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# A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hindpaw oedema and NOS activity

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**1** Intraplantar injection of carrageenan (150 $\mu$l, 1–3% w/v) in the rat resulted in a dose-related increase in hindpaw weight (oedema) characterized by a rapid 'early' phase (up to 2.5 h) response followed by a more sustained 'late' phase (2–6 h) response. No change in weight of either the contralateral (i.e. noninjected) hindpaw or hindpaws injected with saline was observed.

**2** Six hours after intraplantar injection of carrageenan (1–3% w/v) hindpaw constitutive (i.e. calcium-dependent) nitric oxide synthase (cNOS) activity (determined *ex vivo* as the conversion of radiolabelled L-arginine to radiolabelled citrulline) was increased (e.g. 2% w/v; $0.64 \pm 0.08$ pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$ c.f. $0.08 \pm 0.04$ pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$ in saline-injected, control animals, $n=4$, $P<0.05$). Carrageenan injection also resulted in the appearance in hindpaw homogenates of inducible (i.e. calcium-independent) nitric oxide synthase (iNOS, e.g. 2% w/v; $0.67 \pm 0.14$ pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$, $n=4$). Hindpaw cyclic GMP concentration was also significantly increased 6 h after intraplantar injection of carrageenan (e.g. 2% w/v; $379.6 \pm 26.8$ fmol $mg^{-1}$ protein c.f. $261.8 \pm 42.2$ fmol $mg^{-1}$ protein, in saline-injected, control animals, $n=4$, $P<0.05$).

**3** Pretreatment (5–25 mg $kg^{-1}$, i.p., 30 min before carrageenan, 2% w/v) of animals with L-$N^G$ nitro arginine methyl ester (L-NAME; isoform nonselective inhibitor of NOS) or 7-nitro indazole (7-NI; inhibitor of neuronal NOS, nNOS) caused dose-related inhibition of both the early (2 h) and late (6 h) phase hindpaw oedema, associated with reduced hindpaw iNOS and cNOS activity and cyclic GMP concentration in animals killed at 6 h. Administration of 7-NI (5–25 mg $kg^{-1}$, i.p.) to animals 2.5 h after intraplantar carrageenan (2% w/v) injection (i.e. at the end of the early phase oedema response) produced dose-related inhibition of the late phase response.

**4** Pretreatment (5–25 mg $kg^{-1}$, i.p., 30 min before carrageenan, 2% w/v) of animals with L-N6-iminoethyllysine (L-NIL, selective inhibitor of iNOS) (5–25 mg $kg^{-1}$) failed to affect the early phase hindpaw oedema response but did produce a dose-related inhibition of the late phase oedema. L-NIL pretreatment also inhibited the carrageenan-induced increase in both hindpaw iNOS and cNOS activity as well as the rise in hindpaw cyclic GMP concentration.

**5** The present experiments demonstrate an anti-inflammatory effect of 7-NI as evidenced by inhibition of carrageenan-induced hindpaw oedema in the rat. Inhibition of nNOS (early phase) and iNOS (late phase) at the site of inflammation most probably accounts for the anti-inflammatory activity observed. These data suggest a role for nitric oxide synthesized by the nNOS isoform (most probably within sensory nerves) in this model of inflammation.

**Keywords:** Hindpaw oedema; nitric oxide synthase; nitric oxide; 7-nitro indazole; L-NAME; inflammation

## Introduction

Nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS, Palmer *et al*., 1988). To date, three isoforms of NOS have been identified viz. neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Forstermann & Kleinert, 1995; Ogden & Moore, 1995). Both eNOS and nNOS are activated by calcium and are constitutive isoforms whilst iNOS is calcium-independent and is synthesized *de novo* following exposure of a wide range of cell types to bacterial endotoxin and/or inflammatory cytokines.

In recent years, considerably evidence has accumulated suggesting a role for nitric oxide (NO) as a mediator of inflammation (for reviews, see Nussler & Billiar, 1993; Lyons, 1995; Miller & Grisham, 1995). Thus, NO dilates microvascular blood vessels (Gardiner *et al*., 1990) and promotes microvascular permeability resulting in oedema formation (Hughes *et al*., 1990; Giraldelo *et al*., 1994). In addition, NO increases the synthesis/release of pro-inflammatory mediators such as cytokines and reactive oxygen species (Marcinkiewicz *et al*., 1995) and prostanoids (Sautebin *et al*., 1995). Furthermore, administration of isoform-nonselective inhibitors of NOS such as L-$N^G$ nitro arginine methyl ester (L-NAME) and L-$N^G$ monomethyl arginine (L-NMMA) has been shown to inhibit hindpaw oedema formation in the rat following intraplantar injection of Freunds adjuvant (Stefanovicracic *et al*., 1995), streptococcal wall fragments (McCartney-Francis *et al*., 1993) and carrageenan (Ialenti *et al*., 1992; Medeiros *et al*., 1995). L-NAME pretreatment also inhibits carrageenan-induced pleurisy in the rat (Tracey *et al*., 1995).

Taken together these various observations suggest a pro-inflammatory effect of NO. However, the location and identity of the NOS isoform(s) responsible for NO synthesis at the site of inflammation remains unclear. NO is formed by a number of different cell types important in inflammation including leukocytes, endothelial cells and sensory nerves. Whilst most attention has been centred upon the role of iNOS in inflammation the relative contribution of other NOS isoforms has received scant attention. Indeed, to the best of our knowledge, there have been no attempts to study NOS inhibitors with greater selectivity for nNOS for potential anti-inflammatory activity in standard models such as carrageenan-induced hindpaw oedema. To this end, we have

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now investigated, (i) the time course and dose-dependence of carrageenan-induced hindpaw oedema in the rat, (ii) hindpaw NOS activity and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) concentration (determined *ex vivo*) in animals administered intraplantar carrageenan and (iii) the effect of 7-nitroindazole (7-NI, a selective inhibitor of nNOS *in vivo*; Babbedge *et al*., 1993; Moore *et al*., 1993), L-N6-iminoethyllysine (L-NIL, a selective inhibitor of iNOS; Moore *et al*., 1994) and L-NAME on carrageenan-induced hindpaw oedema in the rat and associated changes in hindpaw NOS activity and cyclic GMP concentration. Some of these results have been presented previously to the British Pharmacology Society (Handy & Moore, 1996).

## Methods

### *Measurement of carrageenan-induced hindpaw oedema in rats*

Rats (male, Wistar, 110–140 g, Tucks Ltd., U.K.) were used in this study. Animals were housed in the Biological Services Unit of this College for 1 week and thereafter acclimatized in the laboratory for 1 h before the commencement of the experiment. Hindpaw weight (injected and non-injected) was determined as a measure of hindpaw oedema formation, before, and at timed intervals (30 min–6 h), after intraplantar carrageenan injection. For this purpose, animals were held firmly and each hindpaw immersed, in turn, into a beaker containing warm (37°C) water placed on a top-pan balance (Mettler Ltd.). Oedema formation was monitored as the difference in hindpaw weight (g) before and after carrageenan injection. 7-NI, L-NAME, L-NIL (5–25 mg $kg^{-1}$) or an appropriate volume of vehicle (arachis oil for 7-NI and 0.9% w/$v^{-1}$ NaCl, saline, for L-NAME and L-NIL) were administered i.p. 30 min prior to intraplantar injection of a standard volume (150 μl) of carrageenan (2% w/v) or 150 min (7-NI only) after carrageenan (2% w/v) injection. At the end of the experiment, animals were stunned by a blow to the head and killed by exsanguination. Both hindpaws were rapidly dissected, weighed and snap frozen in liquid nitrogen before storage at −70°C and assay for NOS and cyclic GMP as described below.

### *NOS assay in hindpaw*

NOS was determined in tissue homogenates as the conversion of [$^3$H]-L-arginine to [$^3$H]-citrulline as described elsewhere (Babbedge *et al*., 1993; Moore *et al*., 1993). Briefly, hindpaws were thawed, weighed and homogenized (1 : 10 w/v in 20 mM Tris-HCl buffer, pH 7.4 containing 2 mM EDTA) in an Ultra-Turrax (type 18/2N) homogenizer. Thereafter, homogenates were centrifuged (10,000 × *g*, 10 min, 4°C) and the supernatant aspirated and immediately assayed for NOS.

Routinely, 'total' NOS activity (i.e. combined eNOS, nNOS and iNOS) was assayed in incubations (15 min at 37°C; total volume, 105 μl) containing tissue supernatant (25 μl), [$^3$H]-L-arginine (0.5 μCi, 120 nM), NADPH (0.5 mM) and $CaCl_2$ (0.75 mM). In experiments to characterize NOS in rat hindpaw homogenates, incubations in which (i) NADPH was omitted, (ii) hindpaw homogenate was exposed to 100 μl Dowex AG50WX-8 $Na^+$ before incubation and (iii) hindpaw homogenate was preincubated (15 min, 0°C) with either L-NAME (1 mM) or valine (1 mM) were performed. For measurement of iNOS activity, $CaCl_2$ was omitted and replaced with EGTA (1 mM). Results (pmol citrulline formed 15 $min^{-1}$ $mg^{-1}$ protein) are expressed either as iNOS activity or constitutive NOS (cNOS, i.e. eNOS and nNOS) activity which was calculated by subtracting the value for iNOS activity from 'total' NOS activity. Assays were terminated by addition of 3 ml HEPES buffer (pH 5.5) containing 2 mM EDTA and incubates applied to 0.5 ml columns of Dowex AG50WX-8 ($Na^+$) followed by 0.5 ml distilled water to remove unchanged [$^3$H]-L-arginine. [$^3$H]-citrulline was quantified by liquid scintillation spectroscopy (Beckman Instruments Ltd.) of a 1 ml aliquot of the combined flow-through. Soluble protein concentration of tissue homogenates was determined by use of the Folin-phenol reagent with bovine serum albumin as standard (Lowry *et al*., 1951).

### *Measurement of hindpaw and cyclic GMP concentration*

Hindpaws were removed and stored at −70°C as described above. On the day of the assay, unthawed hindpaws were placed in liquid nitrogen, pulverized with a lead mallet in a metal stage pre-cooled with liquid nitrogen, and the crushed tissue transferred immediately to 10 ml heated (90–95°C) 50 mM Tris HCl buffer (pH 7.4 containing 4 mM EDTA) for 5 min. Thereafter, tissues were homogenized (Ultra Turrax, type 18/2N) and an aliquot removed for protein measurement. The remaining homogenate was centrifuged (10,000 × *g*, 4°C, 10 min) and the supernatant removed and frozen at −20°C until required.

The concentration of cyclic GMP in hindpaw tissue samples was determined by use of a modification of a commercially available radioimmunoassay kit (Amersham U.K., TRK 500) as described elsewhere (Bland-Ward, 1996). Tissue supernatant for assay was diluted (1 : 5 v/v) in 50 mM sodium acetate buffer (pH 6.2). For this procedure, cyclic GMP antibody was reconstituted in sodium acetate buffer (50 mM containing 0.2% w/v bovine serum albumin, pH 6.2) and aliquots (100 μl) incubated with authentic cyclic GMP (200 μl, 25–800 fmol in 50 mM sodium acetate buffer, pH 6.2) and 10 nCi [$^{125}$I]-cyclic GMP (in 50 mM sodium acetate buffer containing 0.2% w/v bovine serum albumin, pH 6.2) at 4°C for 16 h. Additional tubes were prepared to determine non-specific binding (in the absence of authentic cyclic GMP) and total radioactivity added to each tube. At the end of the incubation period, bound and unbound cyclic GMP were separated by addition of activated charcoal suspension (0.5 ml, 1% w/v in 100 mM potassium phosphate buffer, pH 6.2). After centrifugation (10,000 × *g*, 2 min, 4°C) to precipitate charcoal-bound 'free' cyclic GMP, aliquots of the supernatant (750 μl) containing antibody-bound [$^{125}$I]-cyclic GMP were removed and radioactivity determined with a gamma counter (LKB Wallac Ltd.). Samples and standards were routinely assayed in duplicate and a standard curve was constructed for each individual assay. In control experiments, with a concentration of authentic cyclic GMP (400 pmol incubation) which produced approximately 70% inhibition of binding of [$^{125}$I]-cyclic GMP, the calculated inter- and intra-assay variation of this modified assay was $3.1 \pm 0.7\%$ and $5.1 \pm 0.5\%$, respectively ($n = 12$). Results are shown as fmol cyclic GMP $mg^{-1}$ protein.

### *Statistics*

Statistical significance of differences between groups was determined by ANOVA followed by *post-hoc* Bonferroni test. A probability ($P$) value of 0.05 or less was taken to indicate statistical significance. Results show mean ± s.e.mean with the number of observations indicated in parentheses.

*Drugs and chemicals*

7-NI was purchased from MTM Lancaster Ltd. (U.K.) and L-NIL from Alexis Ltd. (U.K.). Radiolabelled [$^3$H]-L-arginine (sp. act. 62 Ci mmol$^{-1}$) and [$^{125}$I]-cyclic GMP (sp. activity ~2000 $\mu$Ci mmol$^{-1}$) were obtained from Amersham Ltd. (U.K.). All other drugs and chemicals were purchased from Sigma Ltd. (U.K.). 7-NI was dissolved in arachis oil following sonication and warming. L-NAME and L-NIL were dissolved in saline. Drug stock were prepared fresh on the morning of each experiment. Stock suspensions of $\gamma$-carrageenan were shaken for at least 1 h before use. Dowex AG50WX-8 $H^+$ form was converted into the $Na^+$ form by soaking for 2 h in 2 M NaOH followed by repeated washing in water until neutrality.

## Results

*Effect of carrageenan on hindpaw oedema, NOS activity and cyclic GMP concentration*

Mean hindpaw weight before carrageenan injection was $1.5 \pm 0.12$ g ($n = 16$) in the hindpaw to be injected and $1.43 \pm 0.09$ g ($n = 16$, $P > 0.05$) in the contralateral (i.e. non-injected hindpaw). Preliminary experiments indicated that there was no significant difference in hindpaw weight between groups of animals or between left and right hindpaws (data not shown). Accordingly, carrageenan injection was performed into either hindpaw chosen at random. The weight of the non-injected hindpaw did not alter significantly throughout the experiment in rats injected intraplantar with either saline or carrageenan (e.g. 6 h, saline, $0.11 \pm 0.07$ g; 1% w/v carrageenan, $-0.025 \pm 0.02$ g; 2% w/v carrageenan, $-0.01 \pm 0.02$ g and 3% w/v carrageenan, $-0.01 \pm 0.06$ g, all $n = 4$, $P > 0.05$ c.f. hindpaw weight before intraplantar injection).

Intraplantar injection of carrageenan resulted in a concentration-dependent increase in hindpaw weight (Figure 1a). At all concentrations of carrageenan tested the resulting increase in hindpaw weight was time-related with a rapid increase up to 2.5 h (hereinafter referred to as the 'early phase') followed by a longer period (2–6 h) in which hindpaw weight remained unaltered (hereinafter referred to as the 'late phase'). Hindpaw oedema in response to intraplantar carrageenan injection was confirmed as the end of the experiment by removing and weighing directly both the injected and non-injected hindpaws. No change in the weight of the non-injected hindpaws was detected (saline; $1.52 \pm 0.05$ g c.f. 1% w/v carrageenan; $1.38 \pm 0.03$ g, 2% w/v carrageenan; $1.22 \pm 0.05$ g and 3% w/v carrageenan; $1.29 \pm 0.04$ g, all $n = 4$, $P > 0.05$). In contrast, hindpaws from carrageenan-injected animals increased in weight compared with hindpaws removed from animals injected intraplantar with saline (e.g. saline; $1.52 \pm 0.02$ g c.f. 1% w/v carrageenan; $2.24 \pm 0.03$ g, 2% w/v carrageenan; $2.48 \pm 0.09$ g and 3% w/v carrageenan; $2.38 \pm 0.12$ g, all $n = 4$, $P < 0.05$).

Preliminary experiments to characterize NOS activity in hindpaw homogenates from animals injected with carrageenan (2% w/v) were performed. Preincubation (15 min, 0°C) with L-NAME (1 mM) reduced hindpaw NOS activity by approximately 90% ($0.13 \pm 0.06$ pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$ c.f. $1.29 \pm 0.04$ pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$, $n = 4$, $P < 0.05$), whilst inclusion of the arginase inhibitor, valine (50 mM), was without effect ($1.32 \pm 0.07$ pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$ c.f. $1.29 \pm 0.04$ pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$, $n = 4$, $P > 0.05$). Exclusion of NADPH from the incubation medium abolished NOS activity whilst removal of endogenous L-arginine from hindpaw homogenates by exposure to Dowex resin resulted in slightly elevated NOS activity ($1.44 \pm 0.04$ pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$ c.f. $1.25 \pm 0.04$ pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$, $n = 4$, $P < 0.05$). These experiments confirmed the presence of NOS in hindpaw homogenates from carrageenan-injected rats in this study.

Intraplantar injection of carrageenan (1–3% w/v) into one hindpaw did not influence cNOS activity in the contralateral

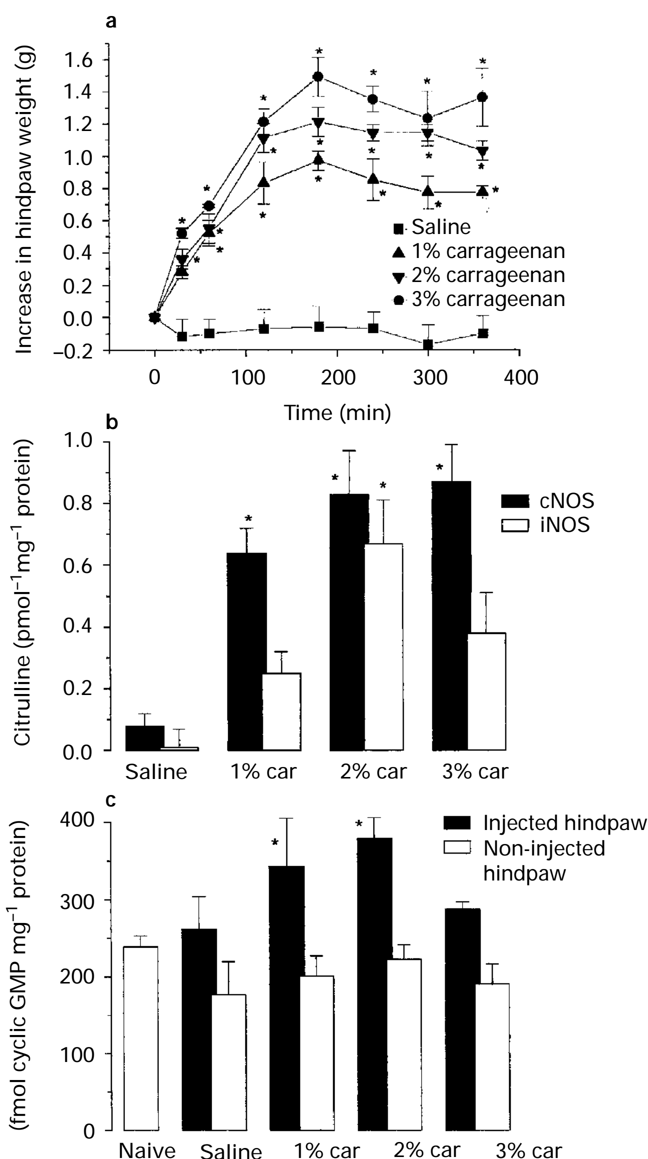


**Figure 1** Effect of intraplantar injection of different concentrations of carrageeenan (all 150 $\mu$l) on: (a) oedema formation determined as increase in hindpaw weight, results are mean with vertical lines showing s.e.mean, $n = 4$. *$P < 0.05$, ANOVA plus Bonferroni test, c.f. saline-injected control animals. (b) Hindpaw cNOS and iNOS activity determined *ex vivo* as the conversion of radiolabelled L-arginine to radiolabelled citrulline. Results show pmol citrulline mg$^{-1}$ protein 15 min$^{-1}$ and are mean±s.e.mean, $n = 4$, *$P < 0.05$, ANOVA plus Bonferroni test, c.f. saline-injected control animals. Carrageenan (car) concentrations are shown at the base of each set of columns. (c) Hindpaw cyclic GMP concentration in carrageenan-injected and contralateral (i.e. non-injected) hindpaws from the same animals. Results show fmol cyclic GMP mg$^{-1}$ protein and are mean± s.e.mean, $n = 6$. *$P < 0.05$ c.f. saline-injected control animals. Carrageenan (car) concentrations are shown at the base of each set of columns. Naive animals received no drug or vehicle injection.

(non-injected) hindpaw (e.g. 2% w/v, $0 \pm 0.03$ pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$ c.f. animal injected intraplantar with saline, $0.06 \pm 0.01$ pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$, $n=4$, $P>0.05$) or cyclic GMP concentration (e.g. 2% w/v; $201.2 \pm 26.2$ fmol $mg^{-1}$ protein c.f. $177.1 \pm 42.7$ fmol $mg^{-1}$ protein, $n=4$, $P>0.05$). No iNOS activity was detectable in homogenates prepared from the contralateral hindpaw.

In contrast, intraplantar injection of carrageenan (1–3% w/v) significantly increased hindpaw cNOS activity and resulted in the appearance of hindpaw iNOS activity (Figure 1b). Compared with animals injected intraplantar with saline, hindpaw cNOS activity was significantly increased at all doses of carrageenan employed whilst iNOS activity was also increased following injection of 2% w/v (but not 1% or 3% w/v) carrageenan. No significant difference in hindpaw iNOS activity was detected in animals injected with either 2% w/v or 3% w/v carrageenan, suggesting that the 2% w/v dose was maximally effective. Based upon these preliminary experiments, carrageenan (2% w/v) was chosen for subsequent experiments to investigate the effect of NOS inhibitors on carrageenan-induced hindpaw oedema.

### *The effect of L-NAME on carrageenan-induced hindpaw oedema, NOS activity and cyclic GMP concentration*

L-NAME (5–25 mg $kg^{-1}$, i.p.) administration caused a dose-related inhibition of carrageenan-induced hindpaw weight gain which, at the highest dose (25 mg $kg^{-1}$, i.p.) used in these experiments, was evident at all time points beyond 1 h (Figure 2a). The percentage inhibition of carrageenan-induced increase in hindpaw weight gain by L-NAME (5, 15 and 25 mg $kg^{-1}$, i.p.) determined 60 min (6 h in parentheses) post-carrageenan injection was $19.0 \pm 3.2\%$ ($13.5 \pm 6.3\%$), $22.2 \pm 6.9\%$ ($20.3 \pm 2.7\%$) and $32.9 \pm 11.1\%$ ($38.7 \pm 8.1\%$) ($n=6$).

Pretreatment of animals with L-NAME (5–25 mg $kg^{-1}$, i.p., 30 min before carrageenan injection) also reduced the carrageenan-induced increase in hindpaw iNOS and cNOS activity (Figure 2b) and cyclic GMP concentration (Figure 2c). At the highest dose of L-NAME used in this study (25 mg $kg^{-1}$, i.p.), hindpaw iNOS activity was completely abolished whilst hindpaw cNOS activity and cyclic GMP concentration were reduced by $86.3 \pm 3.0\%$ and $27.4 \pm 1.9\%$ ($n=6$), respectively.

### *The effect of 7-NI on carrageenan-induced hindpaw oedema, NOS activity and cyclic GMP concentration*

Like L-NAME, pretreatment of animals with 7-NI (5–25 mg $kg^{-1}$, i.p.) also produced dose-related inhibition of both the early (1 h) and late (6 h) phases of carrageenan-induced hindpaw weight gain (Figure 3a). On a weight for weight basis, 7-NI (15 and 25 mg $kg^{-1}$, i.p.) produced significantly ($P<0.05$) greater inhibition of the early phase hindpaw oedema response to carrageenan than L-NAME (e.g. 1 h post-carrageenan, 15 and 25 mg $kg^{-1}$; $35.6 \pm 4.4\%$ and $57.8 \pm 4.4\%$, $n=6$, c.f. $22.2 \pm 6.9\%$ and $32.9 \pm 11.1\%$, $n=6$, $P<0.05$). In contrast, no such difference in the response to 7-NI and L-NAME was apparent in the late phase response to carrageenan injection (e.g. 6 h post-carrageenan, 15 and 25 mg $kg^{-1}$; $22.1 \pm 4.2\%$ and $28.7 \pm 6.3\%$ c.f. $20.3 \pm 2.7\%$ and $38.7 \pm 8.1\%$, $n=6$). Interestingly, administration of 7-NI (5–25 mg $kg^{-1}$, i.p.), not before but 2.5 h after intraplantar injection of carrageenan (2% w/v), also resulted in a dose-related inhibition (e.g. 6 h; $11.6 \pm 6.8\%$, $24.3 \pm 2.9\%$ and $31.1 \pm 3.5\%$, $n=6$) of the late phase hindpaw weight gain.

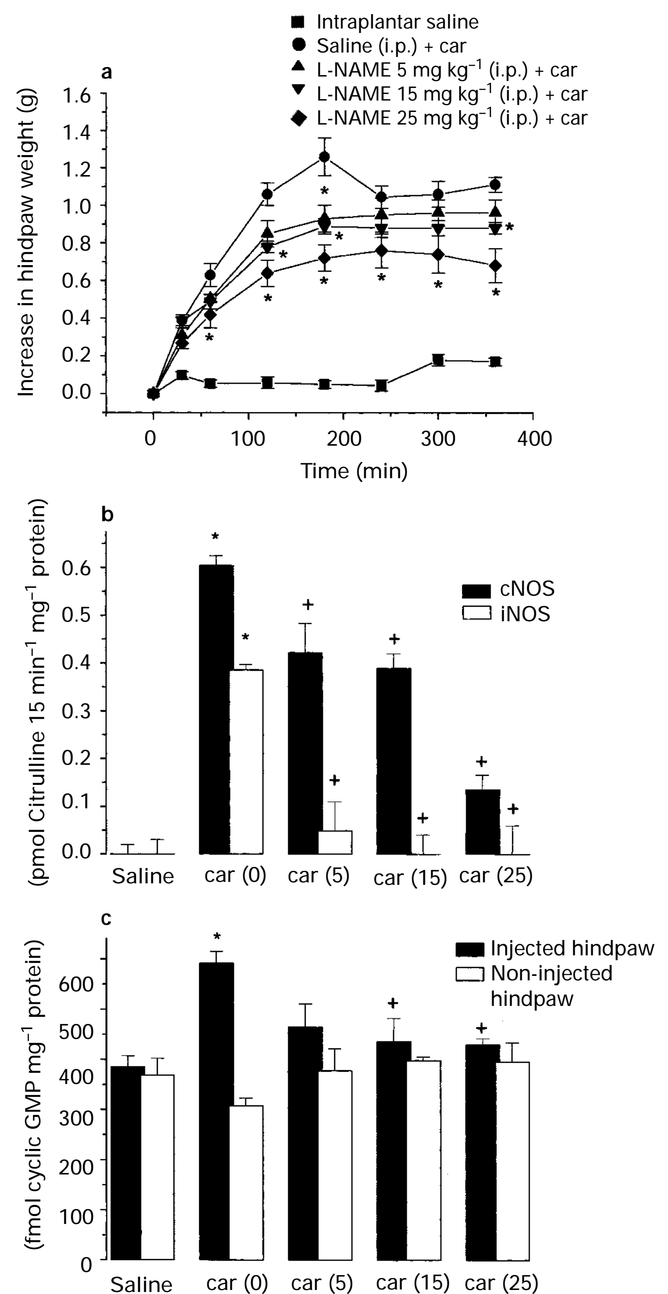


**Figure 2** Effect of L-NAME (5–25 mg $kg^{-1}$, i.p.) administered 30 min before intraplantar injection of carrageenan (car, 150 $\mu$l, 2% w/v) on: (a) oedema formation determined as increase in hindpaw weight, results are mean with vertical lines showing s.e.mean, $n=6$. $^{*}P<0.05$, ANOVA plus Bonferroni test, c.f. animals injected with saline (i.p.) plus carrageenan. (b) Hindpaw cNOS and iNOS activity determined *ex vivo* as the conversion of radiolabelled L-arginine to radiolabelled citrulline. Results show pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$ and are mean $\pm$ s.e.mean, $n=6$. $^{*}P<0.05$ c.f. saline-injected control animals; $^{+}P<0.05$ c.f. animals injected with saline (i.p.) plus carrageenan. (c) Hindpaw cyclic GMP concentration in carrageenan-injected and contralateral (i.e. non-injected) hindpaws from the same animals. Results show fmol cyclic GMP $mg^{-1}$ protein and are mean $\pm$ s.e.mean, $n=6$. $^{*}P<0.05$ (Student's $t$ test) c.f. saline-injected control animals; $^{+}P<0.05$ (ANOVA plus Bonferroni test) c.f. animals injected with saline (i.p.) plus carrageenan. In (b) and (c) doses of L-NAME administered are as indicated (in parentheses) at the base of each set of columns.

Administration of 7-NI (5–25 mg $kg^{-1}$, i.p.) reduced the carrageenan-induced increase in cNOS activity and at higher doses (15 and 25 mg $kg^{-1}$) abolished the carrageenan-induced increase in hindpaw iNOS activity (Figure 3b). Furthermore, a

significant reduction of hindpaw cyclic GMP concentration was observed in animals injected with 7-NI (25 mg $kg^{-1}$, i.p.) (Figure 3c).

### *The effect of L-NIL on carrageenan-induced hindpaw oedema, NOS activity and cyclic GMP concentration*

L-NIL (5–25 mg $kg^{-1}$, i.p.) injection resulted in a dose-related inhibition of late phase (but not early phase) carrageenan-induced hindpaw weight gain (Figure 4a). Pretreatment of animals with L-NIL (5–25 mg $kg^{-1}$, i.p., 30 min before carrageenan injection) also reduced the carrageenan-induced increase in both hindpaw iNOS and cNOS activity (Figure 4b) as well as the rise in hindpaw cyclic GMP concentration (Figure 4c). At the highest dose of L-NIL used in this study (25 mg $kg^{-1}$, i.p.), hindpaw iNOS activity was abolished whilst hindpaw cNOS activity and cyclic GMP concentration were reduced by 69.0±1.4% and 54.5±7.2% ($n$=6), respectively.

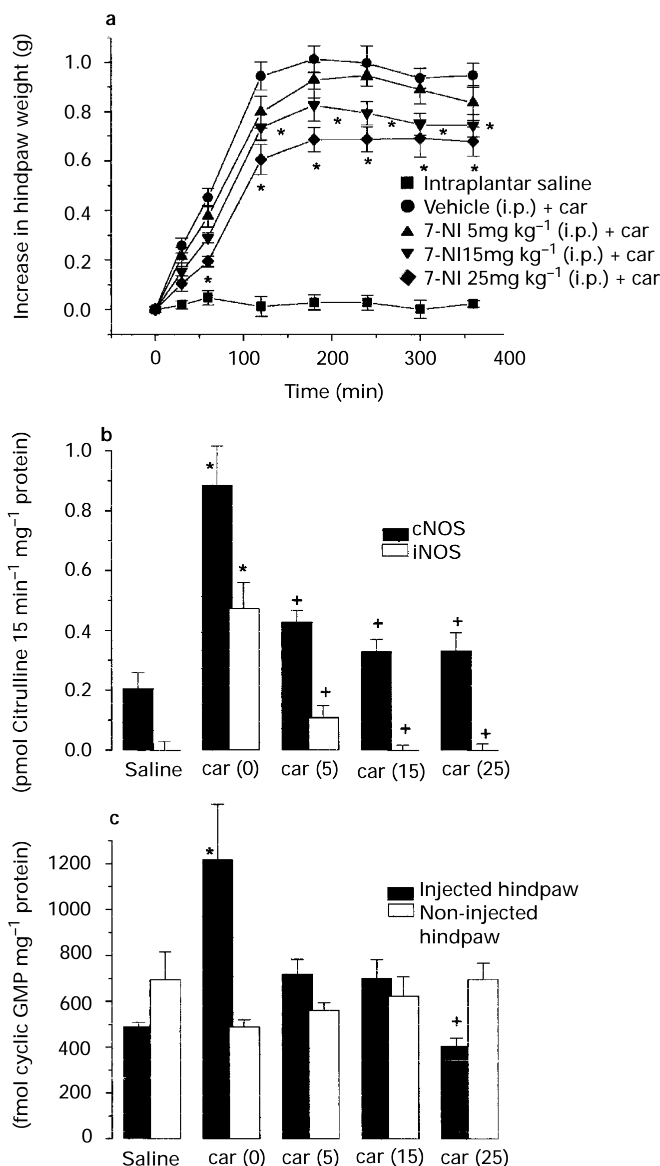


**Figure 3** Effect of 7-NI (5–25 mg $kg^{-1}$, i.p.) administered 30 min before intraplantar injection of carrageenan (car, 150 $\mu$l, 2% w/v) on: (a) oedema formation determined as increase in hindpaw weight (g), results are mean with vertical lines showing s.e.mean, $n$=6. *$P$<0.05, ANOVA plus Bonferroni test, c.f. animals injected with saline (i.p.) plus carrageenan. (b) Hindpaw cNOS and iNOS activity determined *ex vivo* as the conversion of radiolabelled L-arginine to radiolabelled citrulline. Results show pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$ and are mean±s.e.mean, $n$=6. *$P$<0.05 (Student's $t$ test) c.f. saline-injected control animals; $^{+}P$<0.05 (ANOVA plus Bonferroni test) c.f. animals injected with saline (i.p.) plus carrageenan. (c) Hindpaw cyclic GMP concentration in carrageenan-injected and contralateral (i.e. non-injected) hindpaws from the same animals. Results show fmol cyclic GMP $mg^{-1}$ protein and are mean±s.e.mean, n=6. *$P$<0.05 (Student's $t$ test) c.f. saline-injected control animals. $^{+}P$<0.05 (ANOVA plus Bonferroni test) c.f. animals injected with saline (i.p.) plus carrageenan. In (b) and (c) doses of 7-NI administered are as indicated (in parentheses) at the base of each set of columns.

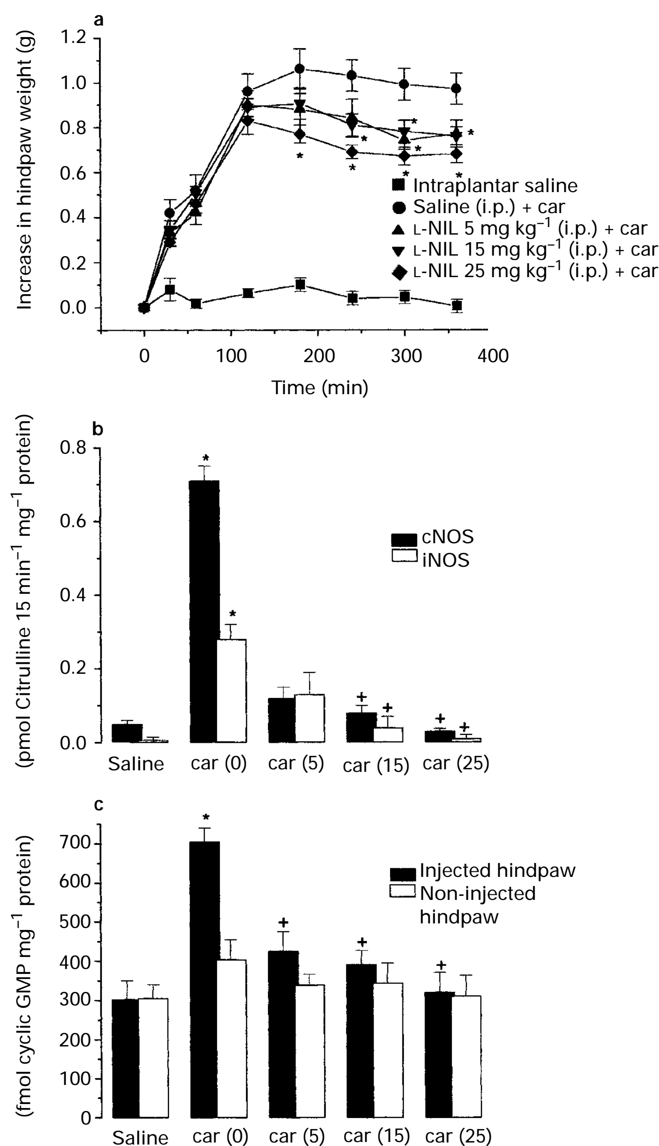


**Figure 4** Effect of L-NIL (5–25 mg $kg^{-1}$, i.p.) administered 30 min before intraplantar injection of carrageenan (car, 150 $\mu$l, 2% w/v) on: (a) oedema formation determined as increase in hindpaw weight (g), results are mean with vertical lines showing s.e.mean, $n$=6. *$P$<0.05, ANOVA plus Bonferroni test, c.f. animals injected with saline (i.p.) plus carrageenan. (b) Hindpaw cNOS and iNOS activity determined *ex vivo* as the conversion of radiolabelled L-arginine to radiolabelled citrulline. Results show pmol citrulline $mg^{-1}$ protein 15 $min^{-1}$ and are mean±s.e.mean, $n$=6. *$P$<0.05 (Student's $t$ test) c.f. saline-injected control animals; $^{+}P$<0.05 (ANOVA plus Bonferroni test) c.f. animals injected with saline (i.p.) plus carrageenan. (c) Hindpaw cyclic GMP concentration in carrageenan-injected and contralateral (i.e. non-injected) hindpaws from the same animals. Results show fmol cyclic GMP $mg^{-1}$ protein and are mean±s.e.mean, $n$=6. *$P$<0.05 (Student's $t$ test) c.f. saline-injected control animals; $^{+}P$<0.05 (ANOVA plus Bonferroni test) c.f. animals injected with saline (i.p.) plus carrageenan. In (b) and (c) doses of L-NIL administered are as indicated (in parentheses) at the base of each set of columns.

## Discussion

### *NO, iNOS and carrageenan-induced hindpaw inflammation*

Intraplantar injection of carrageenan in the rat resulted in an increase in hindpaw weight (indicative of oedema) comprising a relatively rapid early phase (up to 2 h) followed by a more sustained late phase (2 h to 6 h). Previous research has suggested that the early phase inflammation is triggered by the concerted release of histamine, bradykinin and 5-hydroxytryptamine at the inflamed site, whilst the late phase response is due primarily to the formation of pro-inflammatory prostanoids (DiRosa *et al*., 1971).

Recently, a potentially important role for NO generated by the iNOS isoform in this model has been suggested based principally on the detection of iNOS in hindpaw homogenates and increased nitrate/nitrate concentration in inflammatory exudate of hindpaws following carrageenan injection (Salvemini *et al*., 1996). A more general role for iNOS in inflammatory mechanisms has also been suggested based on the demonstration of the presence of this isoform at other sites of inflammation (e.g. mouse air pouch, Vane *et al*., 1994; lipopolysaccharide-evoked uveitis in the rat, Parks *et al*., 1994; carrageenan-induced pleurisy in the rat, Tomlinson *et al*., 1994).

The present study also supports a role for iNOS in carrageenan-induced hindpaw inflammation in the rat. Thus, intraplantar carrageenan administration results within 6 h in the appearance of iNOS activity in the injected hindpaw. The precise cellular location of iNOS in the carrageenan-injected hindpaw has not been addressed in the present study. It seems likely that iNOS generation occurs in macrophages present at the inflammatory site, although other cell types including bone osteoblasts, in which cytokine-induced iNOS formation has been noted (Hukkanen *et al*., 1995), cannot be excluded. Furthermore, administration of the selective iNOS inhibitor, L-NIL, before carrageenan injection failed to influence early phase hindpaw inflammation, but significantly reduced the late phase response and also decreased hindpaw iNOS activity and cyclic GMP concentration. Perhaps surprisingly, L-NIL pretreatment also reduced hindpaw cNOS activity in these experiments. Whilst L-NIL has been shown to exhibit selectivity for iNOS *in vitro* (Moore *et al*., 1994), it is conceivable that significant inhibition of hindpaw cNOS may still be achieved following parenteral administration *in vivo*. Alternatively, it may be argued that L-NIL administration reduces hindpaw NOS activity in these experiments indirectly by alleviating tissue inflammation and thereby reducing the induction/upregulation of iNOS and cNOS. The present data do not allow us to distinguish between these two possibilities. Further experiments to monitor iNOS/cNOS mRNA levels in carrageenan-injected hindpaw inflammation are required. Overall, these results strongly support the possibility that NO derived from iNOS is not involved in the early phase of carrageenan-induced inflammation but does participate in the late phase response.

### *NO, cNOS and carrageenan-induced inflammation*

Whilst considerable evidence suggests a role for iNOS in hindpaw oedema due to carrageenan injection as well as in alternative animal models of inflammation, the involvement of other isoforms of NOS (particularly nNOS) in the inflammatory process has received relatively little attention. Pretreatment of animals with L-NAME resulted in inhibition of both the early and late phase oedema response to carrageenan. Furthermore, hindpaw cNOS and iNOS activity and hindpaw cyclic GMP concentration were reduced in L-NAME pretreated animals 6 h after carrageenan injection, although whether these biochemical changes reflect the direct inhibitory effect of L-NAME on cNOS/iNOS or prevention of the induction of these isoforms due to an anti-inflammatory effect is not clear. Overall, these data, (i) confirm the anti-inflammatory effect of L-NAME noted previously in this and other experimental models (e.g. Ialenti *et al*., 1992; McCartney-Francis *et al*., 1995; Tracey *et al*., 1995; Salvemini *et al*., 1996), (ii) suggest that anti-inflammatory activity is related to inhibiton of hindpaw NOS activity and (iii) imply an important role for NO in both the early and late phases of the hindpaw carrageenan response as suggested previously (Salvemini *et al*., 1996).

Like L-NAME, administration of 7-NI also inhibited both early and late phase hindpaw oedema formation in the present study. Indeed, 7-NI proved to be a more effective inhibitor of the early (but not late) phase hindpaw oedema response to carrageenan that was L-NAME. We have not evaluated the effect of 7-NI on hindpaw blood flow in these experiments. However, previous research has shown that 7-NI, (unlike L-NAME), does not inhibit eNOS in vascular endothelial cells *in vivo* (evidenced by the failure of 7-NI to increase MAP in rat, rabbit or mouse, Babbedge *et al*., 1993; Moore *et al*., 1993) and does not affect skin blood flow in the anaesthetized rat (Kajekar *et al*., 1995). Thus, although we cannot exclude an effect of 7-NI on hindpaw eNOS it seems probable that inhibition of the early phase of carrageenan-induced hindpaw oedema reflects an effect on this compound on nNOS rather than on vascular endothelial cell eNOS. The precise cellular location of the nNOS isoform involved is not known although an involvement of sensory nerves seems likely. Indeed, 7-NI has previously been shown to reduce skin oedema in the rat following saphenous nerve stimulation (i.e. 'neurogenic' inflammation) by a mechanism believed to involve inhibition of neurotransmitter (e.g. substance P, neurokinin A, calcitonin-gene related peptide) release from sensory nerve endings (Kajekar *et al*., 1995). Thus, a similar mechanism may account for the anti-oedema effect of 7-NI in the present experiments. Certainly, the finding that CP-96,345, a selective $NK_1$ receptor antagonist, reduces carrageenan-induced hindpaw oedema in the rat suggests a neurogenic component to this model of inflammation (Birch *et al*., 1992).

### *Carrageenan-induced hindpaw inflammation and induction of cNOS*

Pharmacological assay of the effect of NOS inhibitors on carrageenan-induced hindpaw oedema in the rat strongly suggests the involvement of cNOS isoforms(s) in this event. This conclusion is supported by biochemical measurements of hindpaw cNOS activity. Intraplantar injection of carrageenan has previously been shown to trigger iNOS induction in the rat hindpaw measured 6 h but not 3 h after injection (Salvemini *et al*., 1996). In preliminary experiments, we also observed no change in hindpaw cNOS activity ($0.10 \pm 0.01$ pmol citrulline formed 15 $min^{-1}$ $mg^{-1}$ protein c.f. $0.10 \pm 0.02$ pmol citrulline formed 15 $min^{-1}$ $mg^{-1}$ protein in the non-injected hindpaw, $n=6$, $P>0.05$) and no detectable iNOS activity 3 h after intraplantar carrageenan injection in the rat (Handy & Moore, unpublished). For this reason, biochemical analysis of hindpaw NOS activity in this study was undertaken 6 h after carrageenan administration.

Intraplantar injection of carrageenan significantly increased hindpaw cNOS coupled with the appearance of iNOS activity. Although iNOS and/or cNOS mRNA or protein levels were not assessed in these experiments it seems likely that increased hindpaw iNOS and cNOS enzyme activity reflects *de novo* synthesis (i.e. induction) of both iNOS and cNOS. Numerous examples of the 'induction' or 'upregulation' of cNOS isoforms have appeared in the literature. For example, peripheral axotomy in the rat markedly increased nNOS expression in dorsal root ganglia, whilst salt loading had a similar effect on this isoform in the hypothalamus (Hokfelt *et al.*, 1994). Similarly, eNOS expression is increased in various tissues following treatment with oestradiol (Weiner *et al.*, 1994) or in pancreatic islet capillary endothelial cells exposed to high glucose concentration (Suschek *et al.*, 1994). However, perhaps of more relevance here, are the findings of increased cNOS activity in lung samples from patients with inflammatory lung disease (Belvisi *et al.*, 1995) and in colon from patients with inflammatory bowel disease (Ruan *et al.*, 1995). To the best of our knowledge there have been no previous studies on the induction of cNOS isoform(s) in an animal model of inflammation.

### *7-NI – a dual inhibitor of nNOS and iNOS* in vivo *?*

Whilst inhibition of nNOS most probably accounts for the ability of 7-NI to inhibit the early phase of carrageenan-induced hindpaw oedema, the mechanism(s) underlying the effect of this compound on the late phase response are less clear. Since administration of 7-NI at the completion of the early phase response (i.e. 2.5 h after intraplantar carrageenan injection) also inhibited the late phase oedema response it is tempting to suggest that inhibition of iNOS accounts for the anti-inflammatory effect of 7-NI in the late phase. This conclusion is consistent with *in vitro* experiments in which 7-NI, in addition to inhibition of nNOS (e.g. $IC_{50}$, 0.7 $\mu$M, rat cerebellum, Moore *et al.*, 1993), has also been described as a relatively potent inhibitor of iNOS enzyme activity (e.g. $IC_{50}$, 5.8 $\mu$M, rat lung iNOS; Bland-Ward & Moore, 1995). Furthermore, in the present experiments, 7-NI administered to carrageenan-injected animals greatly reduced and indeed at higher doses abolished iNOS enzyme activity as determined *ex vivo* in hindpaws removed 6 h after carrageenan injection. Whatever the precise mechanism of action of 7-NI, the present results are, to the best of our knowledge, the first to demonstrate inhibition of iNOS following administration of 7-NI in the intact animal. In this light, it would clearly be of interest to determine whether 7-NI administration also inhibits iNOS activity associated with, for example, septic (endotoxic) shock in experimental animals.

In conclusion, results obtained in the present study suggest that (i) NO synthesized by an nNOS isoform (most probably located in sensory nerves) plays an important part in the early phase response to carrageenan in this model of inflammation, (ii) NO synthesized by an iNOS isoform (perhaps located in inflammatory leukocytes) contributes to the late phase response and (iii) compounds with the ability to inhibit both nNOS and iNOS reduce both phases of the carrageenan response and thus may prove viable targets for the development of novel anti-inflammatory compounds.

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