (i.e. non-injected) hindpaw were determined. Results (mean $\pm$ s.e.mean, $n = 6$) are shown as the difference in nociceptive threshold (arbitrary units) before ('pre-') and 3 h after ('post-') intraplantar injection of carrageenan. Statistical comparisons between groups was determined as the difference in 'post-pre' values.

Measurement of formalin-induced hindpaw licking behaviour in the mouse

The formalin-induced hindpaw licking assay was performed as described previously (Moore *et al*., 1991; Morgan *et al*., 1992). Experiments were conducted between 12:00 and 17:00 h in a purpose-built, thermostatically-controlled (22°C) laboratory and in subdued lighting conditions. On the day of the experiment, animals were weighed, marked for identification purposes and transported to the laboratory 60 min prior to the start of the experiment in order to habituate to the environment. Throughout the pre-trial period, animals were allowed food and water *ad libitum*.

Mice were treated orally (time, 0 min) with nitroaspirin, aspirin (both 50 – 300 mg kg^{-1}) or vehicle (0.5% w v^{-1} CMC; 1 ml kg^{-1}) followed 15 min thereafter by intraplantar injection of formalin (10 μl, 5% v v^{-1} in saline) into the right hindpaw and immediate transfer to transparent plexiglass observation chambers (dimensions: $20 \times 20 \times 12$ cm). The duration of time spent licking the injected hindpaw was monitored and recorded over the periods, 0 – 5 min ('early' phase of licking) and 15 – 30 min ('late' phase of licking) after formalin administration. Mirrors positioned behind the chambers enabled observation of the right hindpaw when it was obscured from direct view. Results are shown as time spent licking the injected hindpaw in s and are mean $\pm$ s.e.mean ($n = 3 – 20$). At the end of the experiment animals were killed by cervical dislocation and exsanguination.

Acetic acid-induced abdominal constriction assay

The acetic acid-evoked abdominal constriction test was employed as described previously (Moore *et al*., 1991; Morgan *et al*., 1992). Mice were injected i.p. with freshly prepared acetic acid (2% w v^{-1} in saline; pH 2.7; 10 ml kg^{-1}) and transferred immediately to individual observation cages. The number of abdominal constrictions was monitored over the following 30 min. The abdominal constrictions ('writhes') elicited by mice consisted of contractions of the abdominal muscles which progressed posteriorly and usually ended with simultaneous flexor extension of both hind limbs with arching of the back. Abdominal constrictions of two to three mice were counted at the same time using hand tally counters.

In these experiments, mice were injected with nitroaspirin, aspirin (both 2.5 – 100 mg kg^{-1}, p.o.) or vehicle (0.5% w v^{-1} CMC; 10 ml kg^{-1}, p.o.) 15 min prior to acetic acid administration followed 15 min thereafter by an i.p. injection of acetic acid and evaluation of abdominal constrictions as described above. In some experiments, nitroaspirin or aspirin (50 mg kg^{-1}, p.o.) were administered at timed intervals (15 – 360 min) prior to injection of acetic acid. Results show number of abdominal constrictions per 30 min test period and are mean $\pm$ s.e.mean ($n = 6$). At the end of the observation period animals were killed by cervical dislocation and exsanguination.

Tail withdrawal ('flick') assay

The tail withdrawal assay was performed essentially as described originally by D'Amour and Smith (1941). For these experiments, animals were restrained in a perspex tube and a radiant heat source applied to the tail (lower surface). In preliminary experiments, the radiant heat was adjusted to attain a mean baseline tail withdrawal latency of 2.5–3.5 s in control animals. In order to avoid tissue damage exposure to the noxious stimulus was automatically terminated at 10 s. Baseline latencies were measured on two consecutive occasions before drug administration and the mean value recorded. Thereafter, mice received nitroaspirin, aspirin (both 2.5–100 mg kg^{-1}, p.o.) or vehicle (0.5% w v^{-1} CMC; 10 ml kg^{-1}, p.o.). Tail withdrawal latencies (mean of two consecutive measurements) were monitored 15 min thereafter. Results (in s) show mean$\pm$s.e.mean ($n=10$). At the end of the experiment animals were killed by cervical dislocation and exsanguination.

Statistics

The statistical significance of differences between groups was determined by ANOVA followed by *post hoc* Dunnett's test. A probability (P) value of 0.05 or less was taken to indicate statistical significance.

Materials

Nitroaspirin (NCX-4016; 2-acetoxy-benzoate 2-(2-nitroxy-methyl) phenyl ester) was obtained from NiCOX Ltd. (Sophia-Antipolis, France). Aspirin and carrageenan were purchased from Sigma Ltd. Aspirin and nitroaspirin were suspended in 0.5% w v^{-1} carboxymethylcellulose (CMC). Carrageenan was suspended in saline. In all cases a reasonable suspension was achieved after warming (45°C) and with constant sonication and agitation.

Results

Anti-inflammatory activity of nitroaspirin

Intraplantar injection of carrageenan evoked a time-dependent increase in hindpaw volume (indicative of oedema formation) comprising of a relatively rapid 'early' phase (up to 180 min) followed by a more sustained plateau or 'late' phase (180–360 min) (Figures 1 and 2). Pre-treatment of animals with nitroaspirin or aspirin administered either i.p. (Figure 1a) or p.o. (Figure 2a) resulted in a dose-related inhibition of carrageenan-evoked hindpaw oedema. The relative potency of nitroaspirin and aspirin varied depending upon the route of administration and the 'phase' of the oedema response. When administered i.p the anti-inflammatory effect of nitroaspirin was considerably greater than that of aspirin in the 'late' phase of the oedema response (measured at 180 min) with calculated ED$_{50}$ values of 21.3 mg kg^{-1} and >100 mg kg^{-1} (equivalent to 64.3 μmol kg^{-1} and >555 μmol kg^{-1}), respectively (Figure 1). In contrast, nitroaspirin and aspirin exhibited broadly similar anti-inflammatory activity in the 'late' phase when administered p.o. (see Figure 2). Thus, at the highest dose used (100 mg kg^{-1}, 360 min), orally administered nitroaspirin (aspirin in parenthesis) inhibited oedema formation by 46.9$\pm$1.6% (47.2$\pm$3.8%, both $n=6$, $P<0.05$). Interestingly, both nitroaspirin and aspirin administered i.p. (but not p.o.) also reduced the 'early' phase of carrageenan-evoked hindpaw

oedema. Once again nitroaspirin (ED$_{50}$: 33.3 mg kg^{-1} equivalent to 99 μmol kg^{-1}) was considerably more potent than aspirin (only 39% inhibition at 100 mg/kg^{-1}) when measured 120 min after carrageenan injection.

Anti-nociceptive activity of nitroaspirin

Carrageenan-induced hindpaw hyperalgesia (mechanical stimulation) Intraplantar administration of carrageenan (2% w v^{-1}, 150 μl) caused significant hindpaw hyperalgesia as evidenced by a reduction in the threshold for noxious mechanical stimulation from 9.92$\pm$1.0 to 3.38$\pm$0.37 units, $n=6$, $P<0.05$. In control experiments, intraplantar injection of saline (150 μl) did not alter the noxious mechanical stimulation threshold (data not shown).

Pre-treatment of animals (150 min after intraplantar carrageenan injection; i.e. 30 min before test) with nitroaspirin (25–200 mg kg^{-1}, p.o.) or aspirin (25–200 mg kg^{-1}, p.o.) inhibited carrageenan-induced hyperalgesia in the injected hindpaw without affecting the nociceptive mechanical threshold in the contralateral (i.e. non-injected) hindpaw (Figure 3). The calculated ED$_{50}$ values for nitroaspirin and aspirin as
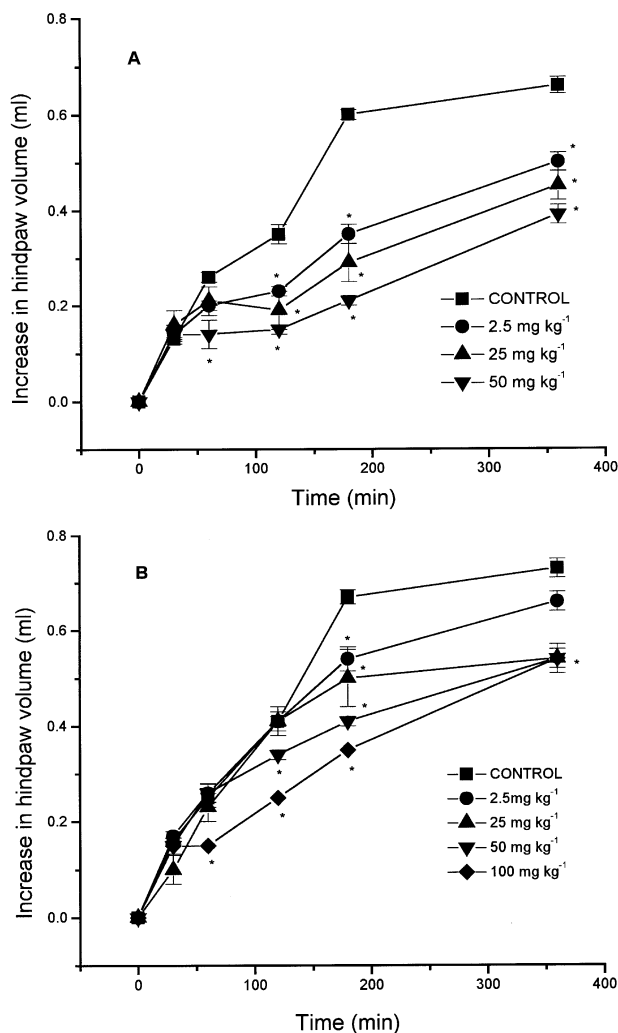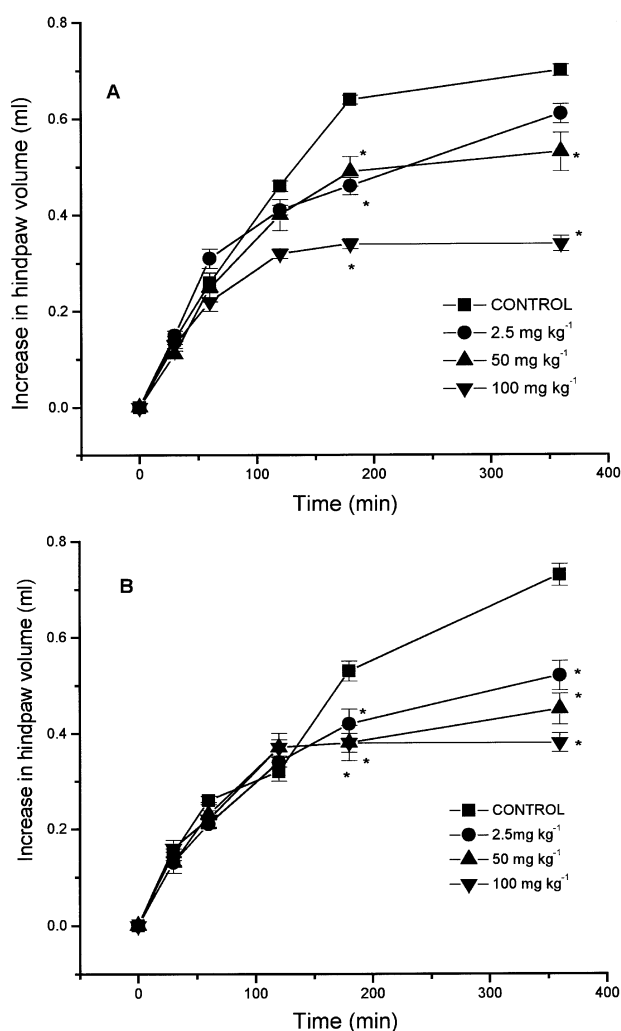

Figure 1 Effect of nitroaspirin (A) and aspirin (B) administered i.p. on carrageenan-induced hindpaw oedema formation in the rat. Results show increase in hindpaw volume (ml) and are mean$\pm$ s.e.mean, $n=6$, *$P<0.05$ (ANOVA plus *post-hoc* Dunnett's test). Where no error bars are shown error lies within the dimensions of the symbol.

Figure 2 Effect of nitroaspirin (A) and aspirin (B) administered p.o. on carrageenan-induced hindpaw oedema formation in the rat. Results show increase in hindpaw volume (ml) and are mean $\pm$ s.e.mean, $n=6$, $*P<0.05$ (ANOVA plus *post-hoc* Dunnett's test).
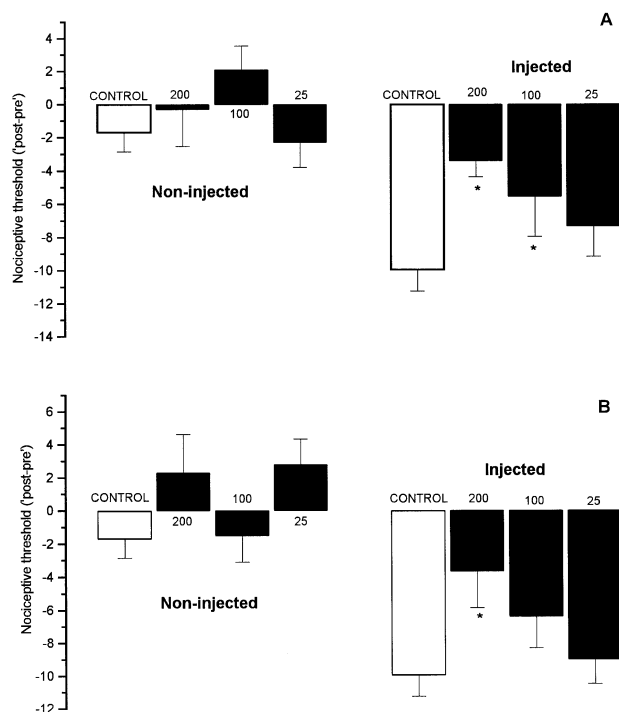

Figure 3 Effect of nitroaspirin (A) and aspirin (B) administered p.o. on carrageenan-induced mechanical hyperalgesia in the rat. Results show change (i.e. 'post–pre') in nociceptive threshold (arbitrary units) to a noxious mechanical stimulus before and at 180 min after intraplantar injection of carrageenan. Drugs (mg kg^{-1}) were administered 150 min after carrageenan injection. Results show mean $\pm$ s.e.mean, $n=6$, $*P<0.05$ (ANOVA plus *post-hoc* Dunnett's test).

inhibitors of mechanical hyperalgesia were 121.6 and 141.2 mg kg^{-1} (equivalent to 365 and 784 μmol kg^{-1}), respectively.

Formalin induced hindpaw licking behaviour

In control, (vehicle-injected) animals, intraplantar injection of formalin evoked a hindpaw licking response of 75.7 ± 6.5 s ($n=20$) in the 'early' phase (0–5 min) and 139.9 ± 15.6 s ($n=20$) in the 'late' (15–30 min) phase.

In contrast to the dose-related ability of both nitroaspirin and aspirin to reduce carrageenan-evoked hindpaw mechanical hyperalgesia in the rat, pretreatment of mice with nitroaspirin or aspirin (50 or 100 mg kg^{-1}, p.o.) failed to affect either phase of the formalin-induced nociceptive licking behaviour (Figure 4). Higher doses of each drug produced more variable results with significant inhibition of 'late' phase licking behaviour apparent at both 200 and 300 mg kg^{-1} of nitroaspirin. Aspirin (200 mg kg^{-1} but not 300 mg kg^{-1}, p.o.) was also anti-nociceptive in the 'late' phase.

Tail withdrawal assay

In these experiments, tail withdrawal latency following application of a topical heat source in control (vehicle-injected)

animals was 2.4 ± 0.15 s ($n=20$). Both nitroaspirin and aspirin (50 and 100 mg kg^{-1}) exhibited anti-nociceptive activity as evidenced by a graded increase in withdrawal latency (Figure 5). Lower doses were without effect.

Acetic acid induced abdominal constrictions

Intraperitoneal injection of acetic acid (2% w v^{-1}, 0.1 ml 10 g^{-1}) resulted in 49.6 ± 1.9 abdominal constrictions ($n=10$) in the 30 min period thereafter. In the present experiments, oral administration of nitroaspirin or aspirin produced a dose-related inhibition of acetic acid induced abdominal constrictions (Figure 6). The calculated ED$_{50}$ values for nitroaspirin and aspirin were 51.2 and 43.7 mg kg^{-1} (equivalent to 154.7 and 242.8 μmol kg^{-1}). The time course of anti-nociceptive activity of nitroaspirin and aspirin was also investigated in this model. Pretreatment of animals with an approximate ED$_{50}$ (i.e. 50 mg kg^{-1}, p.o.) of nitroaspirin or aspirin either 15 min or 180 min prior to acetic acid injection revealed no difference in the resulting anti-nociceptive effect. In contrast, reduced anti-nociceptive activity was apparent in animals pretreated with either compound 360 min prior to acetic acid injection (Figure 7). Thus, these experiments suggest that the time course of anti-nociceptive activity of nitroaspirin and aspirin is similar in this model.

Discussion

Anti-oedema effect of nitroaspirin

The hindpaw oedema which follows intraplantar carrageenan injection involves a complex and time-dependent synthesis/
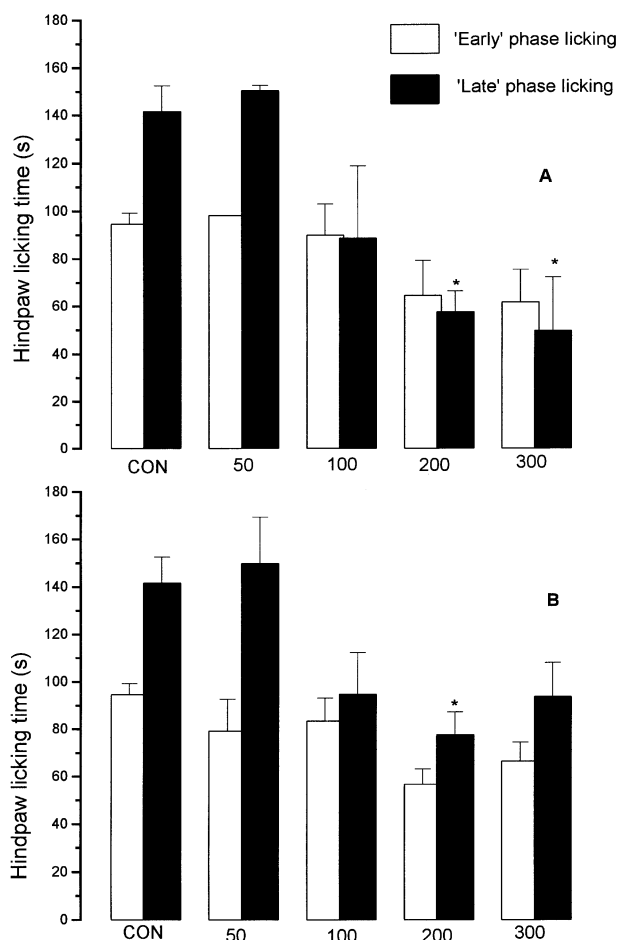
Figure 4 Effect of nitroaspirin (A) and aspirin (B) on mouse formalin-induced hindpaw licking behaviour in the 'early' (0–5 min after intraplantar formalin injection) and 'late' phases (15–30 min). Figures at the foot of each pair of columns indicate dose of drug administered p.o. (mg kg^{-1}). Results shown mean$\pm$s.e.mean, $n=3–20$, *$P<0.05$ by ANOVA and *post-hoc* Dunnett's test.

Figure 5 Effect of nitroaspirin (A) and aspirin (B) on tail withdrawal latency to a noxious thermal stimulus in the mouse. Results show tail withdrawal latency (s) 30 min after p.o. administration of drug. Figures at the base of each column indicate dose administered (mg kg^{-1}, p.o.). Results show mean$\pm$s.e.mean, $n=10$, *$P<0.05$ (ANOVA plus *post-hoc* Dunnett's test).

release of a plethora of different inflammatory mediators. Previous research from this and other laboratories has suggested that the 'early' phase response is triggered mainly by histamine, bradykinin, 5-hydroxytryptamine and nitric oxide (synthesized by the neuronal nitric oxide synthase isoform), whilst the 'late' phase results primarily from the formation of pro-inflammatory prostanoids and nitric oxide (synthesized by the inducible nitric oxide synthase isoform) (DiRosa *et al.*, 1971; Garcia Leme *et al.*, 1973; Handy & Moore, 1998; Holsapple *et al.*, 1980; Salvemini *et al.*, 1996; Vinegar *et al.* (1969).

It has been known for many years that aspirin, and similar NSAID, reduce carrageenan-induced hindpaw oedema in the rat (Flower *et al.*, 1972). Most reports suggest that NSAID preferentially inhibit the 'late' phase response presumably by inhibiting the inducible COX-2 isoform which is believed to be responsible for the generation of pro-inflammatory prostanoids in the later stages of this and other inflammatory models (Vane & Botting, 1998). In the present experiments, both aspirin and nitroaspirin caused dose related inhibition of the 'late' phase of oedema regardless of the route of administration (i.e., i.p. or p.o.) employed. These results, therefore, confirm a preliminary report in which single, equimolar doses of nitroaspirin and aspirin reduced carrageenan-induced hindpaw oedema in the rat (Takeuchi *et al.*, 1998).
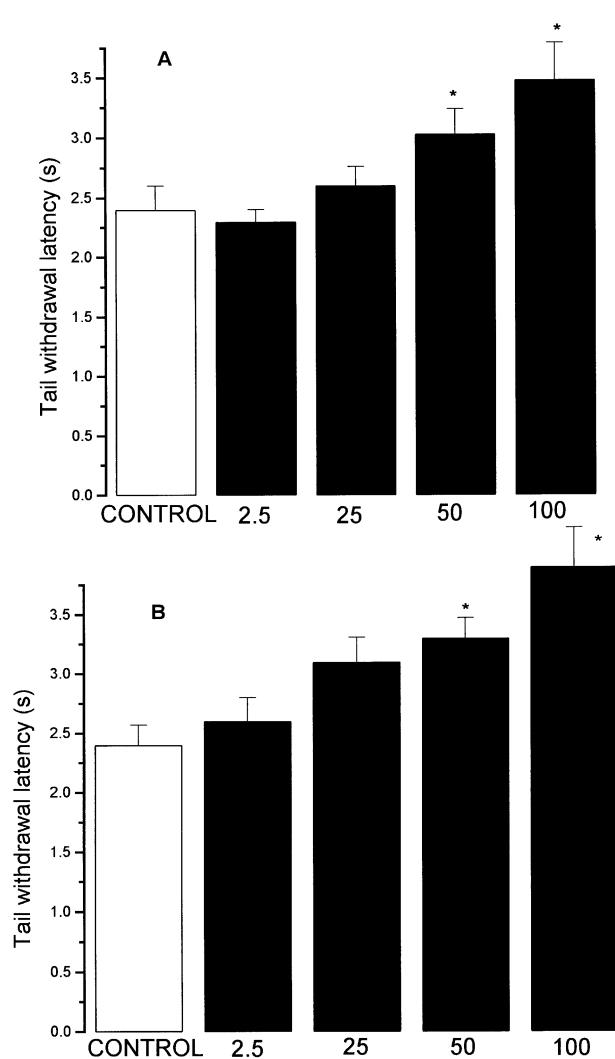
As nitroaspirin and aspirin exhibit similar COX inhibitory activity *in vivo* (Cuzzolin *et al.*, 1996; Wallace *et al.*, 1999a), it seems likely that the 'late' phase inhibitory effect of both drugs depends upon inhibition of hindpaw prostanoid formation presumably by an effect on COX-2 activity, which is likely to be the predominant isoform at this stage in the response (Seibert *et al.*, 1994; Vane *et al.*, 1998). Interestingly, nitroaspirin or aspirin administered i.p. (but not p.o.) also reduced the 'early' phase response. Although some reports suggest that COX-2 may be present as early as 60–90 min after induction of an inflammatory response (Seibert *et al.*, 1994) the possibility that nitroaspirin (and aspirin) may, additionally, reduce hindpaw constitutive COX-1 activity to bring about an anti-oedema effect in the 'early' phase should be considered. Certainly, aspirin reduces inflammation in a carrageenan-induced pleurisy model in the rat over a considerably longer time period than either NS-398 or nimesulide (relatively selective COX-2 inhibitors) (Gilroy *et al.*, 1998), which suggests a pro-inflammatory role for prostanoids generated by both COX-1 and COX-2 at least in this model (Gilroy *et al.*, 1998).
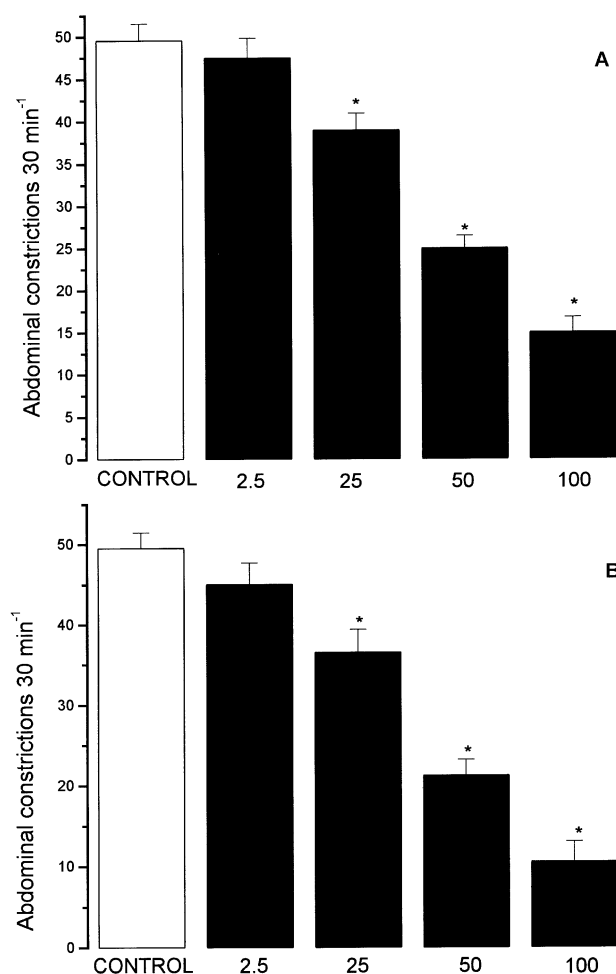
Figure 6 Effect of nitroaspirin (A) and aspirin (B) on acetic acid induced abdominal constrictions in the mouse. Results show number of abdominal constrictions over a 30 min period following i.p. acetic acid injection. Drugs were administered p.o. 15 min prior to acetic acid injection. Figures at the base of each column indicate dose administered (mg kg^{-1}, p.o.). Results show mean$\pm$s.e. mean, $n=10$, *$P<0.05$ (ANOVA plus *post-hoc* Dunnett's test).

Figure 7 Effect of nitroaspirin and aspirin (both 50 mg kg^{-1}, p.o.) on acetic acid induced abdominal constrictions in the mouse. Results show number of abdominal constrictions over a 30 min period following i.p. acetic acid injection. Drugs were administered p.o. either 15, 180 or 360 min prior to acetic acid injection. Results show mean$\pm$s.e.mean, $n=6$, *$P<0.05$ (ANOVA plus *post-hoc* Dunnett's test).

The relative potency of nitroaspirin *vis-a-vis* aspirin is clearly dependent both upon the time course of the carrageenan response (i.e. 'early' c.f. 'late' phase) and upon the route of drug administration. Thus, nitroaspirin (administered i.p.) is more potent than aspirin (by comparison of ED$_{50}$ values; 8.6$\times$on a mol for mol basis) than aspirin in the 'late' phase. Similarly, nitroaspirin (administered i.p.) also exhibits greater anti-inflammatory activity than does aspirin in the 'early' (time, 60 min) phase (e.g. nitroaspirin; 46% inhibition at 50 mg kg^{-1} equivalent to 151 μmol kg^{-1} c.f. aspirin; 40% inhibition at 100 mg kg^{-1} equivalent to 555 μmol kg^{-1}). However, it should be noted that the improved anti-oedema potency of nitroaspirin (c.f. aspirin) is no longer apparent when drugs are administered orally. In this context, a NO-releasing derivative of mesalamine (5-aminosalicylic acid) has recently been reported to exhibit greater anti-inflammatory activity than the parent compound as an inhibitor of colitis in the rat (Wallace *et al.*, 1999b).

Assuming that nitroaspirin acts simply as a 'pro-drug' for anti-inflammatory aspirin it might be expected that the two compounds would exhibit similar potency in these experiments. This is not the case. The reason(s) underlying this discrepancy in potency are not clear and have not been
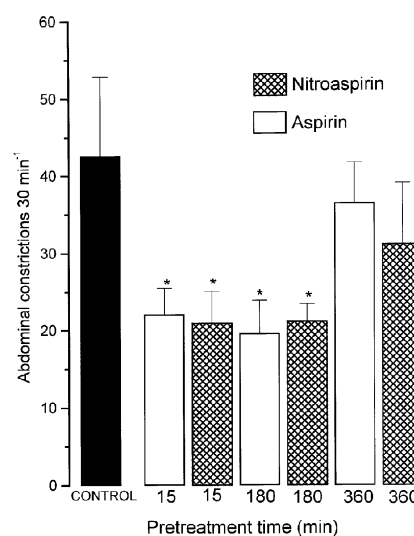
directly addressed in the present study. However, we believe that some comment is appropriate. Perhaps the simplest explanation is that i.p. administered nitroaspirin is accumulated more readily or over a longer period in the carrageenan-inflamed hindpaw than is aspirin. Unfortunately, to the best of our knowledge, there have been no published reports which compare the accumulation and dispersal of nitroaspirin/aspirin (and their metabolites such as salicylate) at the site of inflammation. However, the observation that i.p. injected nitroaspirin in the rat results in a delay in the time required to achieve peak plasma salicylate concentration (6 h) when compared with aspirin (3 h; Tagliaro *et al.*, 1996) suggests that differences in pharmacokinetic profile may be important in determining the relative anti-inflammatory potency of nitroaspirin *vis-à-vis* aspirin. Alternatively, nitroaspirin may exhibit additional pharmacological activity (c.f. aspirin) by virtue of what is presumed to be a slow release of nitric oxide (see del Soldato *et al.*, 1999). In this context, nitroaspirin (but not aspirin) has recently been shown to inhibit caspase 1 activity and, thus, interleukin-1β formation (Fiorucci *et al.*, 1999) and also reduces nuclear factor κB (NF-κB) activation (Minto *et al.*, 1997) although it should be noted that an inhibitory effect of aspirin on NF-κB activation has also been reported (Yin *et al.*, 1998). The NO-releasing derivative of mesalamine has also been demonstrated to reduce leucocyte adherence to the vascular endothelium (Wallace *et al.*, 1999b). In each case, nitric oxide has been shown to be the active moiety within the nitroaspirin molecule. Whether nitroaspirin affects leucocyte function at the site of inflammation *in vivo* or whether other, as yet unidentified, COX-independent mechanisms contribute to the anti-oedema effect of this compound would appear to warrant further study.

Anti-nociceptive effect of nitroaspirin

Nitroaspirin and aspirin exhibited anti-nociceptive activity in all four animal models studied. However, both the absolute

and the relative potency of the two compounds varies depending upon the experimental model used. Thus, both nitroaspirin and aspirin exhibited greater anti-nociceptive activity in animal models of 'inflammatory' pain (e.g. carrageenan-induced hindpaw hyperalgesia; acetic acid induced abdominal constrictions) and were less effective in models of 'acute' pain (e.g. tail flick test; responsiveness to mechanical noxious stimulation in the non-injected hindpaw) and in hyperalgesia states dependent more on 'central' as opposed to 'peripheral' sensitisation (e.g. late phase formalin-induced hindpaw licking) (see reviews by Willis, 1992; Woolf, 1993). Interestingly, the time course of the anti-nociceptive effect of nitroaspirin and aspirin (assessed as inhibition of acetic acid induced abdominal constrictions in the mouse) was very similar with no difference in activity detected when drugs were administered either 15 min or 180 min prior to acetic acid injection.

The profile of anti-nociceptive activity of aspirin identified in these experiments is in broad agreement with previous reports in the literature. Thus, published ED_{50} values (43.7 mg kg^{-1} in this study) for aspirin in the mouse abdominal constriction test have ranged from 24 mg kg^{-1} (Santos *et al.*, 1995) through 31.6 mg kg^{-1} (Inoue *et al.*, 1994) to 81.4 mg kg^{-1} (Satyanarana *et al.*, 1995). In addition, similar doses of aspirin to those used in this study reduce the hyperalgesia associated with hindpaw injection in the rat of carrageenan (Calhoun *et al.*, 1992) or other algogens such as yeast (Seegers *et al.*, 1981) but have only marginal effect on the tail flick response to noxious thermal stimulus (Millan *et al.*, 1995) and no effect on either the 'early' or 'late' phase hindpaw licking response to intraplantar formalin injection even at doses as high as 400 mg kg^{-1} (Rahman *et al.*, 1994; Wheeler-Aceto & Cowan, 1991).

Since this is the first study to examine the anti-nociceptive effect of nitroaspirin in a range of behavioural tests similar comparisons with the results of other researchers are clearly not available. However, as was the case for the anti-oedema effect, nitroaspirin was also marginally more potent (mol for mol) than aspirin as an inhibitor both of carrageenan-induced hindpaw hyperalgesia in the rat (2.1 fold comparing ED_{50} values) and acetic acid-induced abdominal constrictions in the mouse (1.6 fold comparing ED_{50} values). It seems likely that inhibition of hindpaw COX-2 enzyme activity plays a major part in the anti-nociceptive activity of nitroaspirin and aspirin. Experiments using highly selective COX-2 inhibitors (e.g. rofecoxib, Ehrich *et al.*, 1999) strongly suggest that the therapeutic benefit of NSAID in inflammatory pain results from inhibition of COX-2 activity although whether the COX-2 isoform involved is sited in the inflamed tissue or in the spinal cord (see Smith *et al.*, 1998) or both remains unclear. In contrast, bearing in mind the brief (30 min) duration of the present experiments, the effect of nitroaspirin and aspirin on acetic acid induced abdominal constrictions is more likely to reflect COX-1 rather than COX-2 inhibition. Whether the albeit modestly augmented anti-nociceptive potency of nitroaspirin (c.f. aspirin) suggests additional COX-independent mechanisms for the nitro derivative requires more detailed analysis.

Conclusion

The present experiments represent the first systematic attempt to compare the anti-oedema and anti-nociceptive activity of aspirin and nitroaspirin. We conclude that these two compounds are qualitatively similar in their actions. However, on a mol for mol basis, nitroaspirin appears to be more potent than aspirin. The possibility that nitroaspirin exhibits additional anti-inflammatory actions over and above those of aspirin is intriguing and merits further investigation.

References

AL-SWAYEH, O.A., CLIFFORD, R.H. & MOORE, P.K. (1999). Nitroaspirin – a novel anti-inflammatory and anti-hyperalgesic agent. *Mediat. Inflamm.*, **8**, S95.

CALHOUN, W., GILMAN, S.C., DATKO, L.J., COPENHAVER, T.W. & CARLSON, R.P. (1992). Interaction studies of tilomisole, aspirin and naproxen in acute and chronic inflammation with assessment of gastrointestinal irritancy in the rat. *Agents Actions*, **36**, 99–106.

CUZZOLIN, L., ADAMI, A., DEGAN, M., CRIVELLENTE, F., BONAPACE, S., MINUZ, P. & BENONI, G. (1996). Effect of single and repeated doses of a new nitroderivative of acetylsalicylic acid on platelet TxA_2 production in rats. *Life Sci.*, **58**, 207–208.

D'AMOUR, F.E. & SMITH, D.L. (1941). A method for determining loss of pain sensation. *J. Pharm. Exp. Ther.*, **72**, 74–79.

DAVIES, N.M., ROSETH, A.G., APPLEYARD, C.B., McKNIGHT, W., DEL SOLDATO, P., CALIGNANO, A., CIRINO, G. & WALLACE, J.L. (1997). NO-naproxen vs naproxen: ulcerogenic, analgesic and anti-inflammatory effects. *Aliment. Pharmacol. Ther.*, **11**, 69–79.

DELSOLDATO, P., SORRENTINO, R. & PINTO, A. (1999). NO-aspirins: a class of new anti-inflammatory and antithrombotic agents. *Trends Pharmacol. Sci.*, **20**, 319–323.

DIROSA, M., GIROUD, J.P. & WILLOUGHBY, D.A. (1971). Studies of mediators of the acute inflammatory response in rats in different sites by carrageenan and turpentine. *J. Pathol.*, **104**, 15–29.

EHRICH, E.W., DALLOB, A., DELEPELEIRE, I., VAN HECKEN, A., RIENDEAU, D., YUAN, W.Y., PORRAS, A., WITTREICH, J., SEIBOLD, J.R., DESCHEPPER, P., MEHLISCH, D.R. & GERTZ, B.J. (1999). Characterisation of rofecoxib as a cyclooxygenase-2 isoform inhibitor and demonstration of analgesia in the dental pain model. *Clin. Pharm Ther.*, **65**, 336–347.

FIORUCCI, S., ANTONELLI, E., SANTUCCI, L., MORELLI, O., MIGLIETTI, M., FEDERICI, B., MANNUCCI, R., DEL SOLDATO, P. & MORELLI, A. (1999). Gastrointestinal safety of nitric oxide derived aspirin is related to inhibition of ICE-like cysteine proteases in rats. *Gastroenterology*, **116**, 1089–1106.

FLOWER, R.J., GRYGLEWSKI, R.J., HERBACZYNSKA-CEDRO, K. & VANE, J.R. (1972). Effects of anti-inflammatory drugs on prostaglandin biosynthesis. *Nature*, **238**, 104–106.

FUNG, H.B. & KIRSCHENBAUM, H.L. (1999). Selective cyclooxygenase-2 inhibitors for the treatment of arthritis. *Clin. Ther.*, **21**, 1131–1157.

GARCIA LEME, J., HAMAMURA, L., LEITE, M.P. & ROCHA E SILVA, M. (1973). Pharmacological analysis of the acute inflammatory response induced in the rat's paw by local injection of carrageenan and by heating. *Br. J. Pharmacol.*, **48**, 88–96.

GILROY, D.W., TOMLINSON, A. & WILLOUGHBY, D.A. (1998). Differential effects of inhibitors of cyclooxygenase (cyclooxygenase 1 and cyclooxygenase 2) in acute inflammation. *Eur. J. Pharmacol.*, **355**, 211–217.

HANDY, R.L.C. & MOORE, P.K. (1998a). A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hindpaw oedema and NOS activity. *Br. J. Pharmacol.*, **123**, 1083–1088.

HANDY, R.L.C. & MOORE, P.K. (1998b). Effect of selective inhibitors of neuronal nitric oxide synthase on carrageenan-induced mechanical and thermal hyperalgesia. *Neuropharmacology*, **279**, 37–43.

HOLSAPPLE, M.P., SCHNUR, M. & YIM, G.K. (1980). Pharmacological modulation of edema mediated by prostaglandin, serotonin and histamine. *Agents Actions*, **10**, 368–373.

INOUE, K., FUJISAWA, H., MOTYONAGA, A., INOUE, Y., KYOI, T., UEDA, F. & KIMURA, K. (1994). Antiinflammatory effects of etodolac; comparison with other nonsteroidal antiinflammatory drugs. *Biol. Pharm Bull.,* **17,** 1577 – 1583.

LAIRD, J.M.A., MASON, G.S., WEBB, J., HILL, R.G. & HARGREAVES, R.J. (1996). Effects of a partial agonist and a full antagonist acting at the glycine site of the NMDA receptor on inflammation-induced mechanical hyperalgesia in rats. *Br. J. Pharmacol.,* **117,** 1487 – 1492.

MILLAN, M.J., SEGUIN, L., HONORE, P., GIRARDON, S. & BERVOETS, K. (1995). Pro- and antinociceptive actions of serotonin (5-HT$_{1A}$) agonists and antagonists in rodents: relationship to algesiometric paradigm. *Behavioural Brain Res.,* **73,** 69 – 77.

MINTO, M., DEL SOLDATO, P., GHEZZI, P. & FRATELLI, M. (1997). A novel acetylsalicylic acid nitroderivative inhibits NF-κB activation and is antiproliferative to L929 cells. *Inflamm. Res.,* **46,** S3.

MONCADA, S., FERREIRA, S.H. & VANE, J.R. (1973). Prostaglandins, aspirin-like drugs and the oedema of inflammation. *Nature,* **246,** 217 – 219.

MOORE, P.K., OLUYOMI, A.O., BABBEDGE, R.C., WALLACE, P. & HART, S.L. (1991). L-N^G nitroarginine methyl ester exhibits antinociceptive activity in the mouse. *Br. J. Pharmacol.,* **102,** 198 – 202.

MORGAN, C.V.J., BABBEDGE, R.C., GAFFEN, Z., WALLACE, P., HART, S.L. & MOORE, P.K. (1992). Synergistic anti-nociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) and flurbiprofen in the mouse. *Br. J. Pharmacol.,* **106,** 493 – 497.

RAHMAN, A.F.M.M., TAKAHISHI, M. & KANETO, H. (1994). Involvement of pain associated anxiety in the development of morphine tolerance in formalin-treated mice. *Jap. J. Pharmacol.,* **65,** 313 – 317.

SALVEMINI, D., WANG, Z.-Q., WYATT, P.A., BOURDON, D.M., MARINO, M.H., MANNING, P.T. & CURRIE, M.G. (1996). Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.,* **118,** 829 – 838.

SANTOS, A.R.S., NIERO, R., FILHO, Y.C., YUNES, R.A., PIZZOLATTI, M.G., DELLEMONACHE, F. & CALIXTO, J.B. (1995). Antinociceptive properties of steroids isolated from Phyllanthus-Corcovadensis in mice. *Planta Med.,* **61,** 329 – 332.

SATYANARANA, K. & RAO, M.N.A. (1995). Synthesis of 4-[5-(substituted aryl) 4,5 dihydro 1H pyrazol-3-yl] 3 phenylsydnones as antinflammatory, antiarthritic and analgesic agents. *Eur. J. Med. Chem.,* **30,** 641 – 645.

SEEGERS, A.J., JAGER, L.P., ZANDBERG, P. & VAN NOORDWIJK, J. (1981). The anti-inflammatory, analgesic and antipyretic activities of non-narcotic analgesic drug mixtures in rats. *Arch. Int. Pharmacodyn. Ther.,* **251,** 237 – 254.

SEIBERT, K., ZHANG, Y., LEAHY, K., HAUSER, S., MASFERRER, J., PERKINS, W., LEE, L. & ISAKSON, P. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase-2 in inflammation and pain. *Proc. Natl. Acad. Sci., U.S.A.,* **91,** 12013 – 12017.

SMITH, C.J., ZHANG, Y., MASFERRER, J.L., SEIBERT, K. & ISAKSON, P.C. (1998). Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 13313 – 13318.

TAGLIARO, F., CUZZOLIN, L., ADAMI, A., SCARCELLA, D., CRIVELLENTE, F. & BENONI, G. (1996). Pharmacokinetics of a new nitroderivative of acetylsalicyclic acid after a single dose in rats. *Life Sci.,* **60,** 101 – 106.

TAKEUCHI, K., UKAWA, H., KONAKA, A., KITAMURA, M. & SUGAWA, Y. (1998). Effect of nitric oxide-releasing aspirin derivative on gastric functional and ulcerogenic responses in rats: comparison with plain aspirin. *J. Pharm. Exp. Ther.,* **286,** 115 – 121.

UKAWA, H., YAMAKUNI, H., KATO, S. & TAKEUCHI, K. (1997). Effects of cyclooxygenase-2 selective and nitric oxide releasing nonsteroidal anti-inflammatory drugs on gastric ulcerogenic and healing responses in experimental aninmals. *Dig. Dis. Sci.,* **43,** 2003 – 2011.

VANE, J.R., BAKHLE, Y.S. & BOTTING, R.M. (1998). Cyclooxygenases 1 and 2. *Ann. Rev. Pharmacol. Toxicol.,* **38,** 97 – 120.

VANE, J.R. & BOTTING, R.M. (1998). Anti-inflammatory drugs and their mechanism of action. *Inflamm. Res.,* **47,** S78 – S87.

VINEGAR, R., SCHREIBER, W. & HUGO, R. (1969). Biphasic development of carrageenin edema in rats. *J. Pharmacol. Exp. Ther.,* **166,** 96 – 103.

WALLACE, J.L. (1997). Nonsteroidal antiinflammatory drugs and gastroenteropathy: the second hundred years. *Gastroenterology,* **112,** 1000 – 1016.

WALLACE, J.L., McKNIGHT, W., WILSON, T.L., DEL SOLDATO, P. & CIRINO, G. (1997). Reduction of shock-induced gastric damage by a nitric oxide releasing aspirin derivative: role of neutrophils. *Am. J. Physiol.,* **273,** G1246 – G1251.

WALLACE, J.L., MUSCARA, M.N., McKNIGHT, W., DICAY, M., DEL SOLDATO, P. & CIRINC, G. (1999a). In vivo antithrombotic effects of a nitric oxide releasing aspirin derivative, NCX-4016. *Thrombosis Res.,* **93,** 43 – 50.

WALLACE, J.L., REUTER, B., CICALA, C., McKNIGHT, W., GRISHAM, M. & CIRINO, G. (1994a). Novel non-steroidal antiinflammatory derivatives with markedly reduced ulcerogenic properties in the rat. *Gastroenterology,* **107,** 173 – 179.

WALLACE, J.L., REUTER, B., CICALA, C., McKNIGHT, W., GRISHAM, M. & CIRINO, G. (1994b). A diclofenac derivative without ulcerogenic properties. *Eur. J. Pharmacol.,* **257,** 249 – 255.

WALLACE, J.L., VERGNOLLE, N., MUSCARA, M.N., ASFAHA, S., CHAPMAN, K., McKNIGHT, W., DEL SOLDATO, P., MORELLI, I. & FIORUCCI, S. (1999b). Enhanced anti-inflammatory effects of a nitric oxide releasing derivative of mesalamine in rats. *Gastroenterology,* **117,** 557 – 566.

WHEELER-ACETO, H. & COWAN, A. (1991). Standardisation of the rat paw formalin test for the evaluation of analgesics. *Psychopharmacology,* **104,** 35 – 44.

WILLIS, W.D. (1992). *Hyperalgesia and Allodynia.* Raven Press, New York.

WOOLF, C.J. (1993). Evidence for a central component of post-injury pain hypersensitivity. *Nature,* **308,** 686 – 688.

YIN, M.-J., YAMAMOTO, Y. & GAYNOR, R.B. (1998). The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature,* **396,** 77 – 80.