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Reduction of carrageenin oedema and the associated c-Fos expression in the rat lumbar spinal cord by nitric oxide synthase inhibitor

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1 Three hours after intraplantar carrageenin (6 mg/150 μl of saline) Fos-like immunoreactivity (Fos-LI) was mainly observed in L4 and L5 segments of the dorsal horn. Both superficial (I–II) and deep laminae (V–VI) neurones were labelled.

2 We have studied the effect of systemic administration of a nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) on carrageenin evoked c-Fos expression and thus the contribution of nitric oxide to this expression.

3 Pre-administration of L-NAME (10, 25, 50, 100 mg kg^{-1}, i.v.) dose-dependently reduced the number of superficial and deep laminae Fos-LI neurones, 100 mg kg^{-1} produced a 63 ± 2% and 72 ± 4% reduction of Fos-LI neurones respectively, $P<0.0001$ for both superficial and deep neurones.

4 Pre-administered L-NAME dose-relatedly reduced the carrageenin-evoked paw and ankle oedema, with 100 mg kg^{-1} of L-NAME resulting in a 74 ± 2% and 103 ± 2% reduction respectively.

5 Post-administration of L-NAME (10 mg kg^{-1}, i.v.) reduced the number of superficial and deep laminae Fos-LI neurones (65 ± 7% and 53 ± 8% reduction respectively, $P<0.01$ for both superficial and deep neurones).

6 Post-administered L-NAME reduced both the paw and ankle oedema (52 ± 8% and 62 ± 10% reduction respectively, $P<0.0001$ for both paw and ankle).

7 Pre-administered D-NAME (100 mg kg^{-1}, i.v.), the inactive isomer of L-NAME, produced a weak reduction of the number of superficial laminae Fos-LI neurones (26 ± 8% reduction, $P<0.05$), without influencing the deep Fos-LI neurones (5 ± 8% enhancement) or the oedema.

8 Systemic L-arginine (1200 mg kg^{-1}) did not reverse the reduction of the total number of Fos-LI neurones induced by 100 mg kg^{-1} of L-NAME, or the effect of L-NAME on the paw and ankle oedema.

9 Intraplantar L-arginine (30 mg) did not reverse the effect of L-NAME (100 mg kg^{-1}) on the total number of Fos-LI neurones. However, the inhibitory effects of L-NAME on the paw and ankle oedema were partially reversed by intraplantar L-Arginine (34 ± 9% and 45 ± 11% reduction of carrageenin oedema respectively) with these effects being significant as compared to the effect of L-NAME alone ($P<0.05$ for both).

10 There is a strong correlation between the reduction of the number of Fos-LI neurones and the oedema by L-NAME, clearly demonstrating a predominant role of peripheral NO in the development of one of the signs of carrageenin inflammation.

Keywords: Carrageenin; c-Fos; nitric oxide synthase inhibitor; spinal cord of rat

Introduction

A recent study, using the purified polyclonal antibody against rat cerebellar nitric oxide synthase, has demonstrated nitric oxide synthase (NOS) activity in laminae I–IV and X of the rat spinal cord, with the majority of NOS positive neurones also staining for NADPH diaphorase (Saito *et al.*, 1994), thus confirming previous studies of spinal NADPH diaphorase (Zhang *et al.*, 1993; Herdegen *et al.*, 1994; see references in Traub *et al.*, 1994). NADPH diaphorase immunoreactive neurones have also been demonstrated in the sensory ganglion of the rat spinal cord (Aimi *et al.*, 1991; Zhang *et al.*, 1993). The mechanisms of the production and roles of nitric oxide (NO) during nociceptive transmission have recently been reviewed (see references in Meller & Gebhart 1993). Previous behavioural and electrophysiological studies have indirectly evaluated the role of NO during nociception using NOS inhibitors, such as N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME); in general NOS inhibitors did not greatly influence noxious electrically evoked spinal neuronal responses (Haley *et al.*, 1992) and noxious thermal behavioural responses (Malmberg & Yaksh 1993). However, systemic (Moore *et al.*, 1993) and intrathecal (Haley *et al.*, 1992; Malmberg & Yaksh 1993; Yamamoto *et al.*, 1993) NOS inhibitors have been shown to reduce noxious responses to formalin. In addition, carrageenin induced hyperalgesia has been shown to be reduced by intrathecal L-NAME (Meller *et al.*, 1994). Collectively, these studies provide evidence for a spinal contribution of NO during nociceptive transmission of more prolonged pain states. Furthermore during hyperalgesia, interactions between NO and N-methyl-D-aspartate (NMDA) receptor-mediated events have been demonstrated (see references in Meller & Gebhart, 1993; Malmberg & Yaksh, 1993; Radhakrishnan & Henry, 1993).

In addition to the spinal role of NO, there is evidence that endogenous NO may have both pro- and anti-inflammatory effects depending on the experimental conditions (see references in Nathan, 1992). Peripheral proinflammatory roles of nitric oxide has been shown in models of inflammation (Lippe *et al.*, 1993; Vane *et al.*, 1994) including carrageenin

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inflammation (Ialenti et al., 1992). In contrast, L-NAME has been shown not to modulate formalin-induced oedema in mice (Moore et al., 1991). Although the mechanisms of the NO contribution to inflammation are not fully elucidated, NOS inhibitors have been shown to reduce bradykinin (Hughes et al., 1990; Teixeira et al., 1993), phospholipase A_2 and substance P (Hughes et al., 1990)-evoked oedema formation. In addition, inhibition of NOS has been shown to reduce bradykinin-evoked inflammation, both in terms of vasodilatation and plasma extravasation (Khalil & Helme, 1992). Since NO has been shown to have both a peripheral and spinal role during nociceptive activation and/or transmission, with NOS inhibitors being antinociceptive, it has been proposed that NOS inhibitors may be putative analgesics.

The aim of our study was to evaluate simultaneously the effects of the well established NOS inhibitor, L-NAME, on the peripheral signs of inflammation induced by carrageenin and the subsequent spinal expression of c-Fos (Draisci & Iadarola 1989; Noguchi et al., 1991; 1992; Honoré et al., 1994a). Intraplantar carrageenin (Winter et al., 1962) produces a classical inflammation (see references in Kocher et al., 1987) and a heat and mechanical hyperalgesia (Joris et al., 1990; see references in Dubner & Ruda, 1992; Ferreira et al., 1993). Intrathecal L-NAME has been shown to reduce the development and maintenance of thermal hyperalgesia produced by carrageenin (Meller et al., 1994). Furthermore, a recent study has suggested a functional relationship between the spinal expression of immediate early genes and nitric oxide (Herdegen et al., 1994).

In this series of experiments we have assessed the role of NO during nociceptive transmission by studying the effects of the NOS inhibitor, L-NAME, on carrageenin inflammation and the expression of spinal c-Fos. In addition the effect of the inactive isomer of L-NAME, N^G-nitro-D-arginine methyl ester hydrochloride (D-NAME) alone, and administration of systemic and intraplantar L-arginine, before systemic L-NAME, on carrageenin inflammation and the expression of c-Fos were studied. Finally, the effect of systemic L-NAME, administered after the injection of carrageenin, on carrageenin inflammation and the expression of c-Fos was also studied.

(A preliminary account of this work has been published in Buritova et al., 1994).

Methods

Experimental animals

Experiments were performed on 74 adult male albino Sprague-Dawley rats (Charles River, France), weighing 225–250 g. Two groups of experiments were performed with statistical comparisons made within each group of experiments but not between groups of experiments.

In the first series of experiments, the effects of varying doses of L-NAME (injectable solution, Sigma, in saline) on c-Fos expression 3 h after carrageenin was studied. L-NAME (10, 25, 50 or 100 mg kg^{-1}, $n = 8$ for each group) was injected intravenously 30 min prior to carrageenin administration (6 mg 150μl^{-1}, in saline). A control group of rats received an equal volume of intravenous saline ($n = 8$). Rats were perfused 3 h post carrageenin injection since we have previously shown c-Fos labelling to be maximal, with an equal distribution in the superficial and deep dorsal horn neurones at this time point (Honoré et al., 1994a).

In the second series of experiments, under the same experimental conditions, the effect of intravenous D-NAME (injectable solution, Sigma in saline) on c-Fos expression 3 h after carrageenin was studied. D-NAME (100 mg kg^{-1}, $n = 5$) was injected 30 min before carrageenin injection (6 mg 150μl^{-1}, in saline). The effects of a pre-administered versus post-administered L-NAME, injected 30 min before ($n = 5$) or 30 min after ($n = 5$) carrageenin, on carrageenin evoked c-Fos expression was studied. Finally the effects of systemic L-arginine (injectable solution, in saline; Sigma, 1200 mg kg^{-1}-i.v., $n = 5$) and intraplantar L-arginine (30 mg/intraplantar, $n = 5$), administered 10 min before L-NAME (100 mg/kg^{-1} i.v.), which was administered 30 min before carrageenin, on c-Fos expression 3 h after carrageenin was studied. A control group of rats received an equal volume of intravenous saline ($n = 5$).

Immunohistochemistry

Three hours after the carrageenin injection, the animals were deeply anaesthetized with pentobarbitone (55 mg kg^{-1}, i.p.; Sanofi) and perfused intracardially with 200 ml of phosphate buffered saline 0.1 M (PBS) folowed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord was then removed and post-fixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Frontal frozen section 40 μm thick were cut and collected in PB to be processed immunohistochemically as free floating sections. The serial sections from the lumbar segment were immunostained for Fos-like protein according to the avidin-biotin-peroxidase method (Hsu et al., 1981). The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton-X (NGST). The sections were then incubated overnight at 4°C in the primary antiserum directed against the c-Fos protein (Oncogene Science Inc.). The Fos antibody, a rabbit polyclonal antibody directed against residues 4–17 of the N-terminal region of the peptide, was used at 1:4,000. The incubated sections were washed 3 times in 1% NGST and incubated in biotinylated rabbit anti-sheep IgG for 1 h at room temperature, washed twice in 1% NGST and incubated for 1 h in Avidin-Biotin-Peroxidase complex (Vectastain, Vector Laboratories). Finally, the sections were washed 3 times in PBS and developed in 1-naphtol ammonium carbonate solution (89.5 ml 0.1 M PB, 10 ml ammonium carbonate (1% in distilled water), 0.5 ml 1-naphtol (N-199-2 Aldrich, 10% in absolute alcohol) and 0.1 ml hydrogen peroxide) for 5 min, and were then washed 3 times in PB to stop the staining reaction. The sections were mounted on gelatine-subbed slides and air dried for the stain to be intensified and made alcohol-resistant through basic dye enhancement in 0.025% crystal violet (42555 Aldrich) in PB for 3 min. After 2 short PB rinses to take off the excess stain, sections were differentiated in 70% alcohol and the differentiation time was evaluated under the microscope. After being air dried, the slides were coverslipped. To test the specificity of the primary antibody, controls were performed; preabsorption with the corresponding synthetic peptide or omission of any stage in the protocol abolished the staining.

Counting of Fos labelled cells

Tissue sections were first examined by darkfield microscopy to determine the segmental level according to Molander et al. (1984), as well as the gray matter landmarks. The sections were then examined under lightfield microscopy at × 10 to localize Fos-LI neurones. Labelled nuclei were counted using a camera lucida attachment. To study the laminar distribution 4 regions were defined: superficial dorsal horn (laminae I–II; superficial), nucleus proprius (laminae III–IV; nucleus proprius), neck of the dorsal horn (laminae V–VI; neck) and the ventral gray (laminae VII–X; ventral).

We have previously shown that the most numerous Fos positive neurones were localized in the L4–L5 segments, two counts per slice were made: (1) the total number of Fos-LI neurones in the gray matter for 10 sections through L4–L5 segments, and (2) the number of Fos-LI neurones per specific laminar region of the spinal gray matter in these 10 sections.

Inflammatory observations

In order to asses the development of the inflammation, we considered one of the signs of inflammation, the peripheral oedema, at the time the animals were killed. The diameters of the ankle and of the paw, both ipsilateral and contralateral to the stimulation were measured. Both the study of Fos-LI and the assessment of inflammatory parameters were performed in the same rats.

Statistical tests

Statistical analysis with analysis of variance (ANOVA) was made to compare the total number of labelled cells, using 1-way ANOVA for the different groups of animals, and 2-way ANOVA for the different groups of animals and the laminar region. For comparisons of the ankle or the paw diameters we used 1-way ANOVA for the different groups of animals. The Fishers' PLSD test was used for multiple comparisons and simple regressions were performed to establish dose-dependent effects. The investigator responsible for plotting and counting the Fos-LI neurones was 'blind' to the experimental situation of each animal.

Results

Expression of spinal Fos-LI by intraplantar carrageenin

Fos-LI neurones were virtually absent (less than 5 neurones per section) in the lumbar spinal gray matter of non-stimulated freely moving rats. In contrast, in carrageenin-stimulated animals, Fos-LI neurones appeared as round structures, stained to a variable degree. To quantify the number of Fos-LI neurones, we took into account all labelled neurones without considering the intensity of the staining. In agreement with our previous study on carrageenin evoked c-Fos expression (Honoré *et al.*, 1994a), Fos-LI neurones were observed in lumbar segments L2–L6, with maximal labelling observed in segments L4 and L5. In the two series of experiments, the total number of Fos-LI neurones after carrageenin were 136 ± 2 and 109 ± 7 per section, respectively. For both series of experiments the Fos-LI neurones were equally distributed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn (first series: $37 \pm 1\%$ and $39 \pm 2\%$ respectively, second series: $45 \pm 3\%$ and $38 \pm 3\%$ respectively), with a small amount of labelling in the ventral horn.

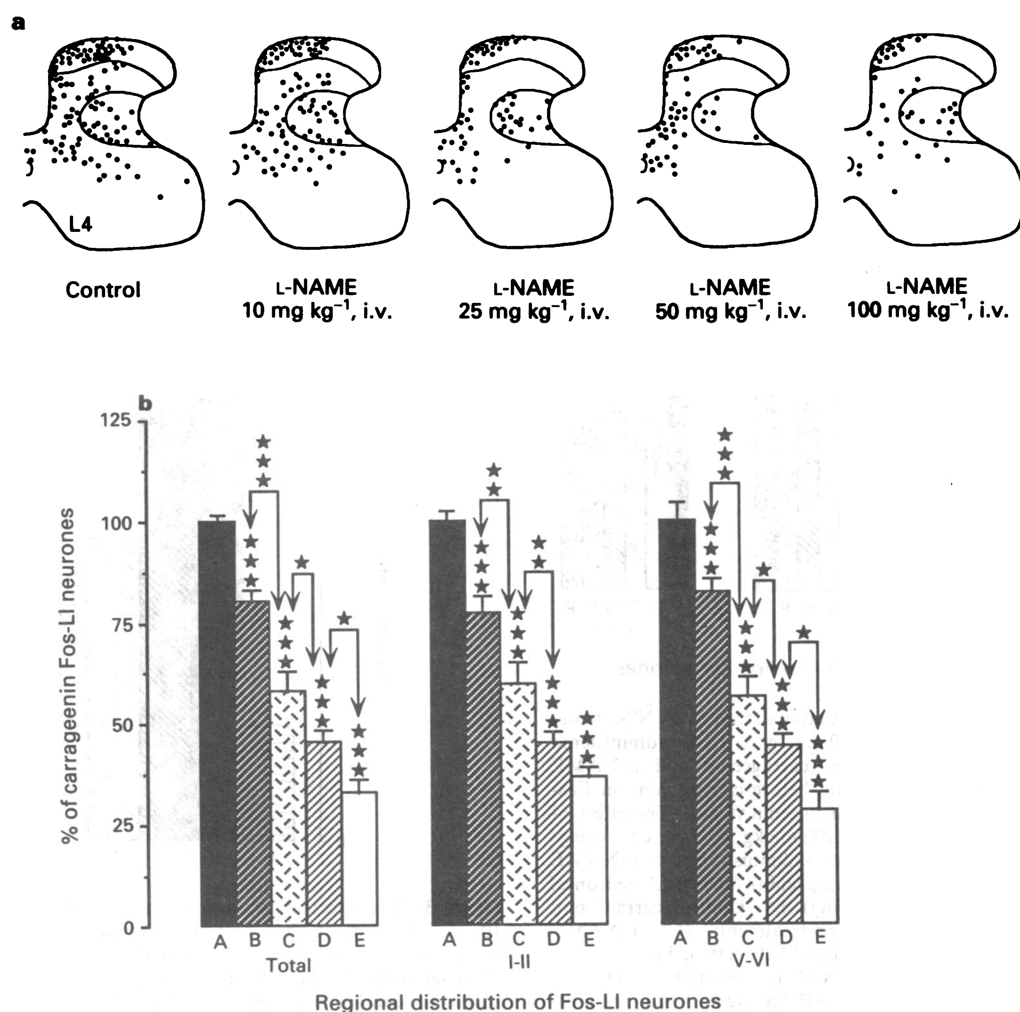

Figure 1 Camera lucida drawings and histograms showing the effect of systemic N^G-nitro-L-arginine methyl ester (L-NAME, 10, 25, 50 or 100 mg kg^{-1}, i.v.) on Fos-like immunoreactivity (Fos-LI neurones), 3 h after intraplantar carrageenin (6 mg). L-NAME was administered 30 min prior to carrageenin. The control group received systemic saline (a) Each diagram includes all labelled neurones in one 40μm section; each dot represents one labelled neurone. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. (b) Systemic pre-administration of L-NAME dose-dependently decreased the number of Fos-LI neurones after carrageenin, in both the superficial and deep laminae. (A) Control carrageenin; (B) L-NAME 10 mg kg^{-1}, i.v.; (C) L-NAME 25 mg kg^{-1}, i.v.; (D) L-NAME 50 mg kg^{-1} i.v.; (E) L-NAME 100 mg kg^{-1} i.v. Results are expressed as percentages of the mean number Fos-LI neurones after carrageenin alone ± s.e.mean in all laminae of the L4–L5 segments (Total), in superficial laminae and in the neck of the dorsal horn in the L4–L5 segments. Significance as compared to carrageenin alone was performed by ANOVA, PLSD Fisher's test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

L-NAME reduces the number of spinal Fos-LI neurones

In the first series of experiments, pre-administered systemic L-NAME (10, 25, 50 and 100 mg/kg) significantly reduced the total number of Fos-LI neurones as compared to the total number of Fos-LI neurones after carrageenin alone (20 ± 3%, $P<0.001$, 42 ± 5%, $P<0.0001$, 55 ± 3%, $P<0.0001$ and 77 ± 3% reduction, $P<0.0001$, respectively, Figure 1). This effect of L-NAME was dose-dependent ($r^2 = 0.643$, $P<0.0001$).

Both the number of superficial (laminae I–II) and deep (laminae V–VI) Fos-LI neurones were reduced to a similar extent by L-NAME (Figure 1), with the effect of L-NAME on both populations of Fos-LI neurones being dose-dependent (superficial laminae, $r^2 = 0.575$, $P<0.0001$ and deep laminae, $r^2 = 0.654$, $P<0.0001$). There was no significant difference between the effect of L-NAME on the superifical and deep Fos-LI neurones.

The effect of L-arginine and L-NAME on the number of spinal Fos-LI neurones

In the second series of experiments, there was a tendency towards a reversal of the effect of 100 mg kg^{-1} of L-NAME on the total number of FLI-neurones by an earlier pre-administration of systemic L-arginine (38 ± 8% reduction, Figure 2), however this effect was not significantly different from the effect of L-NAME alone (57 ± 8% reduction). In addition, there was a tendency towards a reversal of the effect of 100 mg kg^{-1} of L-NAME on the superficial and deep Fos-LI neurones by systemic L-arginine; again this effect was not significantly different from the effect of the highest dose of L-NAME alone (Figure 2).

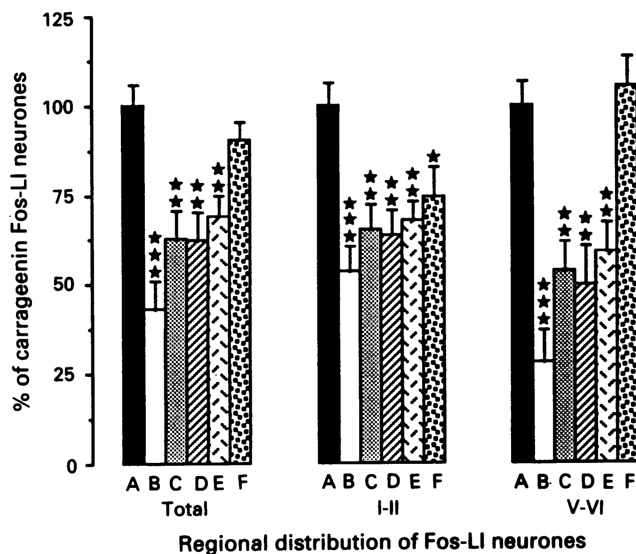

Figure 2 The effects of pre- and post-administration of N^G-nitro-L-arginine methylester (L-NAME, 100 mg kg^{-1}, i.v.), administered either 30 min before or 30 min after carrageenin and D-NAME (100 mg kg^{-1}, i.v.), administered 30 min before carrageenin, on Fos-LI neurones, 3 h after intraplantar carrageenin (6 mg). The effect of pre-administration of L-arginine (1200 mg kg^{-1}, i.v. or 30 mg/intraplantar) given 10 min before the pre-administration of L-NAME (100 mg kg^{-1}, i.v., 30 min prior to carrageenin) on Fos-LI neurones, 3 h after intraplantar carrageenin (6 mg). (A) Control carrageenin; (B) L-NAME 100 mg kg^{-1}, i.v. pretreatment; (C) L-NAME 100 mg kg^{-1}, i.v. post-treatment; (D) L-NAME 100 mg kg^{-1} i.v. + L-arginine 1200 mg kg^{-1}, i.v.; (E) L-NAME 100 mg kg^{-1} i.v. + L-arginine 30 mg intraplantar; (F) D-NAME 100 mg kg^{-1}, i.v. pretreatment. Pre- and post-administered L-NAME reduced the number of Fos-LI neurones to a similar extent. L-Arginine did not reverse the effects of L-NAME on the Fos-LI neurones. D-NAME did not significantly influence the total number of Fos-LI neurones or the number of deep Fos-LI neurones, but weakly reduced the superficial Fos-LI neurones. Results are expressed as percentages of the mean number Fos-LI neurones after carrageenin alone ± s.e.mean in all laminae of the L4–L5 segments (Total), in superficial laminae and in the neck of the dorsal horn in the L4–L5 segments. Significance as compared to carrageenin alone was performed by ANOVA, PLSD Fisher's test *$P<0.05$, **$P<0.01$, ***$P<0.001$).

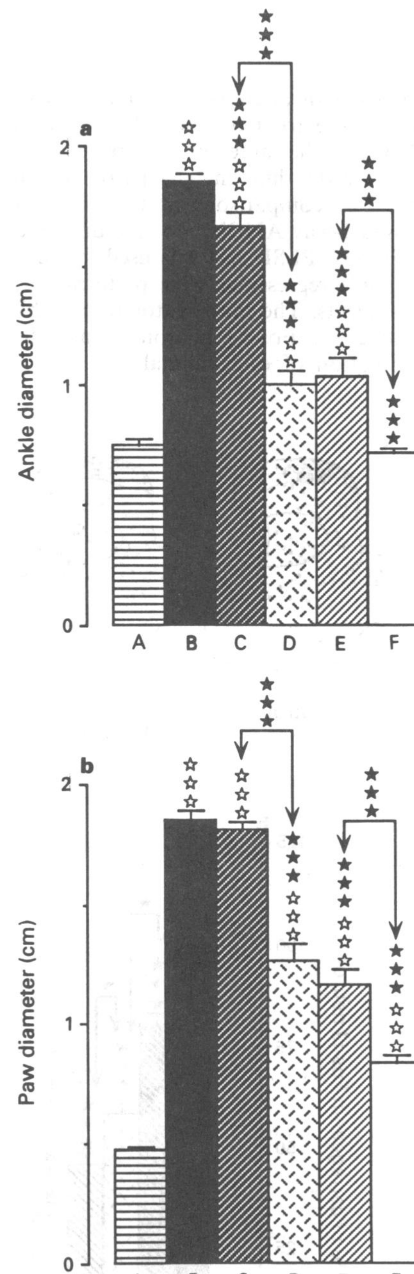

Figure 3 The effect of N^G-nitro-L-arginine methyl ester (L-NAME, 10, 25, 50 or 100 mg kg^{-1}, i.v.) on the ankle (a) and paw (b) oedema, 3 h after intraplantar carrageenin (6 mg). Both the paw and ankle diameter were significantly increased 3 h after carrageenin as compared to control non-stimulated rats. Pre-administered L-NAME dose-relatedly reduced the carrageenin induced paw and ankle oedema. With the highest dose of L-NAME the diameter of the ankle was not different from non-stimulated rats. (A) Non stimulated rat; (B) control carrageenin; (C) L-NAME 10 mg kg^{-1}, i.v.; (D) L-NAME 25 mg kg^{-1}, i.v.; (E) L-NAME 50 mg kg^{-1}, i.v.; (F) L-NAME 100 mg kg^{-1}, i.v. Results are presented as absolute values of paw and ankle diameters ± s.e.mean (centimetres). Significance compared to carrageenin alone and compared to the non-stimulated rats was performed by ANOVA, PLSD Fisher's test, solid and open stars respectively (**$P<0.01$, ***$P<0.001$). Significant differences between different doses of L-NAME are indicated by arrows.

With a prior administration of intraplantar L-arginine (30 mg 50μl^{-1}), there was a tendency towards a reversal of the effect of 100 mg kg^{-1} of L-NAME on the total number of Fos-LI neurones (31 ± 5% reduction, Figure 2). However, the effect of intraplantar L-arginine plus L-NAME was not significantly different from the effect of L-NAME alone (57 ± 8% reduction). Again, similar effects of intraplantar L-arginine plus L-NAME on the superficial and deep Fos-LI neurones were observed, with these effects not being significantly different from those observed with L-NAME alone (Figure 2).

Post-administered L-NAME reduces the number of spinal Fos-LI neurones

In the second series of experiments, post administered L-NAME (100 mg kg^{-1}, i.v.) reduced the total number of Fos-LI neurones (37 ± 7% reduction, $P<0.05$ as compared to carrageenin alone, Figure 2). In addition, the number of superficial and deep Fos-LI neurones were significantly reduced (35 ± 7% and 46 ± 8% reduction respectively, $P<0.05$ for both, as compared to carrageenin alone). Both pre- and post-administered L-NAME reduced the total number of Fos-LI neurones, in addition to the superficial and deep Fos-LI neurones, to a similar extent.

The effect of D-NAME on the number of spinal Fos-LI neurones

Systemic D-NAME (100 mg kg^{-1}) produced no significant effect on the total number of Fos-LI neurones and the deep Fos-LI neurones (10 ± 5% reduction and 5 ± 8% enhancement respectively, Figure 2). However, a weak depressive effect of D-NAME on the superficial Fos-LI neurones was observed (26 ± 8% reduction, $P<0.05$ as compared to carrageenin alone), but this effect was smaller than that observed with the same dose of L-NAME (46 ± 3% reduction, Figure 2).

Carrageenin oedema

The effects of L-NAME (10, 25, 50 and 100 mg kg^{-1}) on one of the signs of carrageenin inflammation, paw and ankle oedema, were studied simultaneously with the expression of c-Fos in all rats. The inflammatory oedema was measured as the increases in the ankle and paw diameters. Three hours after the carrageenin injection, the mean ankle diameter and paw diameter were considerably larger than those of the control non-stimulated rats (281 ± 9% and 357 ± 17% increase respectively, Figure 3).

Pre-administered L-NAME reduced both the ankle and paw oedema in a dose-dependent manner ($r^2=0.565$, $P<0.0001$ and $r^2=0.687$, $P<0.0001$ respectively, Figure 3). With the highest dose of L-NAME (100 mg kg^{-1}), the oedema of the paw and ankle were greatly reduced (55 ± 4% and 84 ± 10% reduction, respectively, Figure 3). The effects of L-NAME on the ankle and paw oedema and spinal expression of c-Fos were strongly correlated ($r^2=0.73$, $P<0.0001$ and $r^2=0.625$, $P<0.0001$, respectively). Post-administration of the highest dose of L-NAME (100 mg kg^{-1}, i.v.) reduced the paw and ankle diameter (52 ± 8% and 62 ± 10% reduction, respectively), with these effects not being significantly different from those observed with pre-administration of 100 mg kg^{-1} of L-NAME (Figure 4).

The effects of L-NAME on the paw and ankle diameters were not reversed by systemic L-arginine (Figure 4). However, the effects of L-NAME on the paw and ankle diameters (55 ± 4% and 84 ± 10% reduction respectively), were partly reversed by intraplantar L-arginine (34 ± 9% and 45 ± 11% reduction of the control paw and ankle oedema respectively, Figure 4). Systemic D-NAME did not reduce the oedema of the paw and the ankle (1 ± 5% and 14 ± 8% facilitation of the control paw and ankle oedema respectively, Figure 4).

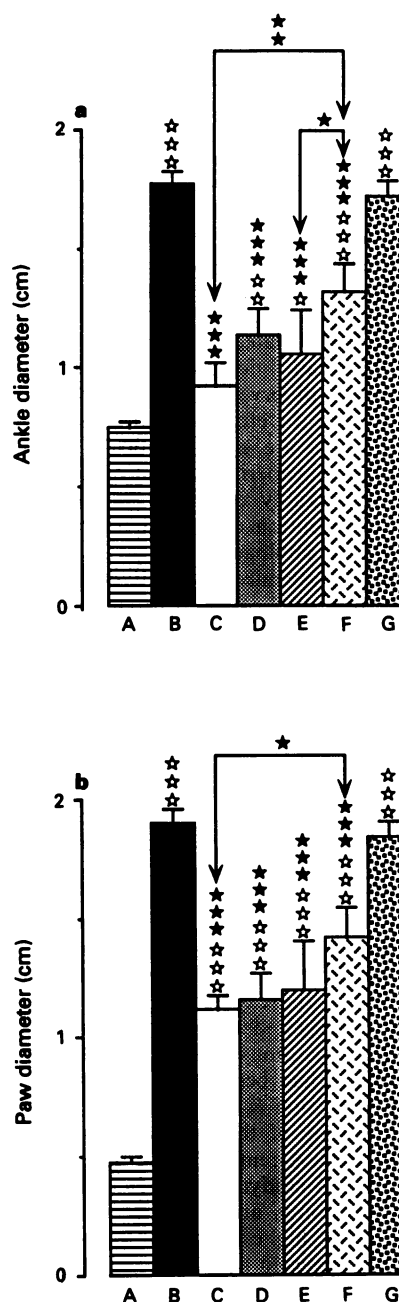

Figure 4 The effects of pre- and post-administration of N^G-nitro-L-NAME, 100 mg kg^{-1} i.v.), pre-administration of D-NAME (100 mg kg^{-1}, i.v.) and pre-administration of L-arginine (1200 mg kg^{-1}, i.v. or 30 mg/intraplantar) plus L-NAME (100 mg kg^{-1}, i.v.), on the ankle (a) and paw (b) oedema, 3 h after intraplantar carrageenin (6 mg). With co-administration of L-arginine and L-NAME, L-arginine was administered 10 min before L-NAME. (A) Non stimulated rat; (B) control carrageenin; (C) L-NAME 100 mg kg^{-1}, i.v. pretreatment; (D) L-NAME 100 mg kg^{-1}, i.v. post-treatment; (E) L-NAME 100 mg kg^{-1}, i.v. + L-arginine 1200 mg kg^{-1}, i.v.; (F) L-NAME 100 mg kg^{-1}, i.v. + L-arginine 30 mg intraplantar; (G) D-NAME 100 mg kg^{-1}, i.v. pretreatment. Both the paw and ankle diameter were significantly increased 3 h after carrageenin as compared to control non-stimulated rats. Pre and post-administered L-NAME reduced the paw and ankle diameters to an equal extent. Systemic L-arginine did not reverse the effect of L-NAME on either the paw or ankle diameter. However, intraplantar L-arginine partially reversed the effect of L-NAME on the paw and ankle diameter. D-NAME did not influence the increased paw and ankle diameters. Results are presented as absolute values of paw and ankle diameters ± s.e.mean (centimetres). Significance compared to carrageenin alone and compared to the non-stimulated rats was performed by ANOVA, PLSD Fisher's test, solid and open stars respectively (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Significant differences between pharmacological groups are indicated by arrows

Discussion

Three hours after intraplantar injection of carrageenin, numerous Fos-LI neurones were observed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn, and to a lesser extent in ventral horn, of lumbar L4–L5 segments of the spinal cord, thus confirming our previous study (Honoré *et al.*, 1994a). Due to experimental constraints, including the necessity to study a large number of experimental groups for a complete pharmacological study, it was not possible to perform all experiments simultaneously and therefore two series of experiments were performed. The c-Fos technique is associated with an inherent variability as illustrated by the small difference between the total number of Fos-LI neurones observed after carrageenin for the two series of experiments, therefore comparisons between the two series of experiments are not statistically valid. However, within each series of experiments the expression of Fos-LI neurones was remarkably consistent, as illustrated by the virtually equal distribution of Fos-LI neurones between the superficial and deep laminae.

Pre-administered systemic L-NAME dose-dependently reduced the number of Fos-LI neurones observed in the superficial and deep laminae of the dorsal horn after carrageenin. In addition, the highest dose of L-NAME studied, given 30 min after intraplantar carrageenin, also significantly reduced the number of Fos-LI neurones in the superficial and deep laminae. There was no significant difference between the effect of the pre and the post-administration of 100 mg kg^{-1} of L-NAME in any of the areas studied. The ability of post-administered L-NAME to reduce carrageenin-evoked c-Fos expression is expected in view of the time course of the associated inflammation, increases in paw temperature and hyperalgesia (Joris *et al.*, 1990, see refs. In Dubner & Ruda, 1992). It is likely that the timing of the post-administered L-NAME, before peak development of inflammation and hyperalgesia, enables it to reduce these events effectively and consequently reduce the expression of c-Fos in a manner similar to pre-administered L-NAME. However our interpretation has to be cautious since a significant increase in spinal c-fos mRNA has been observed as early as 30 min after carrageenin (Draisci & Iadarola, 1989). Nevertheless in this model, the timing of the expression of c-Fos after the increase in c-fos mRNA remains to be determined and more extensive experiments studying the effect of L-NAME given at even later time points are required.

The effects of the highest dose of L-NAME on Fos-LI neurones were not reversed by either systemic or intraplantar L-arginine. Previous studies have indicated that high doses of L-arginine are required to reduce the effects of L-NAME (Malmberg & Yaksh, 1993). In our study L-arginine, at a dose 12 times greater than the highest dose of L-NAME, and given prior to the L-NAME, did not block the effect of L-NAME on c-Fos expression. This lack of effect of L-arginine probably reflects differences in the pharmacokinetics of L-NAME and L-arginine, since L-arginine acts competively with L-NAME for NOS, it is more advantageous for L-arginine to be at the site of action prior to L-NAME. Although in our study L-arginine was administered before L-NAME it is not possible to predict the relative concentrations of the two compounds at their sites of action. Our results with systemic L-arginine are in keeping with an electrophysiological study in which intrathecal L-arginine did not reverse the depressive effects of intrathecal L-NAME on dorsal horn neuronal responses to peripheral formalin (Haley *et al.*, 1992).

The inactive isomer of L-NAME, D-NAME (100 mg kg− 1) did not influence the total number of Fos-LI neurones or the deep Fos-LI neurones; this is in clear contrast to the reduction of Fos-LI neurones by the equivalent dose of L-NAME. Overall, our results with D-NAME indicate that the effect of L-NAME on spinal Fos-LI neurones is specific, due to the inhibition of the nitric oxide synthase enzyme, and are not due to non specific effects. However, a weak reduction of the superficial Fos-LI neurones by D-NAME was observed. Although this effect of D-NAME may be interpretated as evidence for L-NAME acting via mechanisms other than NOS inhibition, the dose of D-NAME studied was high and the effect extremely weak as compared to that observed with the same dose of L-NAME. Furthermore, D-NAME did not influence the deep Fos-LI neurones which were strongly reduced by the same dose of L-NAME.

Since spinal c-Fos expression is strongly associated with noxious spinal transmission (see references in Herdegen *et al.*, 1991; Abbadie *et al.*, 1994; Zieglgänsberger & Tölle, 1993), the ability of L-NAME to reduce such an expression strongly implies a pro-nociceptive role of NO. In this respect it must be emphasised that L-NAME reduced c-Fos expression in the superficial and deep laminae neurones of the dorsal horn, the same laminae in which noxiously evoked neurones are located (Besson & Chaouch, 1987; Willis & Coggeshall, 1991). Furthermore, the effects of L-NAME on c-Fos are reminiscent of the effects of paracetamol, aspirin (Honoré *et al.*, 1994b) and indomethacin (Honoré *et al.*, 1994a) in the same model of inflammatory pain, thus providing additional indirect evidence for a pro-nociceptive role of NO. Our results are in strong agreement with previous studies which have shown L-NAME to reduce both dorsal horn neuronal responses (Haley *et al.*, 1992) and behavioural responses (Malmberg & Yaksh, 1993) to formalin, in addition to reducing carrageenin-evoked hyperalgesia (Meller *et al.*, 1994), suggesting L-NAME diminishes more prolonged nociceptive transmission associated with the development of central hypersensitivity which is associated with inflammatory processing (Dubner & Ruda, 1992).

Three hours after intraplantar injection of carrageenin, both the paw and ankle diameters were considerably larger than control non stimulated rats, the peripheral oedema associated with carrageean indicates the development of inflammation. Both pre and post-administration of systemic L-NAME dramatically reduced the carrageenin oedema, in a clear dose-related manner. These results strongly support a peripheral role of NO in the development of carrageenin-evoked oedema. Our results are in keeping with previous studies which have reported a peripheral role of NO in the development of inflammation (Ialenti *et al.*, 1992; Lippe *et al.*, 1993; Vane *et al.*, 1994) and a previous electrophysiological study in which peripherally administered L-NAME reduced the second phase response, resulting from peripheral inflammation, of dorsal horn neurones to peripheral formalin (Haley *et al.*, 1992).

To substantiate further the role of NO during carrageenin oedema, the effect of L-arginine administered prior to L-NAME on the oedema was studied. The reduction of oedema by the highest dose of L-NAME was not reversed by the prior administration of systemic L-arginine. As discussed above, this lack of effect may reflect the timing of the L-arginine administration or the dose studied. In contrast, the reduction of the oedema by L-NAME was partially attenuated by intraplantar L-arginine. These results suggest that the systemic dose of L-arginine studied was either too low or had poor access to the inflammatory site. Importantly the oedema was not influenced by systemic D-NAME, suggesting the effect of L-NAME on the carrageenin oedema is not due to non specific effects of the drug.

L-NAME has been shown to induce increases in blood pressure and a reduction in blood flow in different organs (Gardiner *et al.*, 1990), and the contribution of these effects to the antinociceptive effect of L-NAME is an important consideration. Since methods for recording blood pressure in freely moving animals are limited, it was not feasible for us to monitor the contribution of changes in blood pressure to the effects of L-NAME on the oedema and c-Fos expression. In addition, any invasive surgical procedures, or even the use of a pneumatic sensory cuff, may influence the spinal c-Fos

expression. However, 7-nitro indazole (7-NI), an NOS inhibitor has been reported to be anti-nociceptive, without influencing blood pressure (Moore *et al.*, 1993). Thus future studies are necessary to determine the effect of 7-NI on carrageenin inflammation and c-Fos expression, in the absence of any possible cardiovascular effects.

In conclusion, L-NAME greatly reduced carrageenin oedema, implicating an important contribution of NO to the development of inflammation. The reduction of the carrageenin oedema by L-NAME was strongly correlated with the decreased spinal expression of c-Fos. These results suggest that the reduction of spinal c-Fos by L-NAME is due to a diminished spinal input and predominantly reflects a peripheral action of systemic L-NAME. However, in view of the results of previous studies using intrathecal L-NAME (Haley *et al.*, 1992; Malmberg & Yaksh, 1993; Meller *et al.*, 1994), our results cannot exclude an additional central effect of systemic L-NAME and thus a spinal contribution of NO to c-Fos expression as previously described (Lee *et al.*, 1992).

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