L-N^G-nitro arginine methyl ester exhibits antinociceptive activity in the mouse

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1 L-N^G-nitro arginine methyl ester (L-NAME, 1–75 mg kg⁻¹) administered intraperitoneally (i.p.) elicits dose-related antinociception in the mouse assessed by the formalin-induced paw licking procedure. Antinociceptive activity is still present 24 h after injection. L-NAME (75 mg kg⁻¹, i.p.) is also antinociceptive in the acetic acid-induced abdominal constriction and hot plate procedures.

2 L-NAME additionally produces a dose-related inhibition of formalin-induced paw licking following intracerebroventricular (i.c.v., 0.1–100 µg per mouse) and oral (p.o., 75–150 mg kg⁻¹) administration.

3 L-Arginine (600 mg kg⁻¹, i.p.) but not D-arginine (600 mg kg⁻¹) or naloxone (5 mg kg⁻¹) reverses the antinociceptive effect of L-NAME in the formalin test.

4 High doses of L-NAME (37.5–600 mg kg⁻¹) but not D-NAME (75 mg kg⁻¹) administered i.p. produce dose-related increases in blood pressure of the urethane-anaesthetized mouse whilst i.c.v. injected L-NAME (0.1 and 100 µg per mouse) is inactive.

5 L-NAME (75 mg kg⁻¹, i.p.) did not inhibit oedema formation in the formalin-injected mouse hindpaw.

6 L-NAME (75 mg kg⁻¹) did not produce any overt behavioural changes in treated mice and failed to influence locomotor activity or the incidence of dipping, crossing, rearing or circling behaviour assessed by a modified 'head-dipping' board procedure. A high dose of L-NAME (600 mg kg⁻¹) reduced dipping behaviour and locomotor activity suggesting a possible sedative effect. D-NAME (600 mg kg⁻¹) was inactive.

7 These results suggest that L-NAME produces an opioid-independent and long-lasting antinociception in the mouse most probably by a direct effect within the central nervous system.

Introduction

Guanidino-monosubstituted derivatives of L-arginine including L-N^G-nitro arginine (L-NOARG) and its methyl ester (L-NAME), L-N^G-monomethyl arginine (L-NMMA) and L-N^G-amino arginine selectively inhibit nitric oxide (NO) biosynthesis by vascular endothelial cells (Rees *et al.*, 1989; Fukuto *et al.*, 1990; Moore *et al.*, 1990). Recently, it has become evident that NO biosynthesis is a widespread phenomenon in the body occurring not only in the vascular endothelium but also in leucocytes, Kupffer cells, hepatocytes, neuroblastoma cells, fibroblasts and the adrenal gland (Moncada *et al.*, 1989; Ishii *et al.*, 1990). In addition, NO or a closely chemically related substance, is released from peripheral non-adrenergic, non-cholinergic (NANC) nerves innervating the mouse and rat anococcygeus (Gillespie *et al.*, 1989; Gibson *et al.*, 1990), guinea-pig trachea (Tucker *et al.*, 1990) and dog ileocolonic junction (Bult *et al.*, 1990). The discovery that NO is also released from rat cerebellar synaptosomes stimulated with N-methyl-D-aspartate or kainate (Garthwaite *et al.*, 1988; 1989) and the identification of a brain synaptosomal enzyme which converts L-arginine into L-citrulline and NO (Knowles *et al.*, 1989) has raised the possibility that NO may additionally function as a neurotransmitter in the central nervous system. However, the biological role or roles of NO in the brain have yet to be investigated. Using L-NAME as a pharmacological tool we report here that NO may have an important function in pain perception in the mouse. Some of these results have been published in preliminary form (Hart *et al.*, 1990).

Methods

Male LACA mice (30–35 g) were used in this study. Animals were allowed food and water *ad libitum* until transported to

the laboratory 1 h before the experiment. All experiments were performed in the period between 13 h 00 min and 17 h 00 min and, except where otherwise indicated, were conducted in normal room light and temperature (22 ± 2°C).

Assessment of antinociceptive effect of L-NAME

The antinociceptive activity of L-NAME was assessed by use of 3 separate procedures: (i) Formalin-induced paw-licking was determined essentially as described by Hunskaar & Hole (1987). Briefly, animals were injected sub-plantar in one hindpaw with formalin (5%, 10 µl). The duration of paw-licking (an index of nociception) was measured 0–5 min and 15–30 min after formalin administration. The first phase response is believed to represent a direct irritant effect of formalin on sensory C fibres whilst the latter phase response is most likely secondary to the development of an inflammatory response and the release of algescic mediators (Hunskaar & Hole, 1987). L-NAME (1–75 mg kg⁻¹) or D-NAME (600 mg kg⁻¹) were injected intraperitoneally (i.p.). In separate experiments, L-NAME was administered either intracerebroventricularly (i.c.v.) as described by Oluyomi (1989) or orally (p.o.). The time interval between L-NAME and formalin administration was 15 min in each case. In order to determine the time course of effect some animals were injected with L-NAME (i.p.) 24 h before the formalin injection and assessment of licking time. Control animals received an equal volume (0.2 ml i.p. and p.o.; 5 µl i.c.v.) of saline (0.9% w/v NaCl). In some experiments, mice injected i.p. with L-NAME (75 mg kg⁻¹) were additionally pretreated (i.p.) with either L-arginine (150–600 mg kg⁻¹), D-arginine (600 mg kg⁻¹) or naloxone (5 mg kg⁻¹) 20 min prior to formalin administration. Such a dose of naloxone has previously been reported to abolish morphine-induced antinociception in the formalin test in mice (Vaccarino *et al.*, 1989). In control experiments, additional animals received either L- or D-arginine (600 mg kg⁻¹) alone. For comparison with other known antinociceptive agents mice were injected i.p. with morphine (2.5–10 mg kg⁻¹)

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or indomethacin ($10\text{--}40\text{ mg kg}^{-1}$) 15 min prior to formalin injection and assessment of licking time. (ii) Acetic acid (0.6%, 10 ml kg^{-1} , i.p.) induced abdominal constrictions were counted over a 10 min period 25 min following i.p. injection of L-NAME ($75\text{--}600\text{ mg kg}^{-1}$), D-NAME (600 mg kg^{-1}) or saline (0.2 ml) and 10 min after acetic acid administration. (iii) Change in foot withdrawal reaction time on a hot plate set to 56°C . Animals were injected with L-NAME (75 mg kg^{-1} , i.p.) or saline (0.2 ml) 15 or 130 min before assessment of reaction time.

Effect of L-NAME on formalin-induced hindpaw oedema

The possibility that the antinociceptive activity of L-NAME in the formalin-induced paw-licking assay may be secondary to an anti-oedema effect was assessed by comparing the weight of the formalin-injected and contralateral (untreated) hindpaws excised at the knee joint at the end of the experiment. Animals were pretreated (i.p.) with L-NAME (75 mg kg^{-1}), indomethacin (40 mg kg^{-1}) or saline (0.2 ml) 15 min prior to subplantar formalin (5%, $10\text{ }\mu\text{l}$) injection. Groups of mice were killed 45 min thereafter.

Effect of L-NAME on mouse blood pressure

Since changes in paw blood flow might be expected to influence the antinociceptive effect of L-NAME assessed by the formalin-induced paw-licking and hot plate assays the effect of L-NAME administration on mouse blood pressure was also investigated. For these experiments, mice were anaesthetized with urethane (10 g kg^{-1} , i.p.) and a cannula inserted into the carotid artery. Blood pressure was monitored continuously with a Bell & Howell pressure transducer connected to a Grass 4-channel pen recorder, L-NAME ($1\text{--}75\text{ mg kg}^{-1}$, i.p.; 0.1 and $100\text{ }\mu\text{g}$ per mouse, i.c.v.), D-NAME (75 mg kg^{-1} , i.p.) or saline (0.5 ml , i.p.; $5\text{ }\mu\text{l}$, i.c.v.) were administered and mean arterial blood pressure (MABP) determined at intervals thereafter.

Effect of L-NAME on animal behaviour

In preliminary experiments, mice were housed singly and observed for any overt changes in behaviour for up to 3 h following i.p. injection of L-NAME (75 or 600 mg kg^{-1}) or saline (0.2 ml). A more objective estimate of the effect of L-NAME on mouse behaviour was performed by use of a modified head dipping board procedure as described by Davis & Wallace (1976). Briefly, mice were injected with L-NAME (75 and 600 mg kg^{-1}), D-NAME (600 mg kg^{-1}) or saline (0.2 ml) and placed 60 min thereafter onto the central square of a 16 square dipping board apparatus positioned approximately 1 metre above the ground. All experiments were performed in neutral red light in a closed and quiet room. Four behavioural parameters (dips, rears, crosses, circles) were monitored over a 3 min test period. In separate experiments, mouse locomotor activity was determined 60 min after L-NAME, D-NAME or saline administration in automated activity cages (Ugo Basile Ltd., Italy). Results are expressed as counts obtained over an initial 10 min period.

Drugs and chemicals

All drugs were purchased from Sigma with the exception of D-NAME which was obtained from Bachem Ltd. Indomethacin was dissolved in 5% (w/v) Na_2CO_3 . All other drugs were dissolved in saline.

Statistical analysis

Results indicate mean $\pm$ s.e.mean with the exception of dipping board and locomotor activity data which are expressed as medians. Statistical significance of differences between groups was determined by unpaired Student's *t* test unless otherwise indicated.

Results

Antinociceptive activity of L-NAME

Formalin-induced paw licking In saline-treated mice the duration of paw licking over the first time period was $103.3 \pm 1.8\text{ s}$ ($n = 8$) and over the second period, $146.7 \pm 3.8\text{ s}$ ($n = 8$), D-NAME (600 mg kg^{-1}) failed to reduce paw licking time in either the early or late phase indicating the absence of significant antinociceptive effect. In contrast, L-NAME ($1\text{--}75\text{ mg kg}^{-1}$) injected i.p. caused a dose-related reduction in paw licking in the second phases of measurement whilst only the highest dose of L-NAME (75 mg kg^{-1}) significantly reduced first phase paw licking time (Figure 1a). L-NAME (75 mg kg^{-1}) reduced paw licking by $30.8 \pm 4.5\%$ in the first phase and $96.3 \pm 3.0\%$ (both $n = 8$, $P < 0.001$) in the second phase. This effect of L-NAME (75 mg kg^{-1}) was not influenced by pretreatment of mice with naloxone (5 mg kg^{-1}) (first phase licking time: $68.5 \pm 2.3\text{ s}$, c.f. $71.2 \pm 1.2\text{ s}$, second phase licking time: $9.1 \pm 1.1\text{ s}$, c.f. $5.6 \pm 3.4\text{ s}$, both $n = 8$, $P > 0.05$). Interestingly, L-NAME (75 mg kg^{-1}) retained both first phase (licking time, $90.9 \pm 1.3\text{ s}$, c.f. $101.9 \pm 1.6\text{ s}$, $n = 6$, $P < 0.05$) and second phase antinociceptive activity 24 h after i.p. administration (licking time, $69.8 \pm 16.3\text{ s}$, c.f. $133.0 \pm 11.4\text{ s}$, $n = 6$, $P < 0.01$).

The antinociceptive potency of L-NAME was also compared to that of indomethacin and morphine (Figure 1b,c). Clearly, L-NAME exhibited greater second phase antinociceptive activity in this model than indomethacin. In contrast to L-NAME, indomethacin did not influence first phase

Figure 1 Antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) (a), indomethacin (b) and morphine (c) in the mouse following i.p. administration. C represents control results obtained in saline-treated animals. Open columns indicate licking time in the first phase (0–5 min) whilst stippled columns indicate licking time in the second phase (15–30 min). Results show mean of $n = 8$; s.e.mean shown by vertical bars. ** $P < 0.05$; * $P < 0.001$.

licking time. Morphine was a more potent antinociceptive agent than L-NAME in both the first and second phases of the response.

L-NAME also exhibited antinociceptive activity following i.c.v. and p.o. administration (Figure 2). For example, 100 μg L-NAME injected i.c.v. or 150 mg kg^{-1} administered p.o. abolished paw-licking in the second phase. Additionally, L-NAME (100 μg , i.c.v.) reduced first phase licking time by $72.2 \pm 7.9\%$ ($n = 8$). In contrast, oral administration of L-NAME (75 mg kg^{-1}) did not significantly influence first phase licking time but did reduce the second phase licking.

L-Arginine (150–600 mg kg^{-1} , i.p.) but not D-arginine (600 mg kg^{-1} , i.p.) pretreatment completely reversed the first phase antinociceptive effect of L-NAME (75 mg kg^{-1} , i.p.) and partially reversed second phase antinociception (Figure 3). Interestingly, L-arginine (600 mg kg^{-1} , i.p.) administered to mice in the absence of L-NAME itself produced a small but significant reduction in paw licking time in the second phase ($111.4 \pm 8.3\text{ s}$, c.f. $148.6 \pm 12.4\text{ s}$, $n = 8$, $P < 0.05$) without affecting the first phase response. D-Arginine (600 mg kg^{-1} , i.p.) was without effect in both phases (data not shown).

Acetic acid-induced abdominal constriction Acetic acid administration in saline-injected mice resulted in the production of 28.0 ± 3.5 abdominal constrictions in the 10 min test period. L-NAME pretreatment reduced the incidence of abdominal constrictions by approximately 40% and 90% at doses of 75 and 600 mg kg^{-1} respectively (Figure 4).

Hot plate assay The latency of foot withdrawal of naive mice on the hot plate set to 56°C was 5.7 s (median). Saline injection failed to influence the latency of foot withdrawal 15 min post-injection (4.3 s; median, $P > 0.05$, Mann-Whitney U test). In contrast, L-NAME (75 mg kg^{-1}) significantly prolonged the time for foot withdrawal both 15 min and 130 min post-injection (Figure 4).

Effect of L-NAME on mouse hindpaw weight

Hindpaw weight of saline-injected mice injected subplantar with formalin increased by $190.7 \pm 6.9\text{ mg}$ ($n = 12$) compared with the contralateral (untreated) hindpaw indicating significant oedema formation. Indomethacin (40 mg kg^{-1}) pretreatment reduced oedema formation by approximately 26%

Figure 2 Antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) in the mouse following i.c.v. (a) or p.o. (b) administration. C represents control results obtained in saline-pretreated animals. Open columns indicate licking time in the first phase (0–5 min) whilst stippled columns indicate licking time in the second phase (15–30 min). Results show mean of $n = 8$ (a) or $n = 6$ (b); s.e.mean shown by vertical bars. * $P < 0.001$.

Figure 3 Reversibility of the antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME, 75 mg kg^{-1} , i.p.) by L-arginine in the mouse. Figures beneath each pair of histograms indicate dose of L-arginine (mg kg^{-1}) administered i.p. 5 min prior to subplantar formalin administration. C represents control results obtained in saline-treated animals. Open columns indicate licking time in the first phase (0–5 min) whilst stippled columns indicate licking time in the second phase (15–30 min). Results show mean of $n = 6$; s.e.mean shown by vertical bars. ** $P < 0.05$, * $P < 0.001$ (compared to results obtained in mice pretreated with L-NAME).

Figure 4 Antinociceptive effect of L-N^G-nitro arginine methyl ester (L-NAME) in the mouse assessed by the acetic acid-induced abdominal constriction assay (a) and the hot plate assay (b). In both cases C represents control results obtained in saline-pretreated animals. Results in (a) indicate number of writhes in a 10 min period and are mean of $n = 6-9$; s.e.mean shown by vertical bars. ** $P < 0.05$, * $P < 0.001$. Results in (b) indicate difference in hot plate latency of mice measured before (t_1) and either 15 min ($t = 15$) or 130 min ($t = 130$) after treatment with L-NAME (t_2). Results show median values, $n = 5-20$, * $P < 0.001$ (Mann-Whitney U test).

Table 1 Effect of L-N^G-nitro arginine methyl ester (L-NAME) on blood pressure of the urethane-anaesthetized mouse

	MABP (mmHg)	
	<i>t</i> = 0	<i>t</i> = 15
L-NAME (i.p.)		
1.0	50.5 ± 2.4	49.7 ± 2.8
10.0	62.0 ± 7.7	78.6 ± 10.6
37.5	46.2 ± 4.0	65.7 ± 7.1*
75.0	68.3 ± 6.9	91.8 ± 10.9*
600.0	61.2 ± 5.4	115.0 ± 5.3*
D-NAME (i.p.)		
75.0	46.7 ± 1.6	50.2 ± 3.5
L-NAME (i.c.v.)		
0.1	51.2 ± 3.8	55.5 ± 4.2
100.0	64.0 ± 6.4	62.4 ± 5.3

Urethane-anaesthetized mice were injected i.p. (mg kg⁻¹) or i.c.v. (μg per mouse) with L-NAME or i.p. (mg kg⁻¹) with D-NAME. MABP was determined before (*t* = 0) and 15 min (*t* = 15) after drug injection. In control experiments no increase in MABP was detected in mice injected with saline (0.5 ml i.p.; 10 μl i.c.v.). Results show mean ± s.e.mean, *n* = 3–6, **P* < 0.05.

(140.8 ± 10.3 mg, *n* = 6, *P* < 0.01). In contrast, L-NAME (75 mg kg⁻¹) failed to influence the weight of formalin-injected hindpaws (170.2 ± 18.4 mg, *n* = 12, *P* > 0.05).

Effect of L-NAME on mouse blood pressure

The resting MABP of urethane-anaesthetized mice was 55.0 ± 2.3 mmHg (*n* = 32). Intravenous injection of L-NAME (1–600 mg kg⁻¹) elicited a dose-related increase in MABP (Table 1). At the highest dose of L-NAME used in this study, MABP was approximately doubled. The threshold dose for vasopressor activity was 10 mg kg⁻¹. In contrast, L-NAME (0.1 and 100 μg per mouse) injected i.c.v. failed to influence MABP (Table 1).

Effect of L-NAME on animal behaviour

Subjective observation of mice injected i.p. with L-NAME (up to 75 mg kg⁻¹) revealed no obvious changes in animal behaviour over a period of 3 h when compared with control animals injected with saline. A high dose of L-NAME (600 mg kg⁻¹) produced no change in animal behaviour until 60 min post-injection. Thereafter, mice remained fixed and motionless although they appeared alert and could be easily aroused. No loss of righting reflex was observed.

L-NAME (75 mg kg⁻¹, i.p.) failed to influence rearing, crossing, circling or dipping forms of behaviour as assessed by the dipping board assay. In addition, no change in locomotor

Table 2 Effect of L-N^G-nitro arginine methyl ester (L-NAME) on dipping board and locomotor activity of mice

	Dips	Rears	Crosses	Circles	LA
Saline	24	39	10	2	1340
L-NAME (75)	16	49	15	2	1229
L-NAME (600)	9*	31	8	5	654*
D-NAME (600)	22	34	12	4	1512

Mice were injected i.p. with L-NAME or D-NAME at doses (mg kg⁻¹) indicated in parentheses. Dips, rears, crosses and circles were monitored over a 3 min period 60 min post-injection. Results show median values, *n* = 5–12, **P* < 0.05 (Mann-Whitney U test). Locomotor activity (LA) was monitored in activity cages immediately post-injection and represents arbitrary counts per 10 min. Results show median values, *n* = 8, *P* < 0.05 (Mann-Whitney U test) subsequent to Kruskal-Wallis analysis of variance.

behaviour in activity cages was detected with this dose of L-NAME. A high dose of L-NAME (600 mg kg⁻¹) did not significantly change rearing, crossing or circling but did reduce dipping behaviour by approximately 50% and additionally depressed overall locomotor count by approximately 35%. These results are summarised in Table 2.

Discussion

The results of the present study indicate that L-NAME (but not D-NAME) produces a potent antinociceptive effect in the mouse assessed by use of three different experimental procedures. Moreover, L-NAME is effective following i.p., i.c.v. or p.o. administration in the formalin assay and its antinociceptive action is reversed by L- but not D-arginine. Interestingly, L- but not D-arginine at a high dose itself elicits a weak antinociceptive action in the formalin test. The rationale underlying this direct effect of L-arginine is unknown but is clearly unlikely to be consequent upon NO production. Since L-NAME is a selective inhibitor of NO biosynthesis in a variety of mammalian tissues and cells including both peripheral (Gibson *et al.*, 1990) and central (Murad *et al.*, 1990) nerves we propose that a similar inhibition of NO formation underlies its observed antinociceptive action.

The precise site of the antinociceptive effect of L-NAME in the mouse remains unclear. That i.c.v. administered L-NAME reduces formalin-induced paw licking time strongly suggests an effect within the central nervous system. Furthermore, L-NAME is antinociceptive in the hot plate assay which is widely believed to be sensitive solely to drugs acting supraspinally (Yaksh & Rudy, 1977).

Since animal models of nociception may be indirectly influenced by drugs acting non-specifically on other body systems we considered it important to exclude other effects of L-NAME in the antinociception observed. For example, intravenous administration of L-NAME reportedly increases blood pressure in the rat (Rees *et al.*, 1990; Dubbin *et al.*, 1990). Thus, it is conceivable that a similar effect in the mouse may contribute to the observed antinociceptive effect of L-NAME in at least two of the three assays employed by altering hindpaw blood flow. We show here that L-NAME administered i.p. elicits a dose-related vasopressor response in the anaesthetized mouse. Interestingly, the threshold dose for significant vasopressor effect was >10 mg kg⁻¹ even though antinociception was observed at doses of L-NAME as low as 1 mg kg⁻¹. Furthermore, i.c.v. injection of L-NAME failed to increase mouse blood pressure although did produce antinociception in the formalin test when injected by this route. Thus, it seems unlikely that the cardiovascular effect of L-NAME contributes significantly to the antinociception.

In addition, the potent antinociceptive effect of L-NAME in the second phase of the formalin test is not secondary to an anti-oedema action since L-NAME pretreatment failed to prevent the increase in hindpaw weight following formalin injection. In contrast, indomethacin significantly inhibited inflammatory oedema formation in the formalin-injected mouse hindpaw. These results seem at variance with those of Antunes *et al.* (1990) who reported that L-NMMA inhibited polyarginine-induced inflammation in the rat hindpaw. The reason for the discrepancy between the two studies is not clear although it may reflect differences between species, nociceptive stimulus or inhibitor of NO formation used. Based upon the present results it therefore seems unlikely that L-NAME exhibits antinociceptive activity in the formalin model secondary to an anti-inflammatory effect.

At doses of L-NAME which produce pronounced antinociceptive activity (up to 75 mg kg⁻¹) no changes in animal behaviour were observed or could be detected by the dipping board procedure. Furthermore, no reduction in locomotor activity was apparent. Thus, at doses within the antinociceptive range, L-NAME has no detectable sedative or

other behavioural effects which might contribute to its antinociceptive effect.

The present results raise two intriguing possibilities: (i) that NO, or a like substance formed from L-arginine, has a physiological role to play in the mouse to promote the pain response most probably by an action within the central nervous system although some effect on peripheral sensory nerves cannot be discounted. (ii) L-NAME, or a like inhibitor

of NO biosynthesis, may at appropriate dose, have clinical application as an analgesic. In this context it is of interest that antinociception in the mouse due to L-NAME is not antagonized by naloxone and is thus independent of endogenous opioid release. Moreover, in contrast to the present generation of clinically used analgesics, the antinociceptive effect of L-NAME, at least in the mouse, persists for up to 24 h.

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(Received July 24, 1990)

Revised August 24, 1990

Accepted August 29, 1990)