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European Journal of Pharmacology 389 (2000) 71–78

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Differential role of nitric oxide pathway and heat shock protein in preconditioning and lipopolysaccharide-induced brain ischemic tolerance

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Received 22 September 1999; received in revised form 3 December 1999; accepted 9 December 1999

Abstract

The purposes of this study were to investigate the role of nitric oxide (NO), nitric oxide synthase (NOS), and 70 kDa heat shock protein in brain ischemic tolerance induced by ischemic preconditioning and lipopolysaccharide. Focal cerebral ischemia was induced in rats by intraluminal middle cerebral artery occlusion. Infarct volume was significantly reduced (1) in rats subjected to 3 min ischemia 72 h prior to 60 min ischemia; (2) in rats administered lipopolysaccharide (0.5 mg/kg; i.p.) 72 h prior to 60 min ischemia compared with controls. The beneficial effect of ischemic preconditioning was unchanged despite prior administration of nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor. Conversely, the protective effect of lipopolysaccharide was nullified by L-NAME. Using immunohistochemical techniques, we observed that (1) ischemic preconditioning but not lipopolysaccharide induces the expression of 70 kDa heat shock protein in cerebral cortex and (2) lipopolysaccharide induces early increased expression of endothelial NOS in cerebral blood vessels. The results suggest that (1) endothelium-derived NO plays a role of a trigger in the brain tolerance induced by lipopolysaccharide, and (2) 70 kDa heat shock protein is involved in the protection afforded by ischemic preconditioning but not by lipopolysaccharide.

Keywords: Brain; Cerebral ischemia; Ischemic tolerance; Preconditioning; Lipopolysaccharide; Nitric oxide (NO); Nitric oxide synthase (NOS); Heat shock protein; Nitro-L-arginine methyl ester; (Rat)

1. Introduction

Brain tolerance to experimental ischemia can be induced by a variety of stimuli such as ischemic preconditioning (Chen and Simon, 1997; Barone et al., 1998) or administration of a low dose of lipopolysaccharide derived from *Escherichia coli* (Tasaki et al., 1997; Dawson et al., 1999). Ischemic preconditioning refers to the phenomenon whereby brief periods of ischemia, inadequate to infarct the brain, protects it from subsequent severe ischemia (Chen and Simon, 1997). Ischemic tolerance involves sequentially both triggering and mediating mechanisms. The time required for the occurrence of tolerance (24–48 h) suggests synthesis of new protective proteins. Heat shock proteins, anti-oxidant enzymes, growth factors, or anti-apoptotic gene products are potential candidates for the role of mediator (Chen and Simon, 1997). Although there is accumulating evidence that suggests a link between heat shock proteins upregulation and tolerance induction, the role of heat shock proteins as mediators has not been definitively proven (Chen and Simon, 1997). Trigger mechanisms are necessary to induce synthesis of protective proteins. Involvement of adenosine or glutamate has been suggested in preconditioning (Heurteaux et al., 1995; Chen and Simon, 1997). Some studies have shown that nitric oxide (NO) may play a role as trigger in the delayed adaptation of the heart to ischemic stress (Bolli et al., 1997; Qiu et al., 1997). More recently, it has been suggested that NO could be involved in anoxic preconditioning in rat hippocampal slices (Centeno et al., 1999). Furthermore, there is increasing evidence suggesting a role of inducible nitric oxide synthase (iNOS) in cardiac tolerance induced by lipopolysaccharide or monophosphoryl lipid A, a non-toxic derivative of endotoxin (McKenna et al., 1995;

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0014-2999/00/$ - see front matter
PII: S0014-2999(99)00893-6

Shindo et al., 1995; Yoshida et al., 1996; Zhao et al., 1997; Maulik et al., 1998).

Since NO is involved in the mechanisms of cerebral ischemia (Iadecola, 1997), NO may also play a key role in ischemic tolerance. To explore the role of NO, endothelial nitric oxide synthase (eNOS), and 70 kDa heat shock protein in this phenomenon, we have studied (1) the effect of inhibition of NOS by nitro-L-arginine methyl ester (L-NAME), and (2) the expression of iNOS, eNOS, and 70 kDa heat shock protein by immunohistochemistry, in two models of brain ischemic tolerance, ischemic preconditioning and lipopolysaccharide-induced tolerance, in a rat model of focal cerebral ischaemia.

2. Materials and methods

2.1. Animals and experimental protocols

Male Wistar rats (IFFA Credo, France), aged 10–12 weeks and weighing 280–320 g were used in the study. All experiments were performed in strict accordance with guidelines of the NIH and the French Department of Agriculture.

2.1.1. Effect of L-NAME alone

On day 1, animals were given L-NAME (3 mg/kg; i.p.) ($n = 6$) or an equivalent volume (0.5 ml) of 0.9% w/v normal saline ($n = 6$). Seventy-two hours later, 60 min ischemia was performed and, 24 h later, the animals were sacrificed.

2.1.2. Effect of L-NAME on ischemic preconditioning

Rats were divided into three experimental groups (six rats per group). In the first group, rats were given L-NAME (3 mg/kg; i.p.), 1 h before being subjected to a 3-min ischemia. In the other groups, they were given an equivalent saline volume 1 h before being subjected to a sham operation or ischemia for 3 min. Seventy-two hours later, 60 min ischemia was performed and, 24 h later, the animals were sacrificed.

2.1.3. Effect of L-NAME on lipopolysaccharide-induced tolerance

Rats were divided into three experimental groups (six rats per group). In the first group animals were given L-NAME (3 mg/kg; i.p.) 1 h prior to receiving lipopolysaccharide (0.5 mg/kg; i.p.). In other groups, they received 0.5 ml saline 1 h prior to receiving lipopolysaccharide (0.5 mg/kg; i.p.) or an equivalent saline volume. Seventy-two hours later, 60 min ischemia was performed and, 24 h later, the animals were sacrificed.

2.2. Method of intraluminal occlusion of MCA

Rats were anesthetized with chloral hydrate (300 mg/kg; i.p.). The core body temperature was monitored with a rectal probe and maintained at 37.0 ± 0.5°C using a heating pad and a heating lamp. The caudal artery was cannulated to monitor blood pressure and to obtain arterial blood gases. Focal cerebral ischemia was induced by reversible intraluminal occlusion of the middle cerebral artery (Longa et al., 1989).

2.2.1. Preconditioning ischemia

Under an operating microscope, the right carotid bifurcation was exposed through a midline cervical incision. The external carotid was ligated distally. The pterygopalatine artery was ligated at its origin. An arteriotomy was made in the external carotid allowing the introduction of a 4–0 surgical nylon monofilament with its tip rounded by heat. The filament was gently advanced into the internal carotid artery 18–20 mm past the carotid artery bifurcation, thereby, occluding the origin of the middle cerebral artery. At 3 min later, the filament was gently withdrawn to permit reperfusion. A duration of 3 min, which induced a transient left hemiparesis, had been chosen for preconditioning ischemia according to the results of a preliminary study which had shown that more prolonged ischemia (5 or 10 min) was of sufficient duration to produce significant brain injuries (unpublished results).

2.2.2. Prolonged cerebral ischemia

The technique has been previously described in detail (Bastide et al., 1999). Briefly, it was similar to that used for preconditioning-ischemia except that the nylon monofilament was inserted into the common carotid artery, which had been ligated more proximally, and then advanced into the internal carotid artery to occlude the middle cerebral artery. One hour later, the filament was gently withdrawn to permit reperfusion.

2.3. Infarct volume measurement

At 24 h after, 60 min ischemia rats were killed by an overdose of pentobarbital. Brains were rapidly removed, frozen, and coronally sectioned. Sections were stained with cresyl violet. The unstained area of the brain sections was

Table 1
Physiologic variables at 60 min ischemia
MABP = mean arterial blood pressure; PC = preconditioning-ischemia; L-NAME = nitro-L-arginine methyl ester; LPS = lipopolysaccharide.

Pretreatment (72 h prior to 60 min ischemia)	pO_2 (mm Hg)	pCO_2 (mm Hg)	pH	MABP (mm Hg)
L-NAME	91 ± 2.6	43 ± 2.4	7.35 ± 0.01	95 ± 8.2
Saline	89 ± 3.3	38 ± 1.5	7.32 ± 0.01	96 ± 4.1
Sham-operation	90 ± 10	41 ± 4	7.38 ± 0.02	101 ± 11
3 min ischemia (PC)	87 ± 16	38 ± 4	7.39 ± 0.02	103 ± 8
L-NAME + PC	92 ± 15	37 ± 5	7.37 ± 0.03	105 ± 13
Controls	88 ± 21	40 ± 3	7.38 ± 0.02	107 ± 17
LPS	89 ± 16	38 ± 4	7.40 ± 0.01	108 ± 11
L-NAME + LPS	91 ± 10	40 ± 3	7.39 ± 0.02	103 ± 14

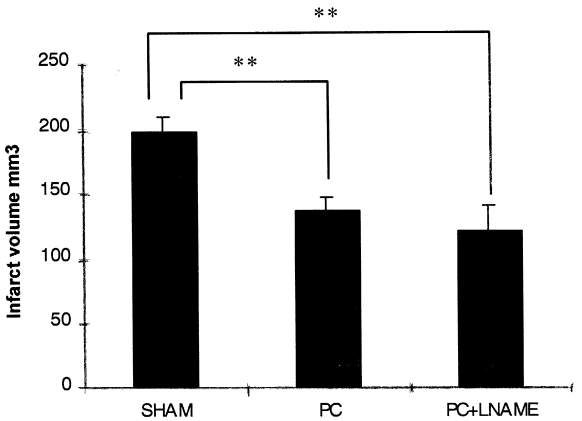

Fig. 1. Effect of L-NAME on ischemic preconditioning-induced tolerance. On the first day, the animals in the SHAM group were subjected to a sham-operation, the PC animals were subjected to 3 min ischemia, and in the group L-NAME + PC the animals received L-NAME (3 mg/kg; i.p.) 1 h prior to being subjected to a 3 min ischemia. Seventy-two hours later, all animals were subjected to 60 min ischemia. True infarct volume was calculated after correcting the total infarct volume for edema. Values are expressed as mean ± S.E.M. Comparison vs. the SHAM group; $^{*}P < 0.05$; $^{**}P < 0.01$.

considered as infarcted. Areas were measured. Infarct volumes and hemisphere volumes were calculated by integration. A corrected total infarct volume was calculated to compensate for the effect of brain edema using the following equation: corrected infarct volume = infarct volume − (right hemisphere volume − left hemisphere volume).

2.4. Immunohistochemistry

2.4.1. Expression of 70 kDa heat shock protein

Rats were divided into five experimental groups (three rats per group). Six rats were given lipopolysaccharide (0.5 mg/kg; i.p.) or an equivalent saline volume. Six rats were subjected to 3 min ischemia or a sham operation. Three rats were subjected to 60 min ischemia to serve as positive control for immunohistochemistry. Seventy-two hours later, animals were anesthetized with pentobarbital and perfused via the ascending aorta with normal saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were removed, post-fixed for 24 h, cryoprotected in 15% sucrose solution, frozen in isopentane, and sectioned (20 μm) on a cryostat. Sections were incubated with the anti-70 kDa heat shock protein antibody SPA 810, directed against the inducible form, in a dilution of 1/100 (Bachelet et al., 1998). After successive rinses and endogenous peroxidase blocking, tissues were incubated with the secondary antibody (biotinylated anti mouse IgG BA 2001,

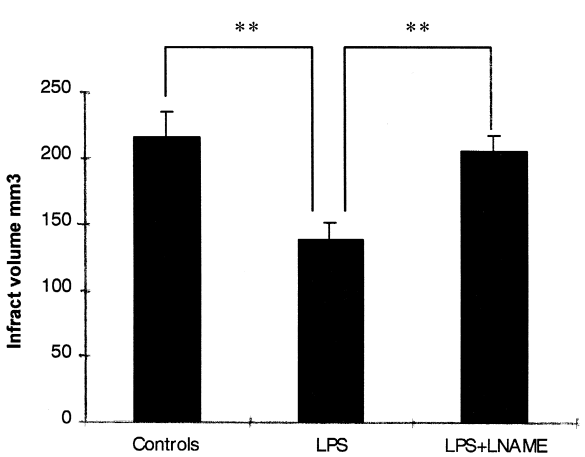

Fig. 2. Effect of L-NAME on lipopolysaccharide-induced tolerance. On the first day, the animals in the control group received normal saline (0.5 ml; i.p.), the LPS animals received lipopolysaccharide (0.5 mg; i.p.), and the animals in the L-NAME + LPS group, animals received L-NAME (3 mg/kg; i.p.) 1 h prior to administration of lipopolysaccharide. At 72 h later, all animals were subjected to a 60 min ischemia. True infarct volume was calculated after correcting the total infarct volume for edema. Values are expressed as mean ± S.E.M. Comparison vs. LPS group; $^{*}P < 0.05$; $^{**}P < 0.01$.

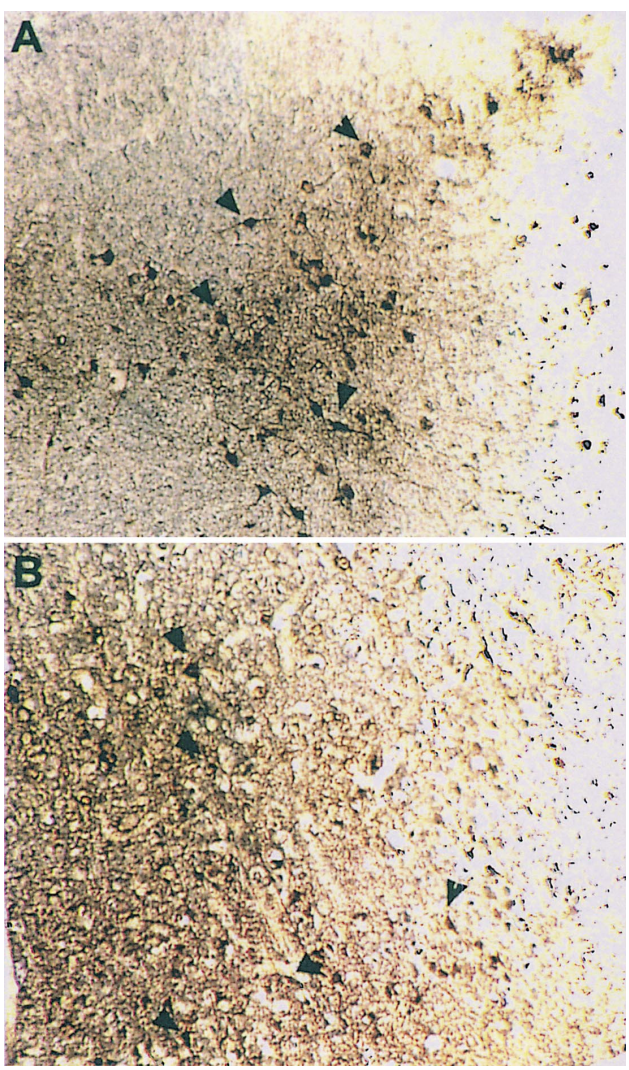

Fig. 3. Representative photomicrographs show 70 kDa heat shock protein immunohitochemistry in cerebral cortex of rats 72 h after (A) 60 min or (B) 3 min ischemia. In A, 70 kDa heat shock protein was expressed in neurons and glial cells in the junctional zone between infarcted and not infarcted areas. In B, 70 kDa heat shock protein was expressed in some cortical neurons of the middle cerebral artery territory. Arrows indicate 70 kDa heat shock protein-positive neurons.

Vector Laboratories, Burlingam, USA). Antigen-antibody complexes were visualised using the avidin–biotin Vectastain AB Kit (Vector Laboratories) and colour development was carried out with diaminobenzidine (Sigma Fast DAB). No specific labeling by 70 kDa heat shock protein antibody was assessed on directly adjacent sections (20 μm space) by (i) incubation with normal mouse serum instead of the primary antibody and (ii) by primary antibody absence. In these two cases, immunostaining was completely abolished.

2.4.2. Expression of eNOS and iNOS in lipopolysaccharide-induced ischemic tolerance

Eight rats were used for these experiments. The animals were given either lipopolysaccharide (0.5 mg/kg; i.p.) ($n = 6$) or normal saline ($n = 2$). After a variable time interval of 12 h ($n = 2$ lipopolysaccharide-treated rats), 24 h ($n = 2$ lipopolysaccharide-treated rats), or 72 h ($n = 2$ lipopolysaccharide-treated rats + 2 saline-treated rats), the animals were anesthetized with pentobarbital and perfused via the ascending aorta with normal saline followed by 4%

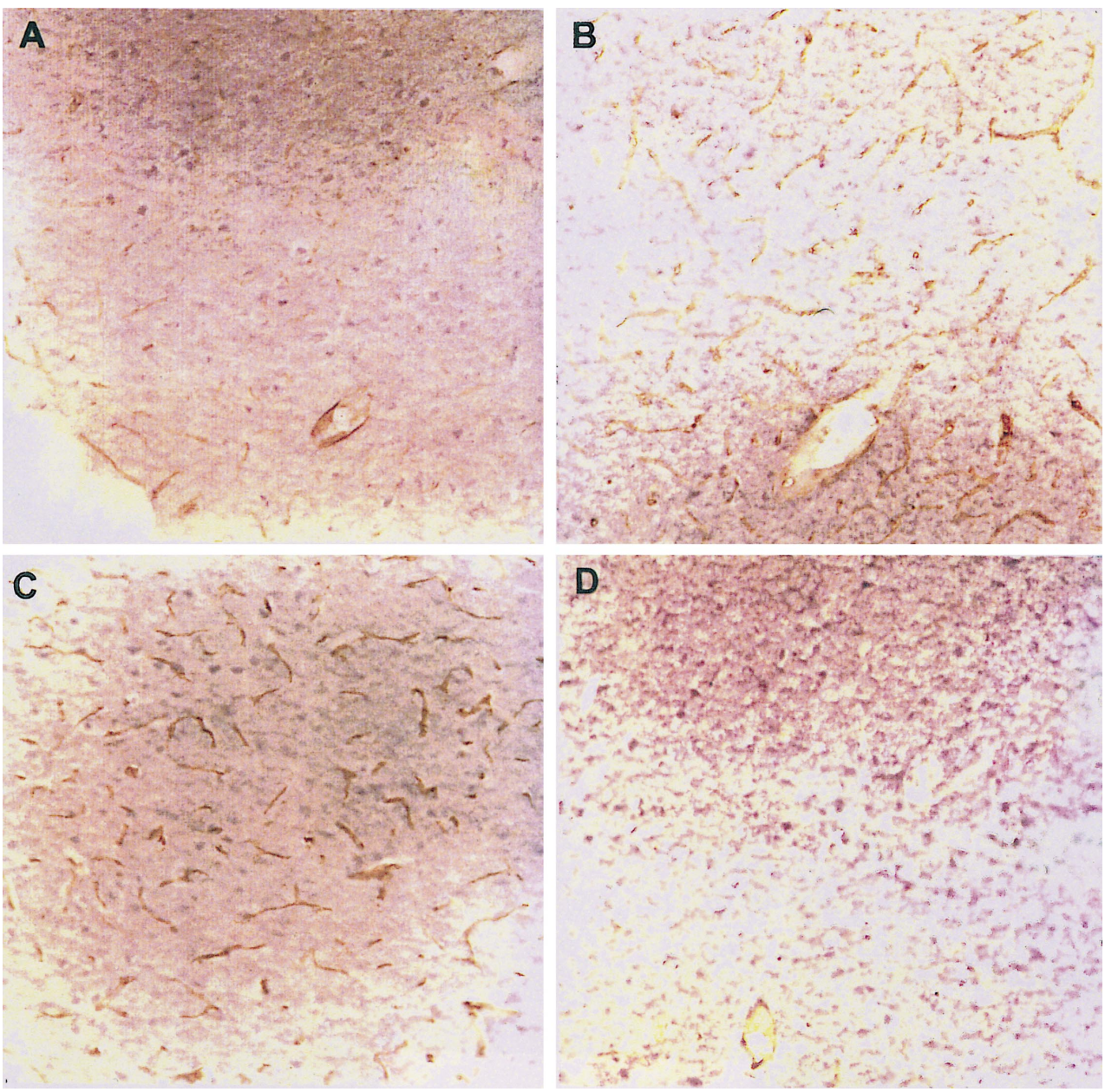

Fig. 4. Representative photomicrographs show eNOS immunoreactivity in middle cerebral artery territory of saline-treated rats (A) and lipopolysaccharide-treated rats 12 h (B), 24 h (C), or 72 h (D) after lipopolysaccharide administration. The intensity of staining for eNOS was increased in B and C as compared with A and D.

paraformaldehyde in phosphate buffer (pH 7.4). The immunohistochemical technique was similar to that used for 70 kDa heat shock protein expression except that we used antibodies specific for iNOS and eNOS in a dilution of 1/500. The intensity of staining for eNOS was expressed in number of + (range 0 to + + +).

2.5. Statistical analysis

Data are expressed as mean ± S.E.M. Blood pressure, blood gases, and infarct volumes were compared with a one way analysis of variance (ANOVA) followed, when significant, by a post-hoc protected least significant difference (PLSD) Fisher test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Mortality and failures of the ischemia occlusion

Of the 68 rats subjected to 60 min ischemia, seven rats died during the surgical procedures or during the reperfusion and 10 other rats did not get cortical infarcts after the prolonged ischemia. These animals were excluded from the study.

3.2. Blood pressure and blood gases

There were no significant differences in blood pressure or blood gases between the different groups (Table 1).

3.3. Effects of inhibition of NO synthesis by L-NAME

L-NAME had no significant effect by itself on infarct volume as proven by the lack of significant differences in infarct volumes between L-NAME treated rats (219 ± 12 mm^3, $n = 6$) and saline-treated rats (227 ± 9 mm^3, $n = 6$).

There was a significant reduction in infarct volume (1) in rats subjected to 3 min ischemia, 72 h prior to 60 min ischemia, compared with sham-operated rats (136 ± 11 mm^3 vs. 197 ± 12 mm^3; −35%; $P < 0.01$); and (2) in rats administered lipopolysaccharide 72 h prior to 60 min ischemia compared with saline-treated controls (139 ± 18 mm^3 vs. 216 ± 8 mm^3; −35%; $P < 0.01$). The differences were due entirely to salvage of the cortex, as pretreatment (preconditioning or lipopolysaccharide) did not significantly lessen the area of caudate infarcted. The beneficial effect of preconditioning remained unchanged despite prior administration of L-NAME (mean infarct volume in L-NAME-treated preconditioned rats was 121 ± 19 mm^3, $n = 6$). The protective effect of lipopolysaccharide was markedly reduced by the prior administration of L-NAME. Indeed, infarct volume in the L-NAME + lipopolysaccharide group (205 ± 12 mm^3) was not significantly different from the infarct volume in the saline-injected group, but was significantly greater than in rats challenged with lipopolysaccharide alone ($P < 0.05$). Figs. 1 and 2 illustrate the results of these experiments.

3.4. Expression of 70 kDa heat shock protein

A 70 kDa heat shock protein was expressed in cortical neurons of the MCA territory 72 h after prolonged 60 min ischemia and after 3 min ischemia. However, no staining was seen in sham-operated rats. No expression of 70 kDa heat shock protein was observed in rats administered lipopolysaccharide or saline 72 h prior. Fig. 3 illustrates the results of these experiments.

3.5. Expression of eNOS and iNOS in lipopolysaccharide-induced ischemic tolerance

Paraformaldehyde-fixed cryostat sections of brains were studied in rats sacrificed 12, 24, and 72 h, respectively, after lipopolysaccharide treatment and in two saline-treated rats. Lipopolysaccharide-treated animals and controls showed no appearance of iNOS cells in the brain at any time of sacrifice (data not shown). In contrast, staining for eNOS was observed in blood vessels with an increased intensity in rats sacrificed 12 h (+ + +) and 24 h (+ + +) after lipopolysaccharide administration as compared with rats sacrificed 72 h after lipopolysaccharide treatment (+) or saline-treated rats (+). Fig. 4 illustrates the results of these experiments.

4. Discussion

The present study confirms that a brief transient focal ischemia, which per itself produces no significant brain injury, renders the involved region of the brain more resistant to a subsequent prolonged ischemia. Such a protective effect was first described in global ischemia models and later in focal ischemia models, with a maximum effect for an interval of 3 days between preconditioning ischemia and prolonged ischemia (Kitagawa et al., 1990; Glazier et al., 1994; Chen et al., 1996; Barone et al., 1998). Our results also confirm that a low dose of lipopolysaccharide (0.5 mg/kg; i.p.) administered 72 h before prolonged ischemia is effective in reducing the infarct size. The protective effect of lipopolysaccharide in the brain has been recently demonstrated (Tasaki et al., 1997; Dawson et al., 1999) with a dose of 0.9 mg/kg (i.v.). In Tasaki's study, the window of lipopolysaccharide-induced protection lasted from 2 to 4 days.

The mechanisms of ischemic tolerance in the brain remain uncertain. However, the fact that the ischemic tolerance requires at least 24 h to become apparent, and persists for 5–7 days, suggests that it is caused by the synthesis of protective proteins. The observation that brain

ischemic tolerance induced by ischemic preconditioning is reduced by cycloheximide, a protein synthesis inhibitor (Barone et al., 1998). Among a number of protein candidates for the role of mediator, the most reliable are heat shock proteins (mainly 70 kDa heat shock protein) (Chen and Simon, 1997) which are believed to play a crucial role in the protection of neurons against ischemia–reperfusion insults by aiding, along with other chaperones, the restoration of the structure and function of the denatured proteins (Rokutan et al., 1998). It has been demonstrated that ischemic preconditioning protective effect in the brain is abolished by quetercin, a non-selective inhibitor of 70 kDa heat shock protein (Nakata et al., 1993). Moreover, heat shock proteins are consistently upregulated in tolerant brains with a time course of 70 kDa heat shock protein expression compatible with the time window of ischemic tolerance (Glazier et al., 1994; Chen et al., 1996). The fact that, in our study, a short 3 min ischemia induced expression of 70 kDa heat shock protein in the cortex of the MCA territory, and delayed protection, supports the hypothesis of 70 kDa heat shock protein involvement in tolerance induced by ischemic preconditioning. Nevertheless, the role of heat shock proteins as mediators of tolerance in ischemic preconditioning remains controversial since other studies have suggested that heat shock proteins expression is not required for ischemic tolerance (Kobayashi et al., 1995; Abe and Nowak, 1996). Thus, it has been demonstrated that cortical spreading depression protects against subsequent global cerebral ischemia in the absence of heat shock protein messenger RNA expression (Kobayashi et al., 1995). To the best of our knowledge, no previous study has investigated the expression of 70 kDa heat shock protein in lipopolysaccharide-induced ischemic tolerance of the brain. However, three studies have examined the expression of 70 kDa heat shock protein in cardiac delayed protection induced by monophosphoryl lipid A, with opposite results depending on animal species and experimental protocols (Baxter et al. 1996; Yoshida et al., 1996; Nayeem et al., 1997). In our model, 70 kDa heat shock protein was not expressed in tolerant cortex 72 h after lipopolysaccharide administration. This result suggests that lipopolysaccharide-induced protection is not related to induction of 70 kDa heat shock protein.

What triggers the synthesis of the protective proteins is unclear. Some studies have suggested that late ischemic preconditioning of the heart may be triggered by the generation of NO (Bolli et al., 1997; Qiu et al., 1997). Recently, Centeno et al. (1999) reported that electrical recovery after anoxia in hippocampal slices was improved by anoxic preconditioning and that NO from constitutive hippocampal NOS could be involved in the induction of neuroprotection. Our results do not support that NO triggers ischemic preconditioning-induced tolerance since inhibition of NOS by administration of L-NAME previous to the preconditioning-ischemia did not abolish the neuroprotective effect. In contrast, the protective effect of lipopolysaccharide is abolished by prior administration of L-NAME. That suggests that NO plays a key role in lipopolysaccharide-induced tolerance. Even though L-NAME has a remaining inhibitory effect on NOS, the timing of L-NAME administration, largely prior to ischemia, suggests a role of trigger rather than a role of mediator.

The source of increased NO generation induced by a low dose of lipopolysaccharide remains uncertain, although our results speak in favour of endothelial origin. Three isoforms of NOS have been identified in the brain, two constitutive, the neuronal isoform (nNOS) and the endothelial isoform (eNOS), and the other inducible (iNOS). There is accumulating evidence that, during Gram-negative shock, lipopolysaccharide triggers the release of cytokines, which in turn induce iNOS, leading to high output of NO and resulting in hypotension and vasoplegia (Stoclet et al., 1998). One can object that doses of lipopolysaccharide used to induce brain tolerance are five to 20 times lower than those required to induce septic shock. However, in rat cardiomyoytes protective doses of lipopolysaccharide are capable of inducing iNOS (McKenna et al., 1995; Shindo et al., 1995). Furthermore, accumulating evidence in different experimental models suggest that cardioprotective doses of monophosphoryl lipid A induce iNOS mRNA, while administration of iNOS selective inhibitors prior to ischemia nullified the protective effect of monophosphoryl lipid A (Yoshida et al., 1996; Zhao et al., 1997; Maulik et al., 1998). Finally, it was recently shown that the dose of lipopolysaccharide used to induce brain tolerance stimulates NO synthesis because increased plasma levels of NO metabolites (nitrates and nitrites) were observed 72 h after lipopolysaccharide treatment (Dawson et al., 1999). For all that, we can not conclude that lipopolysaccharide-induced brain ischemic tolerance necessarily involves induction of iNOS. Indeed, in our experiments, no iNOS immunoreactivity was observed in rat brain within 72 h after lipopolysaccharide administration. Our observations are consistent with previous reports by Salter et al. (1991) and Van Dam et al. (1995) who did not find iNOS in the brains of lipopolysaccharide-treated rats. This suggests that peripheral administration of lipopolysaccharide (as used in our model), unlike the direct administration of lipopolysaccharide into the brain (Okamoto et al., 1997), does not induce significant expression of iNOS in the brain. Consequently, the major source of NO overproduced after peripheral administration of a low dose of lipopolysaccharide used to induce brain tolerance is either peripheral or central, but, in this case, the source is the constitutive form of NOS. It has been previously demonstrated that lipopolysaccharide, in certain experimental conditions, is capable to activate endothelial NOS (Huang et al., 1998). The recent observation from Dawson et al. (1999) that lipopolysaccharide-induced brain tolerance to focal ischemia is, at least partly, dependent on the active maintenance of microvascular patency suggests a vascular mechanism. Our findings that

(1) lipopolysaccharide (0.5 mg/kg; i.p.; administered 72 h prior) induces a delayed enhanced endothelium-dependent relaxation in isolated rat aorta (Pu et al., 1999), and (2) eNOS immunoreactivity in rat brain is increased within 24 h after lipopolysaccharide treatment, supports the hypothesis of eNOS contribution to NO overproduction. Moreover, the fact that expression of eNOS is maximal within 24 h after lipopolysaccharide treatment, and reduced 72 h later, confirms that endothelium-derived NO may play the role of trigger in lipopolysaccharide-induced brain ischemic tolerance.

Although the two methods, ischemic preconditioning and administration of lipopolysaccharide, induce a similar tolerance to focal ischemia and require a similar time interval after the stimulus for tolerance be induced, our results suggest that the underlying mechanisms are, at least partly, different depending on the nature of the stimulus. Involvement of the NO pathway in lipopolysaccharide-induced tolerance opens new ways in the search of therapeutic strategies.

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