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Dose- and time-dependence of L-NAME neuroprotection in transient focal cerebral ischaemia in rats

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1 In this study the effect of the dose and administration time of N^G-nitro-L-arginine methyl ester (L-NAME), an NO-synthase inhibitor, in a model of transient focal cerebral ischaemia in rats was investigated.

2 Two injections of L-NAME were given, of 1, 3 and 10 mg kg^{-1}, 5 min and 3 h after the onset of ischaemia. None of the doses gave any striatal neuroprotection, but 1 and 3 mg kg^{-1} L-NAME reduced the infarcted volume in the cortex (by 26%, $P<0.01$ for 1 mg kg^{-1} and 21%, $P<0.05$ for 3 mg kg^{-1}), whereas 10 mg kg^{-1} had no neuroprotective effect.

3 Single injections of L-NAME 1 mg kg^{-1}, given 5 min or 3 h after ischaemia onset, had similar neuroprotective effects on the cortical infarction as did the repeated injections.

4 L-NAME 1 mg kg^{-1} given 3, 6 or 9 h after ischaemia induction reduced the cortical infarct volume by 19% ($P<0.01$) when given 3 h after ischaemia, by 21% ($P<0.01$) when given at 6 h, and by 16% ($P<0.5$) when given at 9 h, but had no neuroprotective activity when given 12 h after ischaemia.

5 Thus a low dose of L-NAME is neuroprotective in a model of transient focal ischaemia, with a wide therapeutic window, much larger than that found for MK-801.

Keywords: Focal cerebral ischaemia; ischaemia-reperfusion; nitric oxide; N^G-nitro-L-arginine methyl ester (L-NAME)

Introduction

We have demonstrated that the nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (3 mg kg^{-1}) given intraperitoneally 5 min and 3 h after the onset of ischaemia is neuroprotective in a rat model of permanent focal cerebral ischaemia (Buisson *et al*., 1993). However, NOS inhibitors have given conflicting results, in the same model, ranging from reduction to no effect, or even a worsening of the ischaemic damage. These studies differed in the conditions of anaesthesia, the strain of rats, the NO-synthase inhibitor, the time, route and doses of inhibitor (for a review: Verrecchia *et al*., 1995). Transient focal cerebral ischaemia studies in which the effect of L-NAME was examined have also given contradictory results: Ashwal *et al*. (1993) showed that L-NAME (0.1 mg kg^{-1} bolus given 1 h before ischaemia followed by 0.01 mg kg^{-1} min^{-1} for 5 h) reduced the infarcted volume assessed 5 h after ischaemia by 55%; by contrast, Kuluz *et al*. (1993) found that rats pretreated with 15 mg kg^{-1} L-NAME had an increased infarcted volume (137%) 3 days after ischaemia. These conflicting results led us to investigate the dose- and time-dependence of the activity of L-NAME in transient focal cerebral ischaemia.

Methods

All experiments were performed in strict accordance with the guidelines of the NIH and French Department of Agriculture (Licence no. 01352).

Transient focal ischaemia

Male Sprague-Dawley rats (300–350 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.). The tail artery was cannulated to monitor mean arterial blood pressure (MABP) and arterial blood gases. Both common carotid arteries and the left middle cerebral artery (MCA) were occluded for 1 h, as previously described (Margaill *et al*., 1996), and the body temperature was maintained at 37–38°C throughout the procedure. Physiological variables were measured just before ischaemia, 30 min after the onset of ischaemia, and 30 min after reperfusion.

Quantification of infarction

Rats were killed with an overdose of pentobarbitone 24 h after ischaemia onset. Brains were removed and cryostat coronal brain sections were cut and stained with cresyl violet. The striatal and cortical areas of infarction were measured on each section by an image analyser (IMSTAR, Paris, France). Cortical and striatal necrotic volumes were determined by integrating the area measurements corrected for oedema according to Golanov & Reis (1995).

Experimental protocols

Dose-dependent effect of repeated administrations of L-NAME Animals were given L-NAME 1, 3 and 10 mg kg^{-1} or distilled water (L-NAME vehicle) intraperitoneally 5 min and 3 h after the onset of ischaemia.

Effect of a single and repeated injections of L-NAME Animals were given intraperitoneally, distilled water or a single injection of L-NAME (1 mg kg^{-1}) 5 min or 3 h after ischaemia induction, or injections of L-NAME both 5 min and 3 h after the onset of ischaemia.

Therapeutic window of a single injection of L-NAME L-NAME (1 mg kg^{-1}) was given intraperitoneally 3, 6, 9 or 12 h after the onset of ischaemia. Control rats were given distilled water at 3 h ($n=2$), 6 h ($n=2$), 9 h ($n=2$) or 12 h ($n=3$) after ischaemia induction.

Data expression and statistical analysis

Data are expressed as means ± s.e.mean. Statistical comparisons were performed by one-way analysis of variance (ANOVA) with subsequent individual comparisons by a PLSD Fisher test.

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Results

Dose-dependent action of multiple injections of L-NAME

The cortical and striatal infarct volume in control rats ($n=8$) were 251 ± 18 and 42 ± 2 mm^3. L-NAME 1 mg kg^{-1} ($n=10$) given 5 min and 3 h after ischaemia onset resulted in a 26% reduction in the cortical infarct volume (186 ± 11 mm^3, $P<0.01$). Doses of 3 mg kg^{-1} L-NAME ($n=8$) reduced the cortical infarct volume by 21% (198 ± 22 mm^3, $P<0.05$), while 10 mg kg^{-1} L-NAME ($n=5$) did not significantly alter the cortical infarct volume (220 ± 26 mm^3) and 3 of these rats died during the night following ischaemia. Rats given 2 injections of 1, 3 or 10 mg kg^{-1} L-NAME and control rats had similar striatal infarct volumes (41 ± 3, 41 ± 2, 43 ± 3 and 42 ± 2 mm^3, respectively) (Figure 1). The physiological variables were all within normal ranges before and during ischaemia and during reperfusion. There were no differences between the control and treated groups (Table 1).

Effects of a single and repeated injections of L-NAME

The cortical and striatal infarcted volumes in control rats ($n=9$) were 246 ± 11 and 51 ± 3 mm^3. Two injections of L-

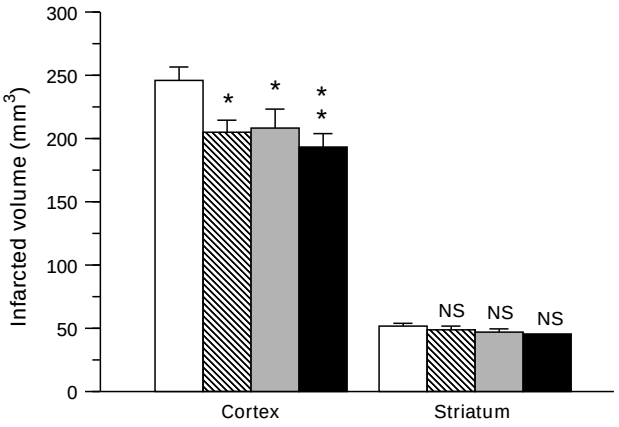

Figure 2 Effect of single and repeated injections of L-NAME on infarct volumes induced by transient focal cerebral ischaemia. L-NAME (1 mg kg^{-1}, i.p.) was given 5 min (hatched column, $n=8$), 3 h (stippled column, $n=9$) or 5 min and 3 h (solid column, $n=6$) after ischaemia induction; vehicle: open column ($n=9$). Values are means$\pm$s.e.mean. Comparison *versus* vehicle: NS: non significant; $^{*}P<0.5$, $^{**}P<0.01$.

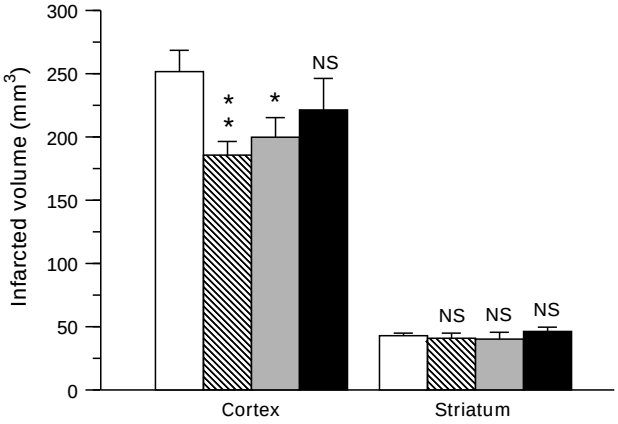

Figure 1 Dose-dependence of the effect of L-NAME on infarct volumes induced by transient focal cerebral ischaemia. Injections were given intraperitoneally 5 min and 3 h after the onset of ischaemia: vehicle (open columns, $n=8$), L-NAME: 1 mg kg^{-1} (hatched columns, $n=10$), 3 mg kg^{-1} (stippled columns, $n=8$), 10 mg kg^{-1} (solid columns, $n=5$). Values are means$\pm$s.e.mean. Comparison *versus* vehicle. NS: non significant; $^{*}P<0.05$; $^{**}P<0.01$.

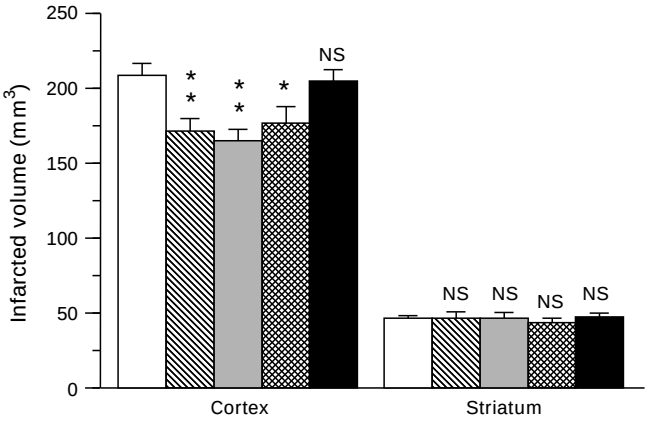

Figure 3 Time-dependence of the effect of L-NAME on infarct volumes induced by transient focal cerebral ischaemia. L-NAME (1 mg kg^{-1}, i.p.) was given 3 h (hatched columns, $n=10$), 6 h (stippled columns, $n=8$), 9 h (crossed-hatched columns, $n=10$), or 12 h (solid columns, $n=11$) after ischaemia induction; control group; open columns ($n=9$). Values are means$\pm$s.e.mean. Comparison versus vehicle: NS: non significant; $^{*}P<0.05$, $^{**}P<0.01$.

Table 1 Physiological variables for rats given L-NAME (i.p.) or its vehicle 5 min and 3 h after the onset of transient focal ischaemia

	MABP (mmHg)	Pa_{O_2} (mmHg)	Pa_{CO_2} (mmHg)	*pH*
Vehicle ($n=8$)				
Preischaemia	91 ± 5	88 ± 3	39 ± 1	7.36 ± 0.01
Ischaemia	81 ± 7	100 ± 5	32 ± 2	7.42 ± 0.02
Postischaemia	79 ± 7	93 ± 2	37 ± 1	7.35 ± 0.01
L-NAME 1 mg kg^{-1} ($n=10$)				
Preischaemia	85 ± 4	89 ± 2	37 ± 1	7.37 ± 0.01
Ischaemia	90 ± 4	96 ± 3	34 ± 1	7.40 ± 0.02
Postischaemia	82 ± 6	90 ± 2	36 ± 1	7.36 ± 0.01
L-NAME 3 mg kg^{-1} ($n=8$)				
Preischaemia	86 ± 4	87 ± 1	39 ± 1	7.36 ± 0.01
Ischaemia	82 ± 4	101 ± 4	32 ± 2	7.41 ± 0.02
Postischaemia	82 ± 9	93 ± 2	37 ± 1	7.35 ± 0.01
L-NAME 10 mg kg^{-1} ($n=5$)				
Preischaemia	86 ± 6	85 ± 2	38 ± 2	7.37 ± 0.01
Ischaemia	85 ± 12	97 ± 4	30 ± 1	7.41 ± 0.01
Postischaemia	81 ± 9	88 ± 4	36 ± 1	7.36 ± 0.01

Data shown are means$\pm$s.e.mean.

Table 2 Physiological variables for rats given single injections of L-NAME (1 mg kg^{-1}, i.p.) 5 min or 3 h after the onset of transient focal ischaemia, or repeated injections

	MABP (mmHg)	Pa_{O_2} (mmHg)	P_{CO_2} (mmHg)	*pH*
Vehicle ($n=9$)				
Preischaemia	100±5	88±3	39±1	7.36±0.01
Ischaemia	104±7	95±2	38±1	7.38±0.01
Postischaemia	97±7	93±2	39±1	7.36±0.01
L-NAME 5 min ($n=8$)				
Preischaemia	96±5	85±2	42±1	7.35±0.01
Ischaemia	104±5	90±3	39±1	7.37±0.01
Postischaemia	96±9	93±2	37±2	7.36±0.01
L-NAME 3 h ($n=9$)				
Preischaemia	104±3	90±3	38±1	7.37±0.01
Ischaemia	110±6	94±3	37±1	7.39±0.01
Postischaemia	96±10	93±3	35±2	7.37±0.01
L-NAME 5 min and 3 h ($n=6$)				
Preischaemia	91±5	82±2	39±1	7.37±0.01
Ischaemia	94±4	87±1	38±1	7.38±0.01
Postischaemia	89±4	86±2	39±1	7.36±0.01

Data shown are means±s.e.mean.

NAME (1 mg kg^{-1}) 5 min and 3 h after MCA occlusion reduced the cortical infarction by 22% (192±11 mm^3, $P<0.01$, $n=6$). Single injections of L-NAME (1 mg kg^{-1}) 5 min or 3 h after MCA occlusion reduced cortical infarction similarly: by 17% (5 min: 205±9 mm^3, $P<0.05$, $n=8$) and 15% (3 h: 208±15 mm^3, $P<0.05$, $n=9$). None of the single or repeated treatments altered the striatal infarct volume. The striatal infarct volumes were 49±3 mm^3 when L-NAME was given at 5 min, 47±2 mm^3 when it was given 3 h, and 43±1 mm^3 when it was given at 5 min and 3 h after ischaemia induction (Figure 2). The physiological variables remained within normal ranges before and during ischaemia and during reperfusion. There were no differences between the control and treated groups (Table 2).

Therapeutic window of a single injection of L-NAME

The cortical and striatal infarct volumes in control rats ($n=9$) were 209±6 mm^3 and 46±2 mm^3. L-NAME (1 mg kg^{-1}) reduced the cortical infarction respectively by 19% when given 3 h after MCA occlusion (170±9 mm^3, $n=10$, $P<0.01$), by 21% when given at 6 h (164±9 mm^3, $n=8$, $P<0.01$) and by 16% when given at 9 h (176±11 mm^3, $n=10$, $P<0.05$). These effects were not significantly different. L-NAME given 12 h after MCA occlusion had no neuroprotective effect in the cortex (203±9 mm^3, $n=11$). L-NAME induced no significant striatal neuroprotection, regardless of when it was given. Striatal infarct volumes were 47±3 mm^3 when L-NAME was given at 3 h, 47±2 mm^3 when given at 6 h, 43±3 mm^3 when given at 9 h and 47±3 mm^3 when given at 12 h after the induction of ischaemia (Figure 3). The physiological variables of the 3 groups were not significantly different, either before or during MCA occlusion, or during early reperfusion (data not shown).

Discussion

Low doses of L-NAME (1 and 3 mg kg^{-1}) reduced the cortical infarct volume induced by transient focal cerebral ischaemia, whereas a higher dose of L-NAME (10 mg kg^{-1}) had no neuroprotective effect and killed 3 of 8 animals. Thus, it appears that high doses of L-NAME are no longer neuroprotective, and may even aggravate the effects of ischaemia. This narrow range of effective doses of L-NAME is consistent with previous results from our laboratory on permanent focal ischaemia (Buisson *et al*., 1992), and also with more recent results in transient focal ischaemia, indicating the neuroprotective effect of low doses (Ashwal *et al*., 1993) and deleterious actions of high doses of L-NAME (Kuluz *et al*., 1993). The worsening of ischaemic damage by high doses of non-selective NOS inhibitors is thought to be due to inhibition of endothelial NOS, leading to vasoconstriction and exacerbation of the ischaemia. NO donors have been shown to be neuroprotective in models of focal ischaemia by increasing cerebral blood flow (Morikawa *et al*., 1994; Zhang & Iadecola, 1994). But marked inhibition of NO production may also be deleterious, as it facilitates the accumulation of polymorphonuclear leukocytes (PMN), an effect thought to exacerbate ischaemia-reperfusion insults (Kubes, 1995). Indeed, it is now known that NO inhibits the functions of PMN such as chemotaxis, superoxide anion generation and degranulation (Kubes *et al*., 1991; Moilanen *et al*., 1993). Thus, inhibition of these beneficial effects of NO by high doses of L-NAME may also help to aggravate ischaemia. The lack of striatal neuroprotection by all the L-NAME doses used may be due to the greater vulnerability of this structure to ischaemia.

As a dose of 1 mg kg^{-1} L-NAME gave the best neuroprotection, this was used to study the relevance of repeated injections of L-NAME. A single injection of L-NAME given 5 min or 3 h after the onset of ischaemia appeared to provide cortical neuroprotection similar to that obtained with repeated injections. Hence, repeated injections do not increase the beneficial effect of L-NAME, which is consistent with the long-lasting action of this NOS inhibitor (Iadecola *et al*., 1994). This experiment also demonstrates that the therapeutic window for L-NAME is at least 3 h in our model. We therefore examined the effect of more delayed L-NAME treatments. The neuroprotective effect of a single 1 mg kg^{-1} dose of L-NAME, even when given 9 h after ischaemia induction, clearly indicates that a low dose of a non-selective NOS inhibitor has a remarkably wide therapeutic window in transient focal ischaemia. This therapeutic window for L-NAME is wider than that observed by Zhang *et al*. (1995) in permanent focal ischaemia (3 h), suggesting that reperfusion causes delayed, deleterious NOS activation in transient focal ischaemia.

The NOS isoform(s) involved in the neuroprotective effect of L-NAME remain(s) to be established. The neuronal calcium-calmodulin dependent NOS isoform (nNOS) has been shown to be deleterious in focal ischaemia. Indeed, selective nNOS inhibitors reduce the infarcted volume induced by permanent (Yoshida *et al*., 1994) and transient (Nagafuji *et al*., 1995) focal cerebral ischaemia. Huang *et al*. (1994) also showed that infarcts after MCA occlusion were smaller in mutant mice in which the nNOS gene was not expressed. Thus inhibition of nNOS may be involved in the neuroprotective effect of delayed L-NAME administration. This isoform was first shown to be activated after exposure to glutamate (Garthwaite *et al*., 1988; Dawson *et al*., 1991),

which leads to activation of N-methyl-D-aspartate (NMDA) receptors and the entry of calcium. Since glutamate accumulation is limited to the period of MCA occlusion in transient focal ischaemia (Uchiyama-Tsuyuki *et al*., 1994; Margaill *et al*., 1996), it seems unlikely that any nNOS activation due to this early, transient glutamate accumulation occurs later. However, calcium entry by pathways unrelated to this glutamate accumulation may participate in the delayed nNOS activation. The protective effect of L-NAME may also be linked to the inhibition of inducible NOS (iNOS). Iadecola *et al*. (1995b) demonstrated that iNOS activity begins to increase 48 h after MCA occlusion in permanent focal ischaemia, suggesting that this isoform may not be involved in the infarction we observed 24 h after ischaemia. But Nagafuji *et al*. (1994) obtained increased iNOS activity 4 h following the onset of ischaemia in the brain microvessels of rats subjected to permanent MCA occlusion. Further, Iadecola *et al*. (1995a) recently demonstrated a peak of iNOS mRNA and increased iNOS immunoreactivity in the wall of small blood vessels as early as 12 h after reperfusion in transient focal ischaemia. Thus, induction of iNOS may occur within 24 in transient focal ischaemia. The neuroprotective effect of L-NAME given 9 h after the onset of ischaemia may be at least partially due to the inhibition of this iNOS.

In conclusion, the present study demonstrates that low doses of L-NAME ($1-3$ mg kg^{-1}), but not a high dose (10 mg kg^{-1}), are neuroprotective in transient focal cerebral ischaemia. The reasons for this biphasic effect of L-NAME are not yet clear, but they may be linked to its vasoconstrictor activity and/or the facilitation of leukocyte accumulation. The feature of particular interest is the very wide therapeutic window (9 h) for 1 mg kg^{-1} of L-NAME, which is much wider than that for the NMDA receptor antagonist, MK-801. We recently found that the therapeutic window for MK-801 was less than 30 min in the same model (Margaill *et al*., 1996). Thus, NOS inhibitors are likely to be more clinically relevant than NMDA receptor antagonists for treating focal cerebral ischaemia followed by reperfusion. The inhibition of both nNOS and iNOS isoforms may be involved in the delayed neuroprotective effect of L-NAME. However, this experiment with a non-selective NOS inhibitor does not indicate the respective contributions of inhibiting the two NOS isoforms to the overall neuroprotection.

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