N^G-nitro-L-arginine methyl ester (L-NAME) has been used extensively as a paradigmatic inhibitor of NO synthase and has been shown to cause antinociception in several experimental models. We describe here how L-NAME produced a dose-dependent antinociceptive effect when injected intraperitoneally in the mouse after acetic acid induced writhings, or intraplantarly in the rat paw pressure hyperalgesia induced by carrageenin or prostaglandin E2. In contrast another NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), had no significant effect per se but inhibited L-NAME systemic induced antinociception in mice and local induced antinociception in the rat paw hyperalgesia test. D-NAME had no antinociceptive effect upon carrageenin-induced hyperalgesia. Pretreatment of the paws with two inhibitors of guanylate cyclase, methylene blue (MB) and 1H-:[1,2,4]-oxadiazolo-:[4,3-a] quinoxalin–1-one (ODQ) abolished the antinociceptive effect of L-NAME. L-Arginine and the cGMP phosphodiesterase inhibitor, MY 5445 significantly enhanced the L-NAME antinociceptive effect. The central antinociceptive effect of L-NAME was blocked by co-administration of L-NMMA, ODQ and MB. The present series of experiments shows that L-NAME, but not L-NMMA, has an antinociceptive effect. It can be suggested that L-NAME causes the antinociceptive effect by stimulation of the arginine/ NO/ cGMP pathway, since the antinociceptive effect of L-NAME can be antagonized by L-NMMA and abolished by the guanylate cyclase inhibitors (MB and ODQ). In addition, the NO synthase substrate, L-arginine and the cGMP phosphodiesterase inhibitor, MY5445 were seen to potentiate the effects of L-NAME. Thus, L-NAME used alone, has limitations as a specific inhibitor of the arginine-NO-cGMP pathway and may therefore be a poor pharmacological tool for use in characterising participation in pathophysiological processes.

Key words: Carrageenin analgesia, NG-nitro-L-arginine methyl ester (L-NAME), Nitric oxide (NO), Guanosine 3′,5′-monophophate cGMP, 1H-[1,2,4]-oxadiazolo-[4,3-a] quinoxalin–1-one (ODQ), Inducible NO synthase (iNOS), Methylene blue (MB), NG-monomethyl-L-arginine (L-NMMA)

L-NAME causes antinociception by stimulation of the arginine-NO-cGMP pathway

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Introduction

L-NAME (N^G-nitro-L-arginine methyl ester), has been shown to cause antinociception by spinal, supraspinal, local (intraplantar) or systemic administration.[1–8] As L-NAME is considered an specific nitric oxide (NO) synthase inhibitor,[2,24] these experiments were taken to support the hypothesis that stimulation of the arginine/NO/cGMP pathway enhances nociception at various levels of the sensory system. On the other hand, there are several reports indicating that cholinergic or opioidergic stimulation of the arginine/NO/cGMP pathway causes central, spinal or peripheral analgesia,[9–12] and some peripheral analgesics cause antinociception by stimulation of this pathway.[13–16] Furthermore, the central analgesic, arginine, seems to be associated with NO-cGMP stimulation.[17–19] Thus there appears to be an apparent contradiction amongst the various experiments made to ascertain the role of the arginine/NO/cGMP pathway in nociception. A great deal of information has been derived with the use of L-NAME as a methodological tool. However L-NAME can be seen to be either analgesic or hyperalgesic in the same test[20]

whilst another NO synthase inhibitor N^G-monome-thyl-L-arginine (L-NMMA) does not show antinociception in the same test as L-NAME, i.e. the formalin test in mice.[2] Recently, L-NAME has been reported to stimulate inducible NO synthase (iNOS) gene expression.[21] Because of these apparent contradictory results, we evaluated the possibility that the analgesic effect of L-NAME was due to stimulation of the arginine/NO/cGMP pathway. In the present study L-NAME was initially assayed in two tests in which nociception involves an inflammatory stimulus: the acetic acid induced writhings in mice and in the rat paw pressure hyperalgesia test induced by intra-plantar administration of carrageenin. In these tests L-NAME showed an antinociceptive effect which was significantly inhibited by pretreatment of the animals with L-NMMA.

To further investigate the antinociceptive effect of L-NAME and in order to avoid the oedema formation and facilitate successive injections of drugs, PGE_2 instead of carrageenin was used to induce hyper-algesia in the rat paw pressure test. In this test the peripheral antinociceptive synergism between L-NAME and the NO synthase substrate, arginine, and the cGMP phosphodiesterase inhibitor, as well the effect of two inhibitors of guanylate cGMP activation, methylene blue (MB) and 1H-[1,2,4]-oxadiazolo-[4,3-a] quinoxalin–1-one (ODQ) was evaluated. Finally, since L-NAME has been shown to cause analgesia by intracerebroventricular administration,[2] we tested L-NAME co-administration with inhibitors of the arginine/NO/cGMP pathway, L-NMMA, MB and ODQ.

Materials and Methods

Animals

The experiments were performed on male Wistar rats (150–180 g) and albino Swiss mice (22–30 g). The animals were housed under natural light, with free access to food and water. Intracerebroventricular (i.c.v.) injections in rats were made following the method described by Côrrea and Graeff.[22] When single doses of the various drugs were used, they were based on dose response pilot experiments. All experimental procedures conformed to the IASP guidelines on the use of animals in pain research. Rats were used once only.

Nociception tests

(a) Writhing test in mice.
This test was based on the frequency of abdominal contortions evoked by an intraperitoneal injection of 10 ml/kg of 0.6% acetic acid.[8] L-NAME, L-arginine, or vehicle was injected 15 min before acetic acid administration, and the number of writhing events were counted for 20 min after the nociceptive challenge. For antagonism studies, mice were treated as above, except that L-NMMA was administered 15 min before L-NAME.

(b) Hind paw hyperalgesia test in rats.
Our modification of the Randall-Selitto rat paw pressure test was used to measure hyperalgesia.[23] In the test, a pressure of 20 mmHg is continuously applied to the hind paw of the rat until the animal presents a typical freezing reaction (reaction time). After measurement of the basal reaction time (control), hyperalgesia was induced either by an intraplantar injection of carrageenin (Cg, 100 μg) or PGE_2. The intensity of hyperalgesia was quantified as the difference in reaction time (delta reaction time) measured 3 h after administration of Cg, from the control reaction time assessed before injection of the hyper-algesic stimulus. The term nociception is used in this paper to describe the presence of an overt standard behaviour induced by the application of a noxious stimulus in a normal tissue or a non-noxious stimulus in previously sensitised tissue. The term hyperalgesia is used when a non-noxious stimulus causes nociception when applied to a sensitized tissue either by an inflammatory stimulus like carrageenin or an hyper-algesic mediator like prostaglandin E2.

Drugs

Carrageenin (Viscarin) was purchased from Marine Colloids, EUA. L-NAME (N-nitro-L-arginine methyl ester, Wellcome, UK) and L-NMMA (N^G-monomethyl-L-arginine acetate) were purchased from Sigma (St Louis, MO, USA) and methylene blue (MB) was from Reagen (Brazil) and ODQ (1H-[1,2,4]-oxadiazolo-[4,3-a] quinoxalin–1-one) was from Tocris Cookson Inc. (St Louis, USA).

Statistics

All results are presented as means (SEM of five rats or six to 12 mice per group. Results are presented as means and standard errors of the means of groups of at least five animals in each group. Differences between responses were evaluated by ANOVA, followed by the Bonferroni *t*-test. Results with $P<0.05$ were considered significant.

Results

Blockade of L-NAME effect by L-NMMA in two tests of nociception induced by inflammatory stimuli

(a) Mice writhing test: nociceptive behaviour induced by intraperitoneal acetic acid administration. L-NAME administered intraperitoneally reduced the number of abdominal constrictions in a dose-dependent manner
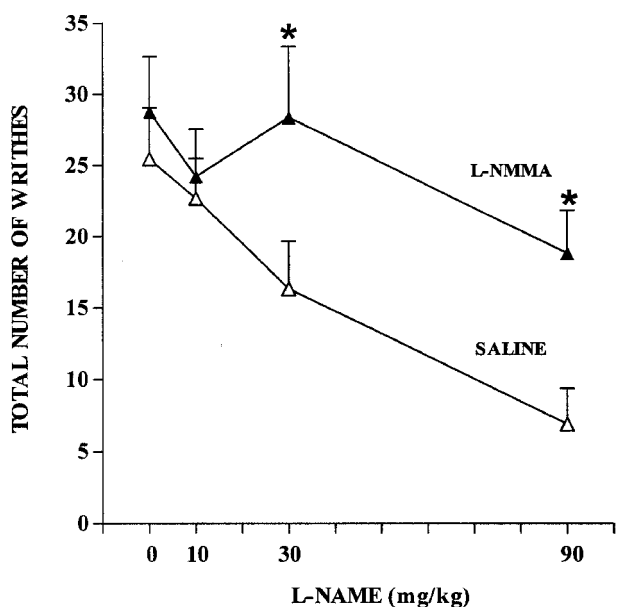
FIG. 1. Inhibition by L-NMMA (10 mg/kg, i.p.) of the anti-nociceptive effect of intraperitoneal administration of L-NAME on the acetic acid writhing test in mice. The symbols are the mean ± SEM of 6–12 mice/group. *indicates significant differences ($P < 0.05$) in comparison with the control L-NAME group treated with intraperitoneal saline.

by approximately 40% and 73% at doses of 30 and 90 mg/kg, respectively. L-NMMA (10 mg/kg i.p.), significantly blocked the antinociceptive action of L-NAME, but had no effect on its own (Fig. 1).

(b) Rat paw pressure test: hyperalgesia induced by carrageenin inflammation.

Intraplantar administration of L-NAME (50 and 300 μg) produced a significant inhibition of carrageenin-induced hyperalgesia of up to 40% for the highest dose used (Fig. 2). L-NAME-induced peripheral hyperalgesia was significantly inhibited by pre-treatment of the paws with 100 μg of L-NMMA or 500 μg of MB. Neither MB, nor L-NMMA (up to 500 μg /paw) had hyperalgesic effects.

Rat paw pressure test: hyperalgesia induced by PGE$_2$

(a) Antagonism L-NMMA, MB or ODQ of L-NAME-induced antinociception.

Pilot experiments showed that doses higher than 200 μg/paw were needed to give significant antinociception. Fig. 3 shows a significant antinociceptive
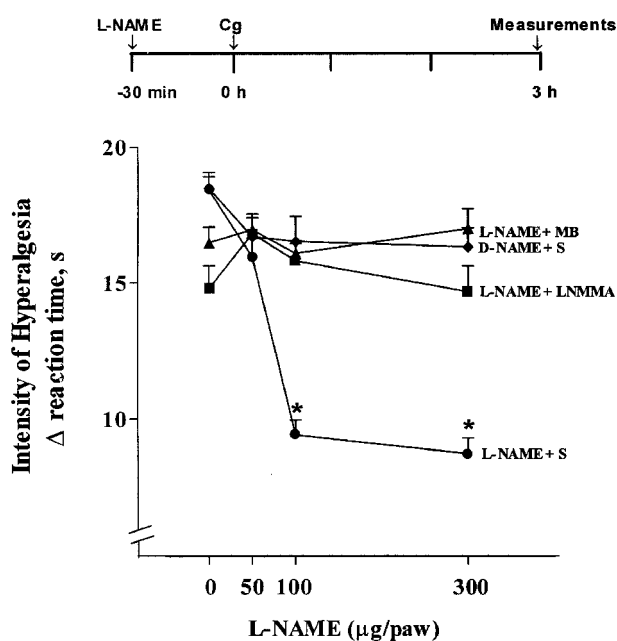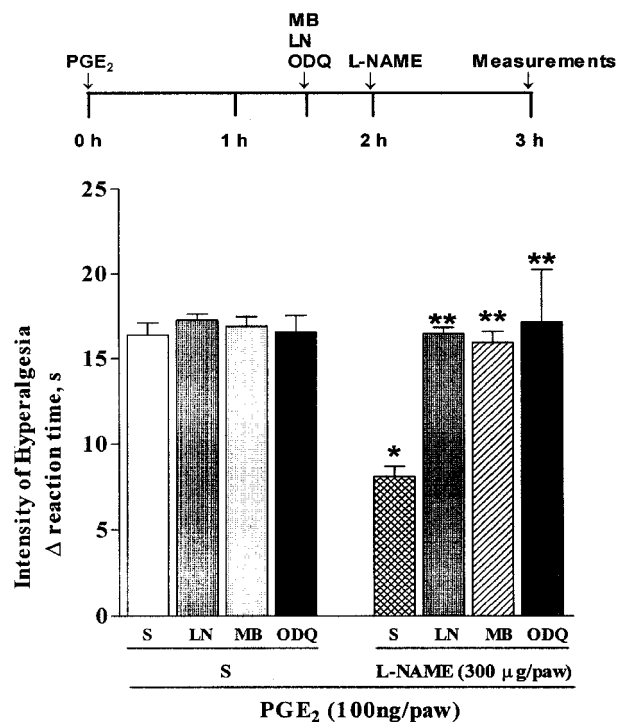

FIG. 2. Antinociceptive effect of intraplantar administration of L-NAME but not of D-NAME on carrageenin-induced hyperalgesia and the blockade of antinociception by local L-NMMA and methylene blue (MB) co-administration. L-NMMA (100 μg/paw), MB (500 μg/paw) or saline (S) were injected 30 min before carrageenin (Cg, 100 μg/paw). L-NAME or D-NAME (50–300 μg/paw) were also injected 30 min before Cg, and the intensity of hyperalgesia was measured 3 h after the hyperalgesic stimulus (see injections diagram). The symbols are the mean + SEM of five rats/group. *indicates significant differences ($P < 0.01$) in comparison with the control (saline, O) or treatment with L-NAME, MB, D-NAME.

FIG. 3. Blockade by L-NMMA (LN) and guanylate cyclase inhibitors (MB and ODQ) of the antinociceptive effect of L-NAME on PGE$_2$ induced hyperalgesia. PGE$_2$ was injected at time zero, saline (S) or LN, MB,ODQ, at time 1.5 h and L-NAME or S 2 h after PGE$_2$. The insert shows the antinociceptive effect of L-NAME (60 = a, 180 = b and 300 μg per paw = c). The bars and symbols are the mean ± SEM of five rats/group. The asterisks mean significant differences ($P < 0.05$): (a) *in comparison with the control PGE$_2$; (b) **in comparison with L-NAME-treated groups.
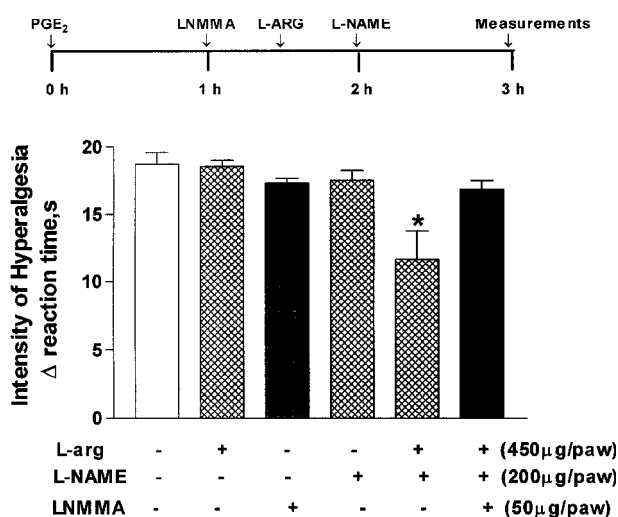
FIG. 4. Antinociceptive synergism between L-NAME and L-arginine. The measurements were made 3 h after the intraplantar injections of PGE_2 (100 ng/paw). L-NMMA, L-Arginine (L-Arg) and L-NAME were injected into the paw 1, 1.5 and 2 h after the PGE_2 challenge, respectively. The bars are the mean ± SEM of five rats/group. The asterisks mean significant differences ($P < 0.05$): (a) *in comparison with the control PGE_2 treated either with L-arg, L-NAME, or saline (S); (b) **in comparison with the group which received L-NMMA + L-arg + L-NAME.
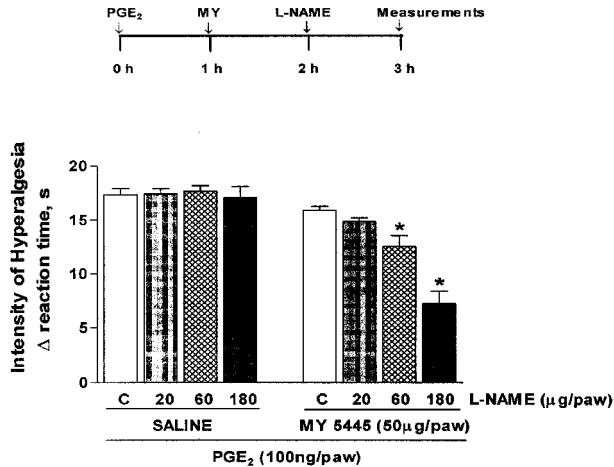

FIG. 5. Potentiation of intraplantar injections of L-NAME by the cGMP phosphodiesterase inhibitor, MY 5445. The measurements were made 3 h after the intraplantar injections of PGE_2 (100 ng/paw). MY 5445 and (or) L-NAME were injected into the paw 1 and 2 h after PGE_2, respectively. The bars are the mean ± SEM of five rats/group. The asterisk means significant differences ($P < 0.05$) compared with respective doses of L-NAME. There was no significant differences between the controls groups (C) treated with saline or MY 5445.
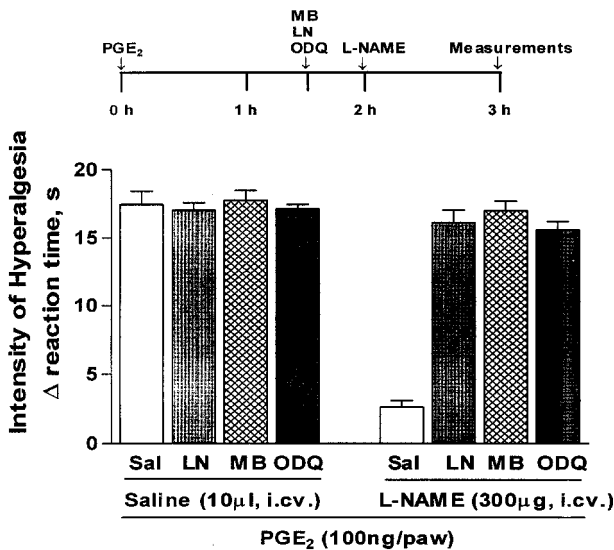
effect upon hyperalgesia induced by PGE_2 of a dose of 300 μg of L-NAME. Pretreatment of the paws with L-NMMA (LN), MB or ODQ prevented the antinociceptive effect of L-NAME.

(b) Arginine and MY 5445 enhancement of L-NAME effects.

Figure 4 shows that the association of L-arginine and L-NAME treatments caused a significant antinociception compared with single treatments. There was no difference among controls groups. L-NMMA significantly inhibited the antinociceptive effect of the association of L-arginine and L-NAME treatment. The association of the same doses of L-NAME and D-arginine did not cause antinociception as compared with the single treatment (data not shown).

Figure 5 shows that MY 5445 enhanced, in a dose-related manner, the antinociceptive effect of L-NAME. A dose of 180 μg, which did not produce antinociception in our experiments, produced an effect similar to 300 μg when the paws were pretreated with MY 5445 (compare with Fig. 3).

(c) Blockade of the intracerebroventricular antinociceptive effect of L-NAME by co-treatment with L-NMMA, MB or ODQ.

L-NAME, when administered i.c.v. at a dose of 300 μg per rat, produced potent antinociception in paws rendered hyperalgesic by PGE_2 (Fig. 6). This L-NAME-induced anti-hyperalgesic effect was abolished by co-i.c.v. administration of L-NMMA (300 μg), MB (400 μg) and ODQ (8 μg). Neither L-NMMA, nor MB,

FIG. 6. Antinociceptive effect of intracerebroventricular administration of L-NAME (N^G-nitro-L-arginine methyl ester) on PGE_2-induced hyperalgesia, and its blockade by i.c.v. L-NMMA (LN, 300 μg,), MB (400 μg) or ODQ (8 μg). These drugs and vehicle were co-injected with L-NAME 2 h after PGE_2 (100 ng/paw). The intensity of hyperalgesia was measured 1 h after the i.c.v. injections. The bars are the mean, SEM of five rats/group. *,**mean significant differences ($P < 0.01$) in comparison with the saline- (Sal) or L-NAME-treated group, respectively.

nor ODQ had any effects on PGE_2 induced hyperalgesia.

Discussion

L-NAME has been shown by several laboratories[1-8] to have a peripheral and central antinociceptive action.

Here, we confirm their observations using two tests of nociception. In the acetic acid writhing test, systemic administration (i.p.) of L-NAME but not L-NMMA caused an antinociceptive effect (Fig. 1). In addition, we showed that L-NMMA inhibited the antinociceptive activity of L-NAME in this test as well as in the rat paw pressure test in which hyperalgesia was induced either by an inflammatory stimuli like Cg or by an hyperalgesic mediator, PGE$_2$ (Figs 2–4). The D-isomer of NAME showed no antinociceptive activity (Fig. 2) in carrageenin-induced hyperalgesia in the rat pressure tests (Fig. 2). It has already been shown that this isomer had no antinociceptive effect in other tests.[2] Co-injection of L-NMMA i.c.v. also inhibited the central analgesic action of L-NAME (Fig. 6).

The observed antinociceptive effect of L-NAME apparently supports the idea that the arginine/NO/cGMP pathway contributes to nociception induced by inflammatory stimuli, particularly because L-NAME is considered to be a selective NO synthase inhibitor.[2,24] L-arginine-derived inhibitors, however, have been found to have bizarre pharmacological effects. For instance, it has been demonstrated that L-NAME is a poor inhibitor of L-arginine transport, whereas L-NMMA and L-NIO substantially inhibit this event.[25] Moreover, L-NAME, but not L-NMMA, is a muscarinic antagonist.[26] On the other hand, L-NMMA, has been shown to behave as a partial agonist, since it antagonizes NO synthesis in some tissues, but stimulates NO synthesis in isolated arterial rings.[27]

Since, in our experiments, L-NMMA in the doses used did not show any effect per se, but blocked the antinociceptive effect of L-NAME, we assume that it may be acting as a NO synthase inhibitor. On the other hand, L-NAME may be acting either as a substrate for or as an iNOS stimulator.

It is known that during carrageenin (but not PGE$_2$) induced hyperalgesia the arginine/NO/cGMP pathway is activated.[28] The absence of activity of the arginine NO cGMP pathway in PGE$_2$-induced hyperalgesia is illustrated here by the fact that neither the NO synthase inhibitors (MB or ODQ) nor the cGMP phosphodiesterase inhibitor, MY5445, have any effect upon control hyperalgesia (Figs 3–5). Nevertheless, L-NAME displayed an antinociceptive effect. Recently, L-NAME has been described as acting as a partial agonist,[27] causing rapid induction of iNOS gene expression.[21] Stimulation of NO synthesis may explain the observed L-NAME antinociception, in models like those used in this investigation. In these models it has been previously shown that either NO donors or drugs which stimulate the arginine NO cGMP pathway cause analgesia.[9,10,13–18] Thus, the simplest comprehensive explanation for the fact that L-NAME antinociception was inhibited by a NO synthase inhibitor, L-NMMA and was abolished by guanylate cyclase inhibitors, MB or ODQ as well as potentiated by either the NO synthase substrate,

arginine or by the cGMP phosphodiesterase inhibitor MY5445 is that L-NAME is activating iNOS. The fact that i.c.v. co-injections of MB or ODQ inhibited the central antinociceptive effect of L-NAME suggests a similar mechanism of action for the peripheral and central action for this agent.

Finally, it must be pointed out that, in contrast with the results presented here, there are several observations indicating that the intraplantar or systemic administration of L-NAME has similar effects to other NO synthase inhibitors in causing antinociception.[4,20] This contradiction may explained by considering that the activation of the arginine/NO/cGMP pathway causes hyperalgesia or analgesia depending on the predominant type of fibres involved in the nociceptive response or depending on the tissue level of NO.[20] From the therapeutic point of view, however, it seems that during inflammatory pain in man, the activation of the arginine/NO/cGMP pathway causes analgesia. This suggestion is in line with the observations that NO donors are either effective as analgesics by themselves or in conjunction with other analgesics.[29–32]

In conclusion, the present study confirms that L-NAME causes analgesia and demonstrated that L-NMMA, another NO synthase inhibitor, significantly blocked both the peripheral and central antinociceptive actions of L-NAME in rats and mice. L-NAME antinociception was also blocked by inhibitors of guanilate-cyclase activation and potentiated by arginine and by a cGMP phosphodiesterase inhibitor. These results allow us to speculate that L-NAME causes antinociception by acting as a partial agonist, thus stimulating iNOS activation in the nociceptive tests used. Furthermore our results draw into question the use of L-NAME alone as a methodological tool to characterise the nociceptive role of the arginine-NO-cGMP pathway in physiopathological processes, in the absence of confirmation with another NO synthase inhibitor.

Acknowledgements

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