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Effect of chronic nitric oxide synthesis inhibition on the inflammatory responses induced by carrageenin in rats

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Abstract

The effect of chronic inhibition of nitric oxide (NO) biosynthesis has been investigated in two models of acute inflammation induced by carrageenin, i.e., paw oedema and pleurisy. Chronic inhibition of NO biosynthesis was achieved by including N^{ω}-nitro-L-arginine methyl ester (L-NAME) in the drinking water to give a dose of approximately 75 μmol/rat/day for 2 and 4 weeks. Control animals received either tap water alone or the inactive enantiomer D-NAME. Since chronic NO inhibition increases blood pressure, rats made hypertensive (2 kidney-1 clip model; 2K-1C) were used to evaluate the effect of hypertension on the carrageenin-induced paw oedema. In a separate set of experiments, L-NAME-treated animals concomitantly received captopril (140 μmol/rat/day) to prevent hypertension. Animals chronically treated with L-NAME (but not D-NAME) for 2 and 4 weeks developed hypertension to the same extent as 2K-1C rats. Carrageenin-induced paw oedema was significantly reduced in animals chronically treated with L-NAME, but not with D-NAME or in 2K-1C rats. Subplantar injection of iloprost completely reversed the inhibition of paw oedema caused by L-NAME. Captopril (140 μmol/rat/day) significantly lowered the high blood pressure levels induced by L-NAME but did not significantly affect the inhibition of paw oedema caused by L-NAME. No changes in vascular permeability, as assessed by Evans blue extravasation, were observed in L-NAME-treated animals. The chronic treatment with L-NAME for 2 and 4 weeks did not inhibit carrageenin-induced leucocyte migration and fluid exudation into the pleural cavity. Although carrageenin-induced paw oedema is reduced in L-NAME-treated rats, this response reflects a decrease in local blood flow rather than an effect on vascular permeability.

Keywords: Iloprost; Nitric oxide (NO); Acute inflammation; Captopril; Hypertension; (2K-1C rat)

1. Introduction

The acute inflammatory reaction is characterised by exudation of fluid and plasma proteins leading to a local oedema formation consisting of leucocyte-dependent and leucocyte-independent components (for review see Garcia-leme, 1989). These vascular changes are produced by different mediators which act mainly by increasing the microvascular permeability to macromolecules in the postcapillary venules thus enhancing plasma protein efflux. The administration of vasodilators such as prostaglandin E_1 (Williams and Peck, 1977; Chahl, 1977; Amelang et al., 1981; Yong and Mayhan, 1992), prostaglandin E_2 (Ikeda et al., 1975), prostacyclin (Williams, 1979), tachykinins (Brain et al., 1985; Gamse and Saria, 1985) and vasoactive intestinal polypeptide (Williams, 1982) markedly potentiates the increased vascular permeability induced by various agents. This potentiation has been explained as a result of an arteriolar vasodilator action of vasodilators further increasing local blood flow (Williams and Peck, 1977). In contrast, the potentiation of oedema formation induced by prostaglandin E_1 in the hamster cheek pouch (Joyner et al., 1979) and canine forelimb (Amelang et al., 1981) has been attributed to a direct action of this agent on the microvascular membrane leading to an increase in the number of large venular gaps which is not influenced by major changes in blood flow.

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Nitric oxide (NO) is a potent vasodilator agent produced by the endothelium (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Palmer et al., 1987) and modulates the microvascular tone in certain vascular beds (Gardiner et al., 1990). Nitric oxide is involved in the modulation of acute inflammation since acute administration of NO inhibitors affects both the interaction of inflammatory cells with the endothelium (Kubes et al., 1991; Kurose et al., 1993) and vascular protein leakage (Hughes et al., 1990; Ialenti et al., 1992; Antunes et al., 1992; Kubes and Granger, 1992; Paul et al., 1994; Giraldelo et al., 1994). In this study, we have investigated the involvement of NO in the inflammatory responses (paw oedema and pleurisy) induced by carrageenin in rats chronically treated with the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and its inactive enantiomer D-NAME. Since the chronic treatment of rats with L-NAME leads to hypertension (Ribeiro et al., 1992), we have carried out experimental studies using animals made hypertensive by the occlusion of the left renal artery (2 kidney-1 clip model). Captopril was also added to the drinking water in order to reduce the increased blood pressure induced by chronic L-NAME administration.

2. Materials and methods

2.1. Measurement of paw oedema

Male Wistar rats (150–200 g) were used. Oedema was induced by a single sub-plantar injection of carrageenin (1 mg/paw) into the left hind paw of the rat under light ether anaesthesia. The total volume injected was always 0.1 ml. The paw volume was measured immediately before the injection and at hourly intervals thereafter using a hydroplethysmometer (model 7150, Ugo Basile, Italy). The results were expressed either as the increase in paw volume (ml) calculated by subtracting the basal volume or by calculating the area under the time-course curve (AUC; ml h) for each group.

2.2. Assessment of vascular response with Evans blue

Evans blue (25 mg/kg, 2.5% w/v in 0.45% NaCl) was injected i.v. immediately before subplantar injection of carrageenin into the left hind paw. Four hours later, the animals were killed and the paws were removed and minced. The minced paws were then incubated with formamide (5 ml) for 48 h at 37°C. The solution was subsequently filtered and the optical density of the filtrate measured at 619 nm in a Uvikon 810 spectrophotometer (Lykke and Cummings, 1969). The amount of Evans blue was expressed as μg/paw and the results presented as the difference in the quantity of Evans blue between the oedematogenic paw and the contralateral paw.

2.3. Measurement of pleurisy

Male Wistar rats (150–200 g) were lightly anaesthetised under ether and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscles were dissected and either carrageenin (0.1, 0.5 and 1 mg/cavity) or sterile saline injected into the pleural cavity. The total volume injected was always 0.2 ml. The skin incisions were closed with a suture and the animals allowed to recover. At 2, 4 and 6 h after the injection of carrageenin, the animals were exsanguinated under ether anaesthesia by cutting the cervical vessels. The chest was carefully opened and the pleural cavity washed with 5 ml of heparinised (10 U/ml) phosphate buffered saline (0.1 M, pH 7.4). The exudate and washings were removed by aspiration and the total volume measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (5 ml) from the total volume recovered. The number of leucocytes in the exudate was counted by conventional techniques.

2.4. Two kidney-1 clip (2K-1C) model

Male Wistar rats (150–180 g) anaesthetised with sodium pentobarbital (Sagatal, 40 mg/kg i.p.) were used. Two kidney-1 clip hypertension was induced by compression of a silver clip reduced to 0.2 mm aperture around the left renal artery.

2.5. Chronic treatment with L-NAME and D-NAME

The chronic treatment with L-NAME was performed as previously described (Ribeiro et al., 1992). Briefly, the animals received L-NAME (or D-NAME) in the drinking water for up to 4 weeks. L-NAME (or D-NAME) was dissolved in the drinking water at a concentration of 1.2 mM to give a daily intake of approximately 75 μmol/rat/day. Control animals received tap water alone. The average daily intake of both water and food did not differ significantly between L-NAME-treated and untreated rats. In some experimental groups, captopril (2.3 mM) was added to the drinking water together with L-NAME to give a daily intake of approximately 140 μmol/rat/day. Systemic arterial pressure was estimated weekly by a tail-cuff method (Zatz, 1990).

2.6. Drugs

N^{ω}-Nitro-L-arginine methyl ester and λ-carrageenin were obtained from Sigma Chemical Co. (USA). Nitro-D-arginine methyl ester and iloprost were obtained

from Bachem (Switzerland) and Schering (Germany), respectively. Captopril was provided by Squibb (USA).

2.7. Statistical analysis

Results are expressed as mean ± S.E.M. for n experiments. Statistical comparison was undertaken by means of Student's unpaired t-test (two-tailed) or analysis of variance (ANOVA) and the Bonferroni corrected P value for multiple comparisons. Values of $P < 0.05$ were considered as significant.

3. Results

3.1. Blood pressure

Fig. 1 shows that addition of L-NAME, but not D-NAME, to the drinking water resulted in a significant elevation of mean arterial blood pressure ($P < 0.05$), as measured 2 and 4 weeks after the treatment ($n = 20$–50). In addition, 2K-1C rats ($n = 30$) developed hypertensive levels which did not differ significantly from the L-NAME-treated rats after 2 and 4 weeks (Fig. 1). The blood pressure of 2K-1C rats was not further increased by concomitant treatment with L-NAME (Fig. 1). The co-administration of captopril ($n = 20$) in the drinking water significantly reduced the increase in blood pressure induced by L-NAME administration (Fig. 1).

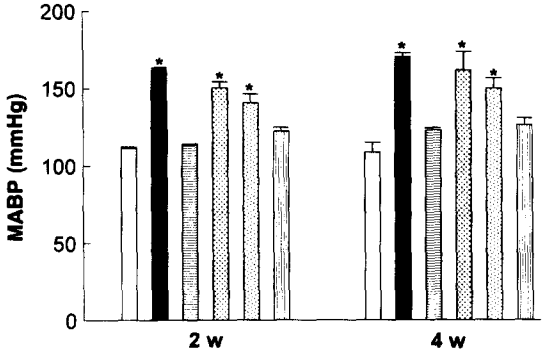

Fig. 1. Addition of L-NAME (approximately 75 μmol/rat/day) to the drinking water for 2 (2 W) and 4 weeks (4 W) markedly enhances the mean arterial blood pressure (MABP; solid columns) compared to the control animals (open columns). D-NAME had no effect on MABP (horizontal striped columns). The addition of captopril to the drinking water for 2 and 4 weeks significantly reversed the increased MABP induced by L-NAME (vertical striped columns). The MABP of 2K-1C rats 2 and 4 weeks following the sub-occlusion of the left renal artery (cross-hatched columns) and for 2K-1C rats receiving L-NAME (white dotted columns) is also shown. Each column represents the mean ± S.E.M. of 15–20 animals. $^*P < 0.05$ compared to control animals receiving tap water alone.

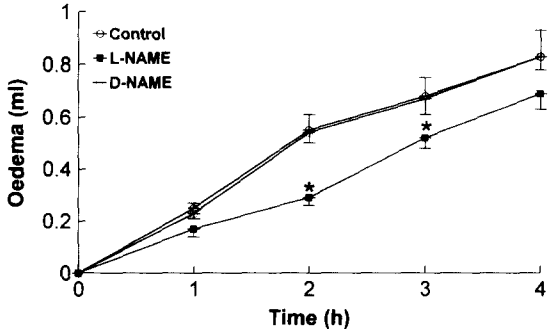

Fig. 2. The inhibition of carrageenin-induced rat paw oedema by chronic treatment with L-NAME for 2 weeks (■) compared to control animals receiving either tap water (○) or D-NAME (□). The procedure for L-NAME administration is described in the Materials and methods section (see also the legend to Fig. 1). The oedema is expressed as the increase in volume (ml) of the injected paw compared to its basal volume. Each point represents the mean of five rats; S.E.M. is shown by the vertical bars. $^*P < 0.05$ compared to control animals.

3.2. Carrageenin-induced oedema

The carrageenin-induced oedema was significantly reduced in animals chronically treated with L-NAME for either 2 weeks (2.6 ± 0.2 and 1.7 ± 0.1 ml h, AUC for control- and L-NAME-treated animals, respectively, $n = 15$, $P < 0.01$; Fig. 2) or 4 weeks (3.2 ± 0.3 and 1.6 ± 0.2 ml h, AUC for control- and L-NAME-treated animals, respectively, $n = 15$–20, $P < 0.01$; Fig. 3). The reduction of carrageenin-induced oedema by chronic L-NAME was not accompanied by significant changes in the protein leakage (at 2 weeks: 291 ± 49 and 268 ± 29 μg Evans blue/paw for control- and L-NAME-treated animals, respectively; at 4 weeks: 312 ± 32 and 243 ± 31 μg Evans blue/paw for control- and L-NAME-treated animals, respectively, $n = 5$). The

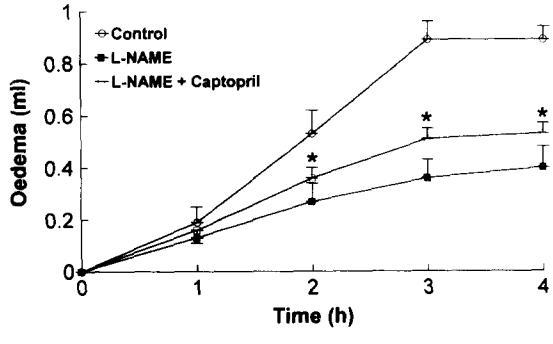

Fig. 3. Captopril does not significantly reverse the L-NAME-induced inhibition of carrageenin (1 mg/paw)-induced rat paw oedema. The symbols represent control animals receiving tap water (○), L-NAME alone (■) and L-NAME + captopril (□). Captopril (140 μmol/rat/day) was added to the drinking water together with L-NAME for 4 weeks. The oedema is expressed as the increase in volume (ml) of the paw compared to its basal volume. Each point represents the mean of ten rats; S.E.M. is shown by the vertical bars. $^*P < 0.05$ compared to control animals.

basal protein leakage was also not affected by chronic treatment with L-NAME (22 ± 3.0, 23 ± 1.7 and 24 ± 1.8 μg Evans blue/paw for control and for L-NAME-treated animals at 2 and 4 weeks, respectively, $n = 5$–10). Chronic administration of D-NAME did not significantly affect the carrageenin-induced oedema ($n = 5$, Fig. 2). The subplantar injection of iloprost (0.3 nmol/paw) significantly potentiated carrageenin-induced oedema (2.1 ± 0.4 and 4.9 ± 0.3 ml h, AUC for control- and iloprost-treated paws, respectively, $n = 5$, $P < 0.01$). At this dose, iloprost impaired the inhibition by L-NAME (4 weeks treatment) of the carrageenin-induced oedema (1.4 ± 0.3 and 5.0 ± 0.7 ml h, AUC for control- and iloprost-treated animals, respectively, $n = 5$).

In contrast to the L-NAME-treated group, the carrageenin-induced oedema was not reduced in 2K-1C rats ($n = 24$; Fig. 4); actually, a significant increase was observed (Fig. 4). In addition, the paw oedema was not further inhibited in 2K-1C rats which received L-NAME for 4 weeks ($n = 5$, Fig. 4) compared to L-NAME-treated animals. Chronic treatment of the animals with captopril for 4 weeks (Fig. 3) did not significantly affect the inhibition by L-NAME of the carrageenin-induced oedema (2.6 ± 0.3, 1.2 ± 0.2 and 1.6 ± 0.2 ml h, AUC for control-, L-NAME- and L-NAME + captopril-treated animals, respectively, $n = 10$).

3.3. Carrageenin-induced pleurisy

Intrapleural injection of carrageenin caused dose-dependent (0.2 ± 0.1, 0.7 ± 0.08 and 1.1 ± 0.2 ml 6 h after 0.1, 0.5 and 1.0 mg carrageenin/cavity, respectively; $n = 5$) and time-dependent (0.6 ± 0.2, 0.7 ± 0.1 and 1.1 ± 0.2 ml for 2, 4 and 6 h, after intrapleural administration of 0.5 mg carrageenin/cavity, respectively; $n = 5$) exudate formation into the pleural cavity. Intrapleural injection of carrageenin also induced dose-dependent (10.8 ± 1.0, 43.3 ± 6.7 and 75.8 ± 9.4 × 10^6 cells/cavity 4 h after 0.1, 0.5 and 1.0 mg carrageenin/cavity, respectively, $n = 5$–15) and time-dependent (12.2 ± 1.7, 37.4 ± 4.7 and 71.0 ± 7.5 × 10^6 cells/cavity for 2, 4 and 6 h, after intrapleural administration of 0.5 mg carrageenin/cavity, respectively, $n = 5$) leucocyte infiltration into the pleural cavity. The dose of 0.5 mg/cavity and the 6 h time point were selected for further studies. Chronic treatment with L-NAME for either 2 or 4 weeks affected neither the formation of pleural exudate nor the leucocyte infiltration (Table 1). At the 6 h time point, intra-pleural administration of carrageenin (0.5 mg/cavity) in control animals ($n = 5$) induced migration of neutrophils (72.6 ± 3.8%), eosinophils (2 ± 0.3%) and mononuclear cells (26.2 ± 3.8%). Chronic treatment with L-NAME for 4 weeks did not alter the cell pattern observed following carrageenin administration (76.2 ± 9%, 2.4 ± 0.6% and 21.4 ± 0.7% for neutrophils, eosinophils and mononuclear cells respectively, $n = 5$).

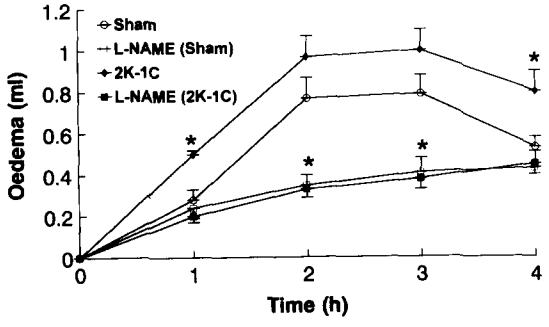

Fig. 4. Two kidney-one clip (2K-1C) rats (♦) did not show diminished carrageenin (1 mg/paw)-induced paw oedema as compared to normotensive sham-operated animals (○). The surgical procedure involved in the ligation of the left renal artery is described in the Materials and Methods section. Four weeks treatment of sham-operated animals with L-NAME significantly inhibited carrageenin-induced oedema (■). The oedematogenic response in 2K-1C rats receiving L-NAME (dose above; □) was not different from sham-operated animals receiving L-NAME. The oedema is expressed as the increase in volume (ml) of the paw compared to its basal volume. Each point represents the mean of five rats; S.E.M. is shown by vertical bars. $^*P < 0.05$ compared to normotensive sham-operated animals.

Table 1
Formation of pleural exudate and leucocyte infiltration induced by carrageenin in rats chronically treated with either L-NAME or D-NAME for 2 and 4 weeks

	Pleural exudate (ml)		Leucocyte counts (cells × 10^6/cavity)	
Treatment	Two weeks	Four weeks	Two weeks	Four weeks
Control	0.84 ± 0.1	0.45 ± 0.12	78.0 ± 8.1	64.6 ± 5.2
D-NAME	0.74 ± 0.2	n.d.	77.0 ± 10.5	n.d.
L-NAME	0.78 ± 0.15	0.4 ± 0.18	68.8 ± 3.6	60.8 ± 5.9

Pleural exudate and leucocyte counts (cells × 10^6/cavity) were evaluated 6 h after the intrapleural injection of carrageenin (0.5 mg/cavity). L-NAME and D-NAME were added to the drinking water at a dose of approximately 75 μmol/rat/day. The counts represent the mean number of leucocytes ± S.E.M. for 8–10 animals. n.d., not determined.

4. Discussion

We have demonstrated here that the chronic administration of L-NAME (but not D-NAME) inhibits carrageenin-induced rat paw oedema, indicating that endogenous NO modulates local oedema formation. However, the mechanism by which NO modulates protein and fluid leakage has not been definitely established. Although it is believed that acute administration of L-NAME reduces albumin extravasation in response to a decrease in local microvascular blood

flow in rat (Hughes et al., 1990; Ialenti et al., 1992), guinea-pig (Teixeira et al., 1993) and rabbit (Mariani-Pedroso et al., 1995) skin as well as oedema formation in the rat hind-paw (Antunes et al., 1990, 1992; Ialenti et al., 1992; Giraldelo et al., 1994), NO per se seems to modulate microvascular permeability in the hamster cheek pouch by a mechanism independent of local blood flow (Mayhan, 1992). However, our results showing that the vasodilator prostacyclin analogue iloprost (Skuballa et al., 1987) reversed the inhibition by L-NAME of the carrageenin-induced oedema indicate that instead of directly affecting permeability, L-NAME probably acts by reducing regional blood flow. This conclusion is supported by the observation that both basal and carrageenin-induced plasma extravasation are not significantly affected by the administration of L-NAME. Whether this discrepancy (inhibition of oedema without concomitant inhibition of protein extravasation) reflects different kinetics of the two events, remains to be further investigated.

In contrast to our observations in the rat hind-paw, in other microcirculatory beds including rat large airways, liver, spleen, pancreas, kidney, stomach and duodenum (Filep and Foldes-Filep, 1993), cat mesenteric circulation (Kubes and Granger, 1992), rat coronary circulation (Filep and Foldes-Filep, 1993) and rat mesenteric venules (Kurose et al., 1993), L-NAME increases microvascular protein efflux indicating that NO has a protective role in the endothelium by minimising the loss of fluid and protein. Whether this discrepancy could be due to atropine-like properties of L-NAME (Buxton et al., 1993) remains to be investigated.

The hypertension induced by chronic treatment with L-NAME is mediated by activation of the renin-angiotensin-aldosterone system (Ribeiro et al., 1992). However, rats made hypertensive by activation of this system (2K-1C) did not show reduced oedema formation indicating that high circulating levels of angiotensin II are not a major component responsible for the modulation of local microvascular blood flow in the rat paws. Furthermore, captopril markedly reduced the increased blood pressure resulting from chronic L-NAME treatment but did not significantly reverse the L-NAME-mediated inhibition of the carrageenin-induced oedema. These results suggest that the reduction in microvascular blood flow in response to L-NAME is not dependent on the increased blood pressure of the animals but probably reflects a local absence of NO production.

Carrageenin-induced oedema in the 2K-1C rats was significantly higher as compared to sham-operated animals. Interestingly, 1K-1C animals present increased blood kinin levels as compared to normotensive controls (Salgado et al., 1986). Since kinins are involved in the carrageenin-induced oedema (Di Rosa et al., 1971), it is possible that the augmented oedematogenic response observed in these animals could be due to increased kinins levels. This could also explain the increased oedematogenic response observed in the L-NAME-treated rats that received captopril.

The inflammatory response induced by carrageenin is dependent on circulating leucocytes (Spector and Willoughby, 1959; Di Rosa et al., 1971). Neutrophils and mononuclear cells synthesise NO (Salvemini et al., 1989) and this mediator is believed to exhibit an inhibitory effect on the adherence and emigration of leucocytes in the postcapillary venules (Kubes et al., 1991). However, the ability of NO inhibitors in suppressing leukocyte accumulation is controversial (Mulligan et al., 1991; Teixeira et al., 1993), probably reflecting sensitivity of the vascular bed to NO. Our finding that chronic treatment with L-NAME affected neither the formation of pleural exudate nor the leucocyte migration induced by carrageenin indicates that NO does not modulate these phenomena in this particular microcirculatory bed.

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