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L-NAME reduces infarction, neurological deficit and blood–brain barrier disruption following cerebral ischemia in mice

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Abstract

The role of nitric oxide (NO) in the development of post-ischemic cerebral infarction has been extensively examined, but fewer studies have investigated its role in other outcomes. In the present study, we first determined the temporal evolution of infarct volume, NO production, neurological deficit and blood–brain barrier disruption in a model of transient focal cerebral ischemia in mice. We then examined the effect of the nonselective NO-synthase inhibitor N^{ω}-nitro-L-arginine-methylester (L-NAME). L-NAME given at 3 mg/kg 3 h after ischemia reduced by 20% the infarct volume and abolished the increase in brain NO production evaluated by its metabolites (nitrites/nitrates) 48 h after ischemia. L-NAME with this protocol also reduced the neurological deficit evaluated by the grip test and decreased by 65% the extravasation of Evans blue, an index of blood–brain barrier breakdown. These protective activities of L-NAME suggest that NO has multiple deleterious effects in cerebral ischemia.

Keywords: Cerebral ischemia, transient focal; Neurological deficit; Blood–brain barrier disruption; Nitric oxide (NO); L-Name (N^{ω}-nitro-L-arginine-methylester)

1. Introduction

In the field of stroke, much attention has been directed this last decade to the role of the free radical, nitric oxide (NO) (Dawson, 1994; Iadecola, 1997; Pelligrino, 1993). The very first studies by the group of Nowicki et al. (1991) and our laboratory (Buisson et al., 1992) showed that non-selective inhibitors of NO-synthase, N^{ω}-nitro-L-arginine (L-NA) and N^{ω}-nitro-L-arginine-methylester (L-NAME), were neuroprotective in models of permanent cerebral ischemia in mice and rat, respectively. These results suggested that NO may play a deleterious role in the neurotoxic cascade leading to neuronal damage after ischemia. However, in the subsequent experiments, the same inhibitors led to contradictory results ranging from reduction (Ashwal et al., 1993; Carreau et al., 1994; Margaill et al., 1997) to no effect (Buchan et al., 1994; Dawson et al., 1992) or even a worsening of ischemic damage (Kuluz et al., 1993; Zhang and Iadecola, 1993). These discrepancies appeared to largely depend on the dosing regimen (Margaill et al., 1997; Meldrum, 1996; Verrecchia et al., 1995). Genetically modified mice have then provided an unique opportunity to evaluate the specific role of each of the three isoforms of NO-synthase (NOS) in stroke. It was thus shown that NO derived from endothelial NOS (eNOS) has a protective function in a model of transient focal cerebral ischemia (Huang et al., 1996). By contrast, NO produced by both neuronal and inducible NOS (nNOS and iNOS) has a detrimental role (Huang et al., 1994; Hara et al., 1996; Iadecola et al., 1997).

Most of the studies dealing with the implication of NO in cerebral ischemia has focused on infarct volumes. By contrast, few studies have investigated the role of NO on the neurological deficit, there again with controversial results. It was reported, in models of transient focal cerebral ischemia, that L-NAME given before ischemia aggravated the neurological deficit (Kuluz et al., 1993) while L-NA given 2 h after ischemia improved neurological function (Gursoy-Ozdemir et al., 2000). Since the evaluation of any stroke therapy is contingent upon a measurable change in functional outcome, further experiments are still needed in this area of NO research.

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Disruption of the blood–brain barrier is another well-documented consequence of cerebral ischemia (Belayev et al., 1996; Cole et al., 1991; Yang et al., 1999). However, information on the contribution of NO in this phenomenon is still limited. A few studies using nonselective inhibitors of NOS suggest that NO may contribute to this pathological alteration (Chi et al., 1994; Gursoy-Ozdemir et al., 2000; Zhang et al., 1995a).

In this context, we first determined the dose of L-NAME that allowed neuroprotection in our model of transient focal cerebral ischemia in mice. We then verified the effect of this dose on post-ischemic NO production. Finally, we studied the effect of L-NAME on the neurological deficit and the blood–brain barrier disruption induced by ischemia. Furthermore, for each outcome, a preliminary experiment was carried out to characterize its time course following ischemia.

2. Materials and methods

All experiments were performed on male Swiss mice weighing 22–25 g (Charles River, L'Arbresle, France) housed under a controlled temperature, 12-h light/dark cycle and allowed access to food and water ad libitum. Animal care was in compliance with French regulations on protection of animals used for experimental and other scientific purposes (D2001-486), as well as with the EEC regulations (O.J. of E.C. L358 12/18/1986).

2.1. Transient focal cerebral ischemia

Mice were anesthetized with an intraperitoneal injection of chloral hydrate (Sigma, Saint Quentin Fallavier, France) at the dose of 400 mg/kg (10 ml/kg). Body temperature was maintained at 37 ± 0.5 °C by means of a heating blanket and a heating lamp during the entire experimental procedure.

Focal cerebral ischemia was induced by occlusion of the left middle cerebral artery using an intraluminal filament technique (Hara et al., 1996) based on modifications of the original rat model (Longa et al., 1989). Through a midline neck incision, the left common and external carotid arteries were isolated and ligatured with a 4-0 silk suture (Ethicon, Issy-Les-Moulineaux, France). A microvascular clip (Zen temporal clip, 13 × 0.4 mm, Ohwa Tsuho, Tokyo, Japan) was temporarily placed across the internal carotid artery. An arteriotomy was fashioned in the common carotid artery just proximal to the carotid bifurcation. A 6-0 nylon monofilament (Ethicon), blunted at tip with an open flame, was introduced through this incision into the internal carotid artery and advanced approximately 13 mm distal to the carotid bifurcation for occlusion of the origin of the middle cerebral artery. The thread was carefully withdrawn 15 min after middle cerebral artery occlusion.

After recovery from anesthesia, animals were returned to their cage placed in an incubator at 29 °C with free access to food and water for remaining duration of observation.

Mice were allowed to recover for 3 h from anesthesia during the reperfusion period. They were then initially tested for obvious neurological deficit using a currently used postural reflex test as previously described (Bederson et al., 1986b; Huang et al., 1994). Neurological scores were as follows: 0, no observable neurological deficit (normal); 1, flexion of torso and contralateral forelimb when lifted by the tail (mild); 2, circling to the contralateral side when held on a flat surface, but normal posture at rest (moderate); 3, spinning or leaning to the contralateral side at rest (severe). Animals showing no sign of neurological deficit were excluded from the study.

In sham-operated mice, the carotid arteries were prepared surgically but the filament was not inserted (Kamii et al., 1994).

2.2. Infarct volume measurement

Mice were killed with an overdose of sodium pentobarbitone, their brain was removed and sectioned into six 1-mm-thick coronal slices using a tissue Chopper (McIlwain, The Mickle Laboratory Engineering, England). Coronal brain slices were immediately immersed into 2% 2,3,5,-triphenyltetrazolium chloride (Sigma) for 20 min at room temperature in the dark followed by fixation in a 4% paraformaldehyde solution (Prolabo, Fontenay-Sous-Bois, France) overnight prior to analysis (Bederson et al., 1986a). The infarction area, outlined in white, was measured on posterior surface of each section using a computer image analysis system (Imstar, Paris, France) and corrected for brain edema according to Golanov and Reis (1995). Infarct volume, expressed in mm^3, was calculated by a linear integration of the corrected lesion areas.

2.3. Brain NO_x assay

Infarct tissue from the brain of ischemic mice or a corresponding segment in sham-operated and non-operated mice were homogenized in 400 μl distilled water and centrifuged at 20,000 × *g*, for 10 min, at 4 °C. Nitrates were first reduced into nitrites: 50 μl of supernatant were incubated for 1 h in the dark with 20 μl of 0.31 M potassium phosphate buffer (pH 7.5), 10 μl of 0.86 mM β-NADPH (Sigma), 10 μl of 0.11 mM FAD in the presence of 20 mU nitrate reductase (Roche Diagnostics, Meylan, France). Proteins were then precipitated by adding 5 μl of 1 M $ZnSO_4$ (Roche Diagnostics) and samples were centrifuged (2000 × *g*, 5 min, 4 °C). The total amount of tissue nitric oxide end products, nitrate plus nitrite (NO_x), was then determined by Griess reaction (Green et al., 1982). Briefly, 100 μl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphtyl)ethylenediamine in distilled water) was mixed with 50 μl of supernatant. After 10-min incubation, absorbances were measured at 540 nm by a spectrophotometer (Milton Roy, Rochester, NY, USA) and converted to NO_x content using a nitrate standard curve treated as the samples. Proteins

in the supernatants were assayed by the method of Bradford (Bradford, 1976), with bovine serum albumin as standard. Data are expressed as pmol NO_x/mg protein.

2.4. Neurological deficit evaluation

Sensorimotor neurological deficits were assessed by a grip test (Hall, 1985). Each mouse was picked up by the tail and placed on a taut string 60 cm long suspended 40 cm above a table. Grip score was measured as the length of time that the mouse remained on the string in some manner (using one or more paws, tail, tail plus paws), for a maximum of 30 s. Each experiment was conducted randomly and blindly.

2.5. Evaluation of blood–brain barrier permeability

The integrity of the blood–brain barrier was investigated using Evans blue extravasation (Chan et al., 1991; Ikeda et al., 1994). Evans blue at 1% in saline (100 μl) was injected in the tail vein and allowed to circulate for 60 min. The chest wall was then opened under sodium pentobarbitone anesthesia (55 mg/kg, i.p.). Animals were perfused transcardially with saline at 100 mm Hg pressure until blue color was absent of the effluent. Brains were removed, tissue samples were dissected out as described above, weighed, placed in 400 μl of pure formamide (Sigma) and incubated for 72 h in the dark at 50 °C. The optical density of the formamide solution was measured at 620 nm. Data are expressed as μg Evans blue/g tissue.

2.6. Experimental protocol

2.6.1. Infarct volume studies

2.6.1.1. Temporal evolution of infarct volume after middle cerebral artery occlusion. Infarct volumes were measured 6 h ($n=7$), 24 h ($n=6$), 48 h ($n=6$) and 72 h ($n=6$) after the onset of ischemia as described above.

2.6.1.2. Effect of L-NAME on infarct volume. L-NAME (Sigma), dissolved in 0.9% saline, was injected at 1 mg/kg ($n=8$), 3 mg/kg ($n=11$), 10 mg/kg ($n=13$) or 30 mg/kg. L-NAME or its vehicle ($n=10$) were administered (i.p.) 3 h after the onset of ischemia and the infarct volumes were determined 48 h after ischemia.

2.6.2. Brain NO_x studies

2.6.2.1. Temporal evolution of brain NO_x content after middle cerebral artery occlusion. Brain NO_x contents were first measured in the early phase of reperfusion, 15 min ($n=10$), 30 min ($n=9$), 45 min ($n=9$), 1 h 15 min ($n=9$) and 3 h 15 min ($n=10$) after the onset of middle cerebral artery occlusion. In a second experiment, brain NO_x contents were measured at the later stage of ischemia, 6 h ($n=7$), 24 h ($n=11$), 48 h ($n=10$) and 72 h ($n=10$) after middle cerebral artery occlusion. In both studies, brain NO_x contents of non-operated ($n=7-14$) and sham-operated mice ($n=5-8$) were also determined.

2.6.2.2. Effect of L-NAME on brain NO_x content. L-NAME 3 mg/kg ($n=6-13$) or its vehicle ($n=7-10$) were injected i.p. 3 h after middle cerebral artery occlusion. NO_x contents were evaluated 24 and 48 h after ischemia in ischemic mice and tissue samples were also taken from non-operated ($n=14$) and sham-operated mice ($n=7-14$) for NO_x assay.

2.6.3. Neurological deficit studies

2.6.3.1. Temporal evolution of the neurological deficit after middle cerebral artery occlusion. Mice were subjected to ischemia as described above and the neurological deficit was evaluated 6 h ($n=7$), 24 h ($n=11$), 48 h ($n=10$) and 72 h ($n=10$) after the onset of middle cerebral artery occlusion. The neurological deficit was also evaluated in non-operated ($n=14$) and sham-operated mice ($n=5-8$).

2.6.3.2. Effect of L-NAME on the neurological deficit. L-NAME at 3 mg/kg ($n=11$) or its vehicle ($n=10$) was given i.p. 3 h after middle cerebral artery occlusion and the grip score was evaluated 48 h after the onset of ischemia. Non-operated mice ($n=12$) were concomitantly studied.

2.6.4. Blood–brain barrier studies

2.6.4.1. Time course of the blood–brain barrier disruption after middle cerebral artery occlusion. Tissue samples were taken from ischemic and sham-operated mice 3 h ($n=4$), 6 h ($n=4$), 24 h ($n=4-5$) and 48 h ($n=3-4$) after the onset of surgical process to evaluate the blood–brain barrier opening. Non-operated mice ($n=6$) were concomitantly studied.

2.6.4.2. Effect of L-NAME on blood–brain barrier disruption. L-NAME at 3 mg/kg ($n=7$) or its vehicle ($n=8$) was injected i.p. 3 h after middle cerebral artery occlusion and the blood–brain barrier permeability was evaluated 24 h after ischemia. Evans blue extravasation was concomitantly studied in non-operated ($n=8$) and sham-operated mice ($n=8$).

2.6.5. Effect of L-NAME on physiological variables

To study the effect of L-NAME on physiological variables, the carotid artery was cannulated to determine mean arterial blood pressure or sample arterial blood gas and pH. Body temperature was maintained at 37 ± 0.5 °C by means of a heating blanket.

The arterial catheter was connected to a pressure transducer (EMKA Technologies) to monitor the mean arterial blood pressure. Once mean arterial blood pressure was stabilized, animals were treated either with L-NAME at 3 mg/kg or its vehicle, and mean arterial blood pressure was

recorded during 2 h. Arterial blood samples were collected before treatments and again 30 min, 1 and 2 h after to analyze pH, partial pressure in arterial oxygen and arterial carbon dioxide (PaO_2 and $PaCO_2$), using a blood gas/pH analyzer (ABL 330, Radiometer, France).

2.7. Statistical analysis

Data are presented as means ± S.E.M. of *n* observations, where *n* represents the number of animals or samples. For the time course studies (except infarct volumes), the effect of surgery was first evaluated by comparing sham-operated groups against the non-operated group using one-way analysis of variance (ANOVA) followed by Dunnett's test. Comparisons between sham-operated and ischemic groups were then performed by one-way ANOVA followed by protected least significant difference (PLSD) Fisher's test. In the temporal evolution of infarct volume and L-NAME studies, a one-factorial ANOVA followed by a PLSD Fisher's test was performed. When measures were repeated (physiological variables), data were analyzed by a two-factorial ANOVA for repeated measures. A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Infarct volume studies

3.1.1. Temporal evolution of infarct volume after middle cerebral artery occlusion

Among the 27 animals subjected to ischemia, 2 died during the initial 6 h of reperfusion. Ten percent of the animals showing no neurological deficits (score 0) evaluated by postural reflex test at 3 h after middle cerebral artery occlusion were immediately excluded from the study. In all the remaining mice, transient occlusion of the left middle cerebral artery induced hemispheric infarction involving the cerebral cortex and the striatum. Total infarct volumes were significantly smaller at 6 h after middle cerebral artery occlusion compared with other groups ($P<0.05$ vs. 24 h, $P<0.01$ vs. 48 h and $P<0.05$ vs. 72 h). There was no significant difference between the infarct volume at 24, 48 and 72 h (Table 1).

Table 1
Temporal evolution of infarct volume after transient focal cerebral ischemia in mice

Time after MCAO	Infarct volume (mm^3)
6 h ($n=7$)	47.2 ± 11.3
24 h ($n=6$)	74.9 ± 7.2[a]
48 h ($n=6$)	82.0 ± 3.1[b]
72 h ($n=6$)	73.2 ± 4.5[a]

MCAO: middle cerebral artery occlusion.
Data are means ± S.E.M.
[a] $P<0.05$ vs. 6 h group.
[b] $P<0.01$ vs. 6 h group.

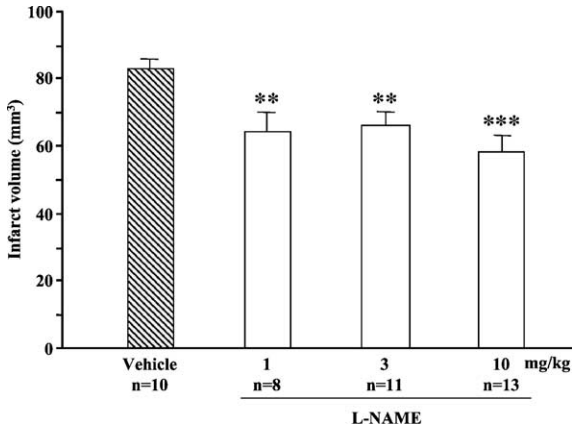

Fig. 1. Effect of L-NAME on infarct volumes induced by transient focal cerebral ischemia. L-NAME was given i.p. 3 h after the onset of ischemia. Data are means ± S.E.M., $^{**}P<0.01$, $^{***}P<0.001$ vs. vehicle-treated group.

3.1.2. Effect of L-NAME on infarct volume

The infarct volume in vehicle-treated mice was 84 ± 3 mm^3 (Fig. 1). L-NAME reduced the infarct volume by 23% at the dose of 1 mg/kg ($P<0.01$), by 20% at 3 mg/kg ($P<0.01$) and by 30% at 10 mg/kg ($P<0.001$). The highest dose, 30 mg/kg, was excluded during the experiment because four of five mice of this group died during the night following ischemia.

3.2. Brain NO_x studies

3.2.1. Temporal evolution of brain NO_x content after middle cerebral artery occlusion

Brain NO_x content in sham-operated mice was not different from that of non-operated mice at each time point in both kinetics (Fig. 2A,B). NO_x content increased by 59% in the infarct area as early as 15 min after middle cerebral artery occlusion ($P<0.05$ vs. sham-operated mice). This increase maintained until 1 h 15 min after middle cerebral artery occlusion ($P<0.05$ vs. sham-operated mice), but was not significant at 3 h 15 min (Fig. 2A). In the late phase of reperfusion, NO_x content was not modified 6 h after middle cerebral artery occlusion compared with sham-operated mice, but a second increase in NO_x was observed 24 h after middle cerebral artery occlusion (67%, $P<0.05$ vs. sham-operated mice) and 48 h after middle cerebral artery occlusion (158%, $P<0.001$ vs. sham-operated mice). NO_x content returned to baseline 3 days after ischemia (Fig. 2B).

3.2.2. Effect of L-NAME on brain NO_x content

The content of NO_x in sham-operated mice (3.4–3.7 pmol/mg protein) was not modified compared with that of non-operated mice (3.9 ± 0.3 pmol/mg protein). In vehicle-treated ischemic mice, brain NO_x was increased by 132% 24 h after middle cerebral artery occlusion ($P<0.001$) and by 43% 48 h after compared with sham-operated mice ($P<0.01$). L-NAME at the dose of 3 mg/kg given 3 h after

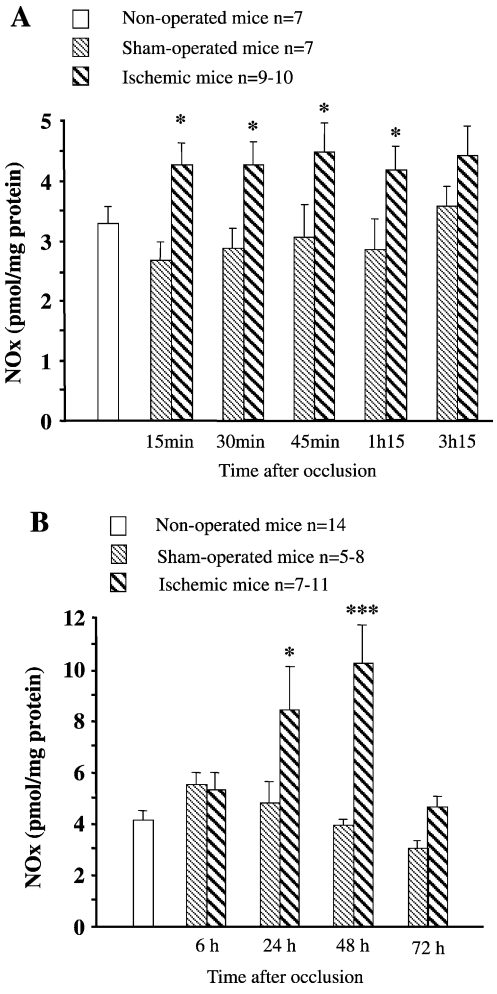

Fig. 2. Temporal evolution of brain NO_x content in the early (A) and late (B) phase after transient focal cerebral ischemia. Data are means ± S.E.M., $^{*}P<0.05$, $^{***}P<0.001$ vs. sham-operated mice.

middle cerebral artery occlusion reduced the increase in NO_x measured 24 and 48 h after middle cerebral artery occlusion compared with vehicle-treated mice ($P<0.001$ and $P<0.01$, respectively). The content of NO_x in L-NAME-treated mice was not significantly different from that of sham-operated mice ($P>0.05$) (Fig. 3).

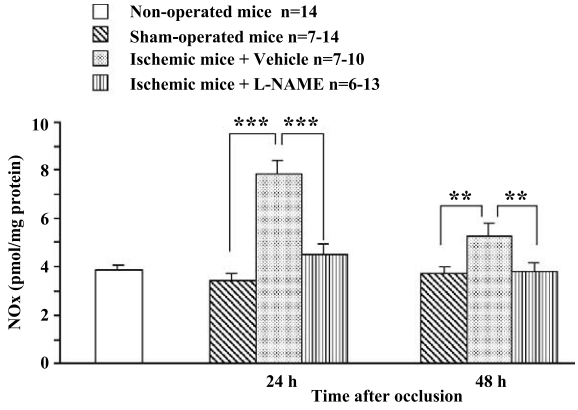

Fig. 3. Effect of L-NAME on NO_x content 24 and 48 h after transient focal cerebral ischemia. L-NAME (3 mg/kg) was given i.p. 3 h after the onset of ischemia. Data are means ± S.E.M., $^{**}P<0.01$, $^{***}P<0.001$.

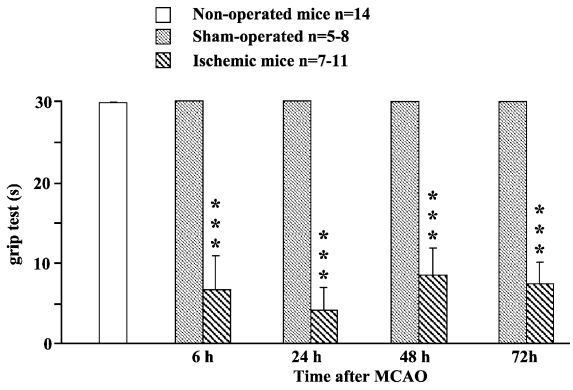

Fig. 4. Temporal evolution of the neurological deficit evaluated by the grip test after transient focal cerebral ischemia. Data are means ± S.E.M., $^{***}P<0.001$ vs. sham-operated mice at each time point.

3.3. Neurological deficit studies

3.3.1. Temporal evolution of the neurological deficit after middle cerebral artery occlusion

The grip score was 30 ± 0 s in sham-operated mice at each time point and was not significantly different from that of non-operated mice (29.7 ± 0.2 s). In mice subjected to transient focal ischemia, the grip score was significantly decreased by 78% as early as 6 h after middle cerebral artery occlusion ($P<0.001$ vs. sham-operated mice) and remained attenuated up to 72 h after ischemia. There was no significant difference between ischemic groups (Fig. 4).

3.3.2. Effect of L-NAME on the neurological deficit

The grip score in non-operated mice was 29 ± 1 s. Transient middle cerebral artery occlusion led to a dramatic decrease in the grip score measured 48 h after ischemia in vehicle-treated mice (3 ± 3 s, $P<0.001$ vs. non-operated

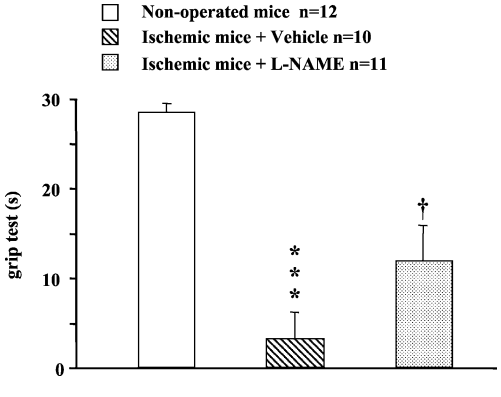

Fig. 5. Effect of L-NAME on the neurological deficit 48 h after transient focal cerebral ischemia. L-NAME (3 mg/kg) was given i.p. 3 h after the onset of ischemia. Data are means ± S.E.M., $^{***}P<0.001$ vs. non-operated mice and $^{\dagger}P<0.05$ vs. vehicle-treated group.

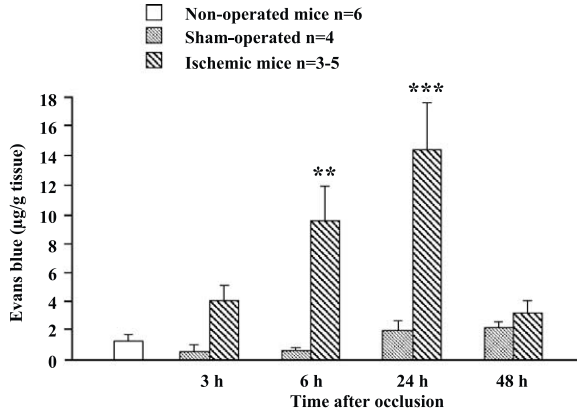

Fig. 6. Time course of blood–brain barrier disruption evaluated by Evans blue extravasation after transient focal cerebral ischemia. Data are means ± S.E.M., $^{**}P<0.01$, $^{***}P<0.001$ vs. sham-operated mice.

mice). L-NAME given 3 h after middle cerebral artery occlusion at the dose of 3 mg/kg induced a fourfold increase in the grip score ($P<0.05$ vs. vehicle-treated mice) (Fig. 5).

3.4. Blood–brain barrier studies

3.4.1. Time course of the blood–brain barrier disruption after middle cerebral artery occlusion

Evans blue contents in sham-operated mice (0.6–2.0 µg/g tissue) were not significantly different from that of non-operated mice (1.3 ± 0.5 µg/g tissue). The level of Evans blue content was significantly increased in the infarct area 6 h after middle cerebral artery occlusion (9.6 ± 2.3 µg/g tissue, $P<0.01$ vs. sham-operated mice) and was maximal 24 h after ischemia (14.4 ± 3.2 µg/g tissue, $P<0.001$ vs. sham-operated mice). Forty-eight hours after middle cerebral artery occlusion, Evans blue content was not significantly different from that of sham-operated mice (Fig. 6).

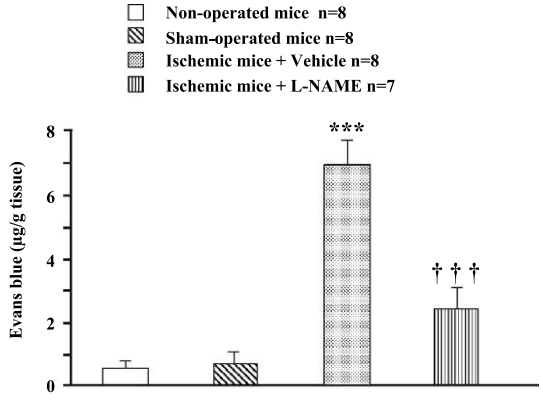

Fig. 7. Effect of L-NAME on Evans blue extravasation 24 h after transient focal cerebral ischemia. L-NAME (3 mg/kg) was given i.p. 3 h after the onset of ischemia. Data are means ± S.E.M., $^{***}P<0.001$ vs. sham-operated mice, $^{\dagger\dagger\dagger}P<0.001$ vs. vehicle-treated group.

Table 2
Effect of L-NAME on physiological variables

		Saline ($n=5–8$)	L-NAME ($n=5–6$)
MABP (mm Hg)	Before treatment	97.8 ± 2.0	89.9 ± 6.9
	30 min after treatment	99.6 ± 3.4	110.2 ± 6.3[a]
	1 h after treatment	97.5 ± 4.0	110.0 ± 5.9[a]
	1 h 30 min after treatment	93.6 ± 4.1	108.5 ± 7.8[a]
	2 h after treatment	88.2 ± 7.7	82.8 ± 4.4
PaO_2 (mm Hg)	Before treatment	107.9 ± 3.0	107.9 ± 3.0
	30 min after treatment	107.9 ± 1.7	105.1 ± 1.8
	1 h after treatment	110.6 ± 5.9	102.8 ± 1.1
	2 h after treatment	112.9 ± 7.3	107.9 ± 2.8
$PaCO_2$ (mm Hg)	Before treatment	35.8 ± 3.6	35.8 ± 3.6
	30 min after treatment	36.8 ± 1.3	38.3 ± 3.2
	1 h after treatment	38.2 ± 2.7	36.0 ± 3.5
	2 h after treatment	36.6 ± 2.4	34.5 ± 1.0
pH	Before treatment	7.32 ± 0.04	7.32 ± 0.04
	30 min after treatment	7.35 ± 0.02	7.38 ± 0.02
	1 h after treatment	7.35 ± 0.02	7.38 ± 0.02
	2 h after treatment	7.37 ± 0.02	7.36 ± 0.03

MABP: mean arterial blood pressure.
L-NAME was given i.p. at 3 mg/kg.
Data are means ± S.E.M.
[a] $P<0.01$ vs. before treatment.

3.4.2. Effect of L-NAME on blood–brain barrier disruption

At 24 h, Evans blue content in the ischemic area of vehicle-treated group (6.9 ± 0.8 µg/g tissue) was significantly higher than that of sham-operated mice ($P<0.001$). In the group treated with L-NAME, Evans blue content of infarct area (2.4 ± 0.7 µg/g tissue) was reduced by 65% ($P<0.001$) compared with that of the vehicle-treated group (Fig. 7).

3.5. Effect of L-NAME on physiological variables

The physiological variables measured before and after administration of L-NAME (3 mg/kg) or its vehicle are summarized in Table 2. Arterial blood gases, pH and mean arterial blood pressure remained within the physiological ranges throughout the experiment. There were no significant differences between the experimental groups except that L-NAME transiently (within 2 h) increased mean arterial blood pressure by about 20 mm Hg.

4. Discussion

The mice model of transient focal cerebral ischemia performed in the present study induced a rapid maturation of infarction together with a moderate increase in NO production during the acute phase following ischemia and a more important generation of NO during the later phase of reperfusion. Moreover, this model led to an early deficiency of neurological function and the disruption of blood–brain barrier. Treatment with the nonselective NOS inhibitor L-NAME administered after the acute NO overproduction abolished the delayed production of NO, reduced the infarct

size, improved the neurological score and prevented blood–brain barrier disruption.

In our model, transient occlusion of middle cerebral artery by the intraluminal suture technique induced hemispheric infarction developing rapidly between 6 and 48 h after the onset of ischemia. Accordingly, the contribution of NO in infarct volume was examined 48 h after middle cerebral artery occlusion by using L-NAME. First, we determined the best dosing regimen in terms of neuroprotection. The treatment schedule is based on our previous studies (Margaill et al., 1997). Furthermore, many investigators suggested that eNOS contribution to the early NO production after ischemia may have a beneficial effect by increasing cerebral blood flow and decreasing platelet aggregation and neutrophil adhesion (Batteur-Parmentier et al., 2000; Cooke and Dzau, 1997). Accordingly L-NAME given at the dose of 3 mg/kg during the acute phase of transient ischemia (5 min after middle cerebral artery occlusion) failed to reduce ischemic infarct and even aggravated the ischemic outcome in models of transient and permanent focal cerebral ischemia (Kamii et al., 1996; Zhang et al., 1995b). Taken together, these studies led us to delay the administration of L-NAME 3 h after the onset of ischemia in order to avoid the deleterious consequence of early eNOS inhibition. Treatment with L-NAME thus occurred after the first increase in NO_x and prior to the second substantial production of NO in our model (see below). With this administration time, L-NAME at low doses (1, 3 and 10 mg/kg) reduced the infarct volume, whereas the high dose, 30 mg/kg, killed four of five animals. This U-shaped dose–response curve of L-NAME is consistent with our previous results on permanent (Buisson et al., 1992) and transient (Margaill et al., 1997) focal ischemia in rat and is postulated to result from detrimental effects due to eNOS inhibition. The neuroprotective effects of L-NAME are unlikely due to the moderate and transient increase in mean arterial blood pressure observed after its administration, since comparable increase in mean arterial blood pressure with phenylephrine was reported to have no influence on the infarct size induced by middle cerebral artery occlusion (Gursoy-Ozdemir et al., 2000). Because the three doses of L-NAME provide similar neuroprotection, the intermediate dose of 3 mg/kg was used in the next experiments.

In the second part of our study, we have extensively investigated the time course of NO production that is poorly characterized in murine models of transient focal cerebral ischemia. Grandati et al. (1997) were the first group to report a delayed increase in brain NO_x content in a model of transient ischemia in mice. However, in this study, NO_x were not measured within the first hours after the onset of reperfusion. In the present study, in order to evaluate NO production, brain NO_x were also preferred to the ex vivo radioenzymatic dosage of NOS activity because the latter technique defines only the potential for NO production in optimized conditions and may not necessarily be a reliable index of actual NO generation in vivo. In our model, a moderate increase in brain NO_x occurred immediately after reperfusion with a return to baseline 3 h 15 min after the onset of ischemia. A second and more substantial increase in NO_x was observed 24–48 h after middle cerebral artery occlusion, and followed by a return to control level at 3 days.

These results are in accordance with previous studies in rats showing a rapid and transient increase in NO production during the first hours after ischemia. In these studies, NO was measured NO directly with a porphyrinic microsensor (Malinski et al., 1993) or indirectly through determination of brain and plasmatic NO_x, brain cGMP concentration and NOS activity (Kader et al., 1993; Kumura et al., 1994). This overproduction of NO in the early phase of ischemia may be mainly due to constitutive nNOS and eNOS. An up-regulation of nNOS mRNA expression and nNOS immunoreactivity was indeed observed acutely (within 10–15 min) after both permanent (Zhang et al., 1994) and transient middle cerebral artery occlusion (Hara et al., 1997; Holtz et al., 2001). By using nNOS knockout mice, Eliasson et al. (1999) reported that activation of nNOS contributes to the increase in immunoreactivity of citrulline (a marker for NOS activity) and of 3-nitrotrotyrosine (a marker for NO-derived peroxynitrite) observed within 3 h after 30 min of middle cerebral artery occlusion in mice. Endothelial NOS protein expression also increases in ischemic brain, 1 h after induction of permanent ischemia (Zhang et al., 1993) and within 15 min after transient ischemia (Holtz et al., 2001). The inducible NOS isoform may also contribute to the early NO production, as (1) iNOS immunoreactivity was present as soon as 15 min in neurons (Holtz et al., 2001) and (2) calcium-independent NOS activity (taken as an index of iNOS activity) was detected 4 h after ischemia in brain microvessels (Nagafuji et al., 1995). Taken together, these results suggest that the acute increase in brain NO_x production following reperfusion in our study may be due to the combined activation of the three isoforms of NOS.

NO_x return to basal level between 3 and 6 h of reperfusion might involved several mechanisms: (1) substrate availability may become rate limiting for NO synthesis with the progression of microcirculation disturbances and edema formation (Hata et al., 2000), (2) the number of NOS-containing neurons decreased when tissue necrosis develops (Margaill et al., 1995; Iadecola et al., 1995), (3) NO may nitrosylate sulfhydryl groups in NOS thereby inactivating it (Stamler et al., 1992), (4) NO may oxidize a critical sulfhydryl group in *N*-methyl-D-aspartate receptor thereby decreasing calcium influx and reducing neuronal NOS activity (Lei et al., 1992), and finally, (5) activation of peptidases in ischemic tissue resulting in NOS deactivation cannot be ruled out (Kader et al., 1993).

In the late phase of ischemia, 24 and 48 h after middle cerebral artery occlusion, the second increase in the NO_x content of the infarct area might be attributed to iNOS. Expression of iNOS mRNA, iNOS immunoreactivity and

iNOS enzymatic activity were indeed demonstrated lately (6–48 h) after transient middle cerebral artery occlusion in rat vascular and infiltrating cells (Iadecola et al., 1996). Furthermore, by using iNOS knockout mice, iNOS was suggested to be responsible for the nitrotyrosine formation in the vascular wall 15 h after reperfusion (Hirabayashi et al., 2000).

According to the temporal evolution of brain NO_x in our model, L-NAME given 3 h after middle cerebral artery occlusion spared the early NO production. By contrast, this treatment abolished ischemia-induced NO overproduction at 24 and 48 h. These data are consistent with the long-lasting action of this inhibitor (Iadecola et al., 1994).

The time course of the neurological deficit evaluated by the grip test showed that the neurological deficit is maximum 6 h after middle cerebral artery occlusion. This temporal evolution is more rapid than that of infarct volume. A lack of correlation between the neurological deficit and the infarct volume has already been reported in the model of middle cerebral artery occlusion (Chabrier et al., 1999; Lolic et al., 1997; Wahl et al., 1992; Yamaguchi et al., 1995); these observations underline the importance to perform both histological and neurological studies.

L-NAME reduced not only the infarct size but also improved the neurological deficit 48 h after ischemia. This finding is in agreement with previous report showing that a similar NOS inhibitor L-NA given 2 h after ischemia improved neurological function in mice subjected to transient ischemia (Gursoy-Ozdemir et al., 2000). Furthermore, it has been demonstrated that the motor deficits produced by middle cerebral artery occlusion in both nNOS and iNOS knockout mice were smaller than in wild type animals (Huang et al., 1994; Iadecola et al., 1997). These finding suggest that both nNOS and iNOS are detrimental to the neurological dysfunction induced by ischemia.

Although disruption of the blood–brain barrier has been widely studied in models of cerebral ischemia in rat (Belayev et al., 1996; Cole et al., 1991; Preston et al., 1993; Yang and Betz, 1994; Zhang et al., 1995a), there is limited information regarding the time course of blood–brain barrier permeability in the murine model, especially its correlation with the evolution of infarct size and the time course of NO production. In our model, extravasation of Evans blue progressively increased between 3 and 24 h after ischemia, but was not detectable at 48 h. These data provide evidence that the blood–brain barrier breakdown does occur in the early phase (within 24 h) after ischemia in our model. Our findings are in accordance with previous works obtained in models of 1 h middle cerebral artery occlusion by the intrafilament technique in mice (Kondo et al., 1997; Yang et al., 1999). The mechanism of this altered permeability in the ischemic brain is not entirely clear. Firstly, it may result from the acute destruction of the endothelium by early production of reactive oxygen species. It is indeed well established that blood–brain barrier is one of the primary targets for reactive oxygen species including NO, superoxide anion O^{2-}, and their reaction product, peroxynitrite (Chan, 1994; Chan et al., 1996; Kondo et al., 1997; Nelson et al., 1992). Secondly, the increase in brain NO_x induced by ischemia–reperfusion is paralleled by an increase in cGMP level (Kader et al., 1993) and cGMP is known to decrease the endothelial cell retraction and to open the gaps between adjacent endothelial cells (Vigne and Frelin, 1992). This supports an important role for the NO–cGMP pathway in increased microvascular permeability (Marsault and Frelin, 1992). Finally in the late stage of ischemia, the opening of the blood–brain barrier might be due to the accumulation of inflammatory leukocytes which secrete cytotoxic substrates such as reactive oxygen species, lysozomal enzymes and cytokines (Soares et al., 1995). The absence of significant Evans blue extravasation at 48 h after ischemia may reflect the restoration of blood–brain barrier at this time point. Such pattern of blood–brain barrier opening and closure was also described in rat model of cerebral ischemia (Preston et al., 1993). However, it cannot be excluded that at this delayed time point Evans blue failed to reach the cerebral microcirculation within infarct area because of elevated pressure in brain due to edema formation and structural destruction of cerebromicrovasculature.

Because NO may participate to the blood–brain barrier disruption, we next studied the effect of L-NAME on this outcome. In our model, L-NAME reduced by 65% the Evans blue level in infarct area 24 h after middle cerebral artery occlusion. This result is in accordance with previous finding that nonselective NOS inhibitors attenuated the disruption of blood–brain barrier and is in favor of the role for NO in the increase in blood–brain barrier permeability (Chi et al., 1994; Gursoy-Ozdemir et al., 2000; Zhang et al., 1995a,b). This protection of the blood–brain barrier by L-NAME may sequentially contribute to reduce cell injury by limiting the entry of potential toxic compounds from blood into the brain parenchyma.

In conclusion, the current study reports that delayed administration of L-NAME at a low dose that abolished NO overproduction reduced not only the infarct volume but also the neurological deficit and the blood–brain barrier disruption. These findings suggest that NO generation during the delayed phase of reperfusion mediates a wide variety of post-ischemic brain insults.

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