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Role of nitric oxide in post-ischemic cerebral hyperemia in anesthetized rats

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Abstract

This study was undertaken to determine the extent to which nitric oxide (NO) mechanisms are involved in cerebral hyperemia following global brain ischemia. The vertebral arteries were cauterized through the first alar foramina in anesthetized male Sprague–Dawley rats and followed by 20-min occlusion of the common carotid arteries. Blood flow from the parietal cerebral cortex was measured using laser-Doppler flowmetry. In saline-treated animals, carotid occlusion reduced cerebral blood flow by approximately 95% with a maximal hyperemia of about 400% observed after 15 min of reperfusion. Pre-treatment with the nonspecific NO synthase inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester; 2, 10 and 50 mg kg^{-1}), produced dose-related depression of post-ischemic hyperemia, whereas D-NAME (10 mg kg^{-1}) was inactive. Pre-treatment with L-arginine (300 mg kg^{-1}, i.v.) prevented L-NAME attenuation of cerebral hyperemia. The selective neuronal NO synthase inhibitor, 7-nitroindazole (30 mg kg^{-1}), was without significant depressant effect. These results suggest that NO (largely from vascular endothelium) is instrumental in development of post-ischemic cerebral hyperemia. © 1998 Elsevier Science B.V.

Keywords: Cerebral ischemia-reperfusion; (Rat); Hyperemia; Nitric oxide (NO); N^G-nitro-L-arginine methyl ester (L-NAME); 7-Nitroindazole

1. Introduction

Nitric oxide (NO) has complex actions in the brain including a multifaceted role in control of the cerebral circulation. Constitutive NO synthase in vascular endothelium or neuronal elements produces NO that contributes to maintenance of resting cerebral blood flow as well as a variety of vasodilator responses in the brain including hyperemia in response to hypercapnia, severe hypoxia, and neuronal activation (Iadecola et al., 1994; Faraci and Brian, 1994; Pelligrino et al., 1996; Iadecola, 1997). Several studies suggest that NO also might be involved in the early hyperemic response that is seen following global cerebral ischemia. However, the models used either do not allow for continuous measurement of cerebral blood flow (Greenberg et al., 1995) or do not produce a large consistent hyperemic response during reperfusion (Prado et al., 1993; Sadoshima et al., 1997).

In this study, we utilized a rodent four-vessel occlusion model of forebrain ischemia combined with continuous measurement of cerebral blood flow using laser-Doppler flowmetry. Experiments were undertaken to determine whether systemic administration of L-NAME (N^G-nitro-L-arginine methyl ester) and 7-nitroindazole produce a dose-related attenuation of post-ischemic hyperemia, and if so, if the attenuation of response might be prevented by pre-treatment with L-arginine. The overall goal of this study was to determine if either NO of either endothelial or neuronal derivation is instrumental in production of post-ischemic cerebral vasodilation in anesthetized rats.

2. Materials and methods

2.1. General

Adult male Sprague–Dawley rats (300–475 g) were anesthetized with pentobarbital (50 mg kg^{-1}, i.p.). The trachea, femoral artery and femoral vein were cannulated, with the animals then positioned in a Kopf rat stereotaxic devise to immobilize the head. Body temperature was maintained at approximately 37°C with a heating pad. Neuromuscular relaxation and anesthesia were maintained

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with intravenous pancuronium (0.1 mg kg^{-1} min^{-1}) and pentobarbital (1.0 mg kg^{-1} min^{-1}) after the animals were placed on positive artificial ventilation with room air using a Harvard small animal respirator. Arterial blood pressure was measured from a femoral artery using a Statham P23 blood pressure transducer. Heart rate was determined from the femoral arterial pulse wave. All physiological responses were recorded on a Grass model 7 polygraph. The animals were treated in a manner consistent with the regulations of the USPHS with experimental protocols approved by the University of Oklahoma Institutional Animal Use and Care Committee.

2.2. Cerebral blood flow measurements

Cerebral blood flow was measured by laser-Doppler flowmetry using a Laserflow (BPM 403A) laser-Doppler flowmeter (Gherezghiher et al., 1991; Koss and Gherezghiher, 1993; Koss, 1994). Blood flow in the parietal cortex was measured through a cranial window, formed by thinning the calvarium with a Dremel drill and dental burr. The laser-Doppler technique exposes a small surface area to coherent diode generated laser light which is reflected from both stationary tissue and blood cells with the moving blood cells producing a Doppler frequency shift which creates Doppler beat-frequencies at the photodetector. The computer processed Doppler beat-frequencies are proportional to the total blood flow within the volume of tissue measured and is dependent upon the relative concentration of blood cells and average blood cell velocity (Shepard and Oberg, 1990; Riva et al., 1994). 'Zero' blood flows were determined in each preparation after sacrifice at the conclusion of the experiment.

2.3. Four-vessel occlusion

Using the method described by Pulsinelli and Brierley (1979), the vertebral arteries were cauterized using an Ellman Surgitron F.F.P.F. (set on the coagulate and partially rectify with power set on 1.5–2) with a fine tip electrode via the alar foramen of the first cervical vertebrae. The carotid arteries were looped using 0-gauge suture exiting the neck on either side of the trachea tube. At the time of occlusion, the carotid arteries were clamped using microvascular clips. Only preparations demonstrating at least 80% reduction of cerebral blood flow were utilized for these studies.

2.4. Experimental protocols

All the animals were exposed to 20 min of ischemia following a 20–35-min stabilization after drug administration. In most of the experimental groups, a 30-min stabilization period followed injection. Where L-arginine plus L-NAME were administered, the L-arginine was injected immediately following baseline and allowed 15 min to stabilize, then the L-NAME was injected followed by 20-min additional stabilization before occlusion.

2.5. Drugs and statistics

Pancuronium bromide was purchased from Sigma (St. Louis, MO). The L- and D-isomers of N^G-nitro-L-arginine methylester (L- and D-NAME), L-arginine and 7-nitroindazole (7NI) were obtained from Research Biochemicals International (RBI; Natick, MA). All drugs given intravenously were prepared daily by dissolving them in physiological saline solution. 7-Nitroindazole was administered intraperitoneally after dissolution in dimethyl sulfoxide (DMSO; Sigma) at a concentration of 60 mg ml^{-1}. DMSO was used in the same volume as a vehicle control.

Statistical evaluation was by analysis of variance (ANOVA) followed by Dunnett's *t*-test with $P < 0.05$ testing for significance. Statistical differences of resting levels of cerebral blood flow, systemic arterial blood pressure and heart rate were determined using Student's *t*-test for paired values.

3. Results

3.1. Post-ischemic cerebral hyperemia

Fig. 1 shows a typical polygraph recording of cerebral blood flow responses to bilateral common carotid artery occlusion in an anesthetized rat in which the vertebral arteries were previously cauterized. In the initial series of control experiments, bilateral carotid occlusion caused a sustained depression of cerebral blood flow in 8 of 13 rats. In subsequent experiments, only 7 of 62 preparations failed to reach the established 80% threshold level. After 20 min of occlusion there was a $94 \pm 3\%$ reduction of cerebral blood flow in the saline treated rats. Release of the carotid occlusion resulted in a cerebral blood flow response of $404 \pm 69\%$ of the pre-occlusion levels with the maximal hyperemia seen at 15 min after release. Return to pre-oc-

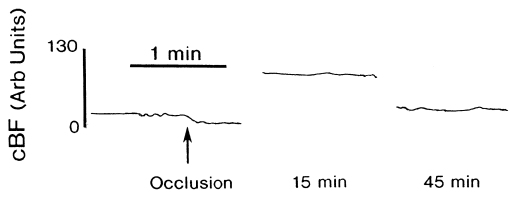

Fig. 1. Typical example of four-vessel cerebral ischemia-reperfusion in a pentobarbital anesthetized Sprague–Dawley rat. Cerebral blood flow (cBF) was measured with a laser-Doppler flowmeter and is expressed in arbitrary (Arb) units. Vertebral arteries were coagulated about 1 h prior to start of illustrated polygraph record. Common carotid arteries were clamped for 20 min (left panel). Records of 1-min reperfusion demonstrate post-occlusive reactive hyperemia (438% of control) seen after 15-min reperfusion (middle panel) and cerebral blood flow level 45 min after release of carotid occlusion (right panel).

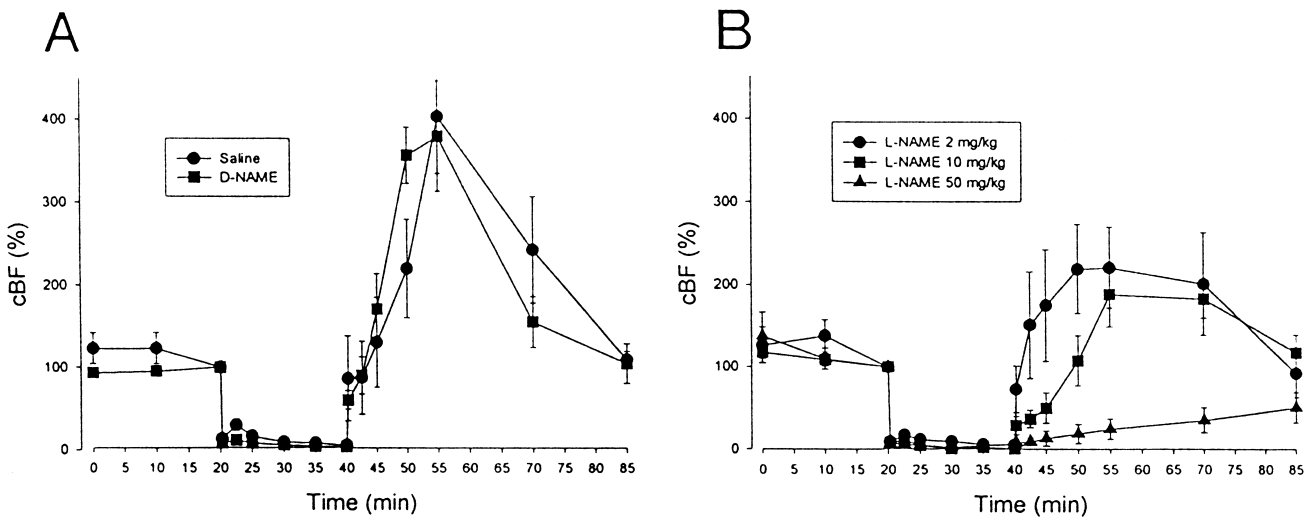

Fig. 2. Composite representation of cerebral blood flow levels before, during and after bilateral common carotid occlusion (between 20- and 40-min time points) in anesthetized rats that underwent prior vertebral artery coagulation. Values are means ± S.E.M. for times indicated. (A) Solid circles represent responses in saline pre-treated controls ($n = 8$). Solid squares represent responses in animals that were administered D-NAME (10 mg kg^{-1}, i.v.) 30 min prior to occlusion ($n = 7$). (B) Effects of intravenous pretreatment with L-NAME on ischemia reperfusion responses in anesthetized rats (solid circles: L-NAME 2 mg kg^{-1}; solid squares: L-NAME 10 mg kg^{-1}; solid triangles: L-NAME 50 mg kg^{-1}). $n = 6$–8 rats per group.

clusion levels was generally achieved within 45 min after return of carotid flow (Fig. 2A). Pre-treatment with D-NAME (10 mg kg^{-1}, i.v.) did not alter this response pattern (Fig. 2A). In contrast, pre-treatment with L-NAME (2, 10 and 50 mg kg^{-1}, i.v.) resulted in a dose-related attenuation of the post-occlusive cerebral hyperemia (Fig. 2B). The larger dose of L-NAME also largely prevented reperfusion to baseline (normalization) of cerebral blood flow levels (Fig. 2B).

As illustrated in Fig. 3, the post-occlusive hyperemia seen after L-arginine (300 mg kg^{-1}, i.v.) was significantly greater ($P < 0.05$) than that of the control saline group (see Fig. 2A). L-NAME (10 mg kg^{-1}, i.v.) did not attenuate post-occlusive hyperemia (compared with saline controls) when administered to animals previously treated with L-arginine. The post-occlusive hyperemia responses in animals treated with the selective inhibitor of neuronal nitric oxide synthase (7-nitroindazole; 30 mg kg^{-1}, i.p.) also was not significantly different from the control groups (Fig. 4). Fig. 5 contrasts maximal post-occlusive cerebral hyperemia seen 15 min after return of carotid blood flow in seven experimental groups. Only L-NAME produced attenuation of hyperemic responses.

3.2. Basal cerebral blood flow and blood pressure

Pre-treatment with L-NAME (10 and 50 mg kg^{-1}, i.v.) and with 7-nitroindazole (30 mg kg^{-1}, i.p.) resulted in a

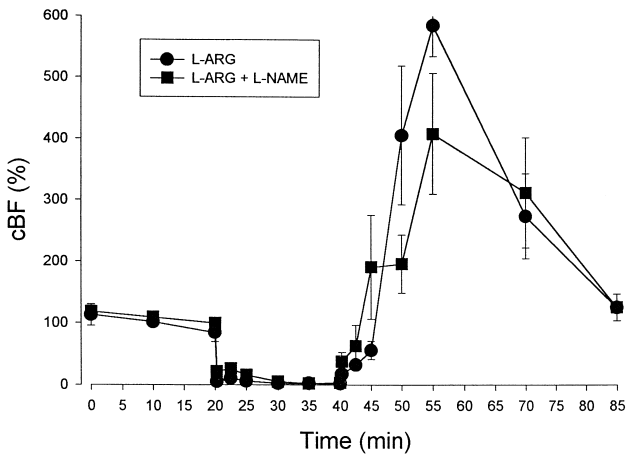

Fig. 3. Effects of L-arginine (300 mg kg^{-1}, i.v.) alone (solid circles, $n = 6$) and L-arginine plus 10 mg kg^{-1} of L-NAME (solid squares, $n = 7$) on ischemia-reperfusion cerebral blood flow responses in anesthetized rats. Carotid clamps applied between 20- and 40-min time periods. Values represent means ± S.E.M.

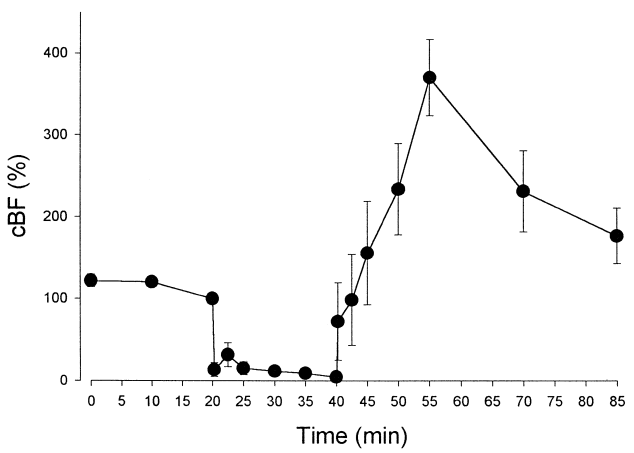

Fig. 4. Ischemia-reperfusion responses on cerebral blood flow in anesthetized rats pre-treated intraperitoneally with 7-nitroindazole 30 min prior to carotid artery clamping (between 20- and 40-min time periods). Values represent means ± S.E.M. for five preparations.

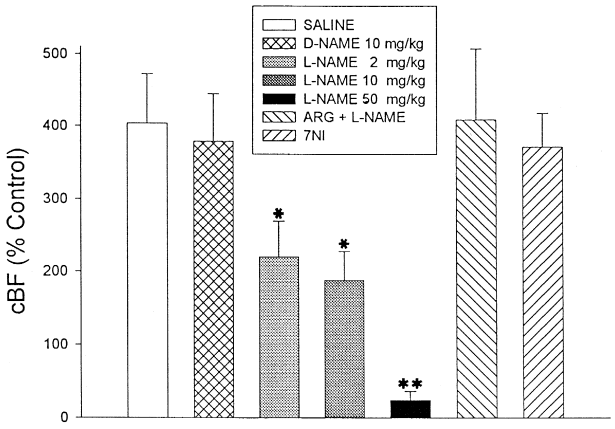

Fig. 5. Comparison of post-occlusive cerebral reactive hyperemia levels in seven experimented groups of anesthetized rats. Responses measured 15 min after re-opening of carotid artery blood flow. Values represent mean responses + S.E.M. for 5–8 rats per group. $* P < 0.05$; $** P < 0.01$ vs. saline group.

significant depression of basal cerebral blood flow (Table 1). The reduction of resting cerebral blood flow was comparable for both the non-selective nitric oxide synthase inhibitor (L-NAME at 50 mg kg^{-1}) and the selective neuronal nitric oxide inhibitor (7-nitroindazole at 30 mg kg^{-1}). Treatment with the lowest dose of L-NAME, D-NAME and L-arginine did not alter resting cerebral blood flow. All three doses of L-NAME produced a significant elevation of systemic arterial blood pressure, whereas L-arginine caused a significant reduction of blood pressure (Table 1). Bilateral occlusion of the common carotid arteries produced the expected reflex elevation in arterial blood pressure with no significant differences between any of the experimental groups (data not shown). Also, there were no significant differences between resting arterial pressures prior to and 15 min after the 20-min period of carotid occlusion, except for rats receiving the two higher doses of L-NAME. In these two groups of animals, blood pressure was lower 15 min after reperfusion than that seen immediately prior to carotid clamping. However, these arterial pressure levels were not lower than seen prior to L-NAME administration which may indicate some degree of recovery from the L-NAME-induced hypertension.

4. Discussion

Global cerebral ischemia followed by reperfusion often is characterized by pronounced hyperemia. For example, reperfusion following 15 min of total ischemia in the dog brain causes a three-fold increase in blood flow at about 5 min with return to basal levels by 30 min (Snyder et al., 1975). In cats, re-transfusion after prolonged hemorrhage results in reactive hyperemia lasting 10–25 min (Carter and Atkinson, 1973). Reperfusion after global cerebral ischemia (four-vessel) in Wistar rats produces a reactive hyperemia with a duration of 5–15 min (Pulsinelli et al., 1982). Todd et al. (1986), also using the four-vessel occlusion technique, reported a consistent cerebral hyperemic response in Sprague–Dawley rats that peaked between 5–15 min and was fully recovered by 30 min after reperfusion. In the present study, using four-vessel occlusions of 20-min duration, reperfusion resulted in a 3- to 4-fold increase of cerebral blood flow which peaked at about 15 min and usually returned to baseline by 45 min after release of the carotid artery occlusion.

After initial description by Pulsinelli and Brierley (1979), the four-vessel rodent occlusion model of forebrain ischemia has been employed widely as a technique in studies of cerebral ischemic mechanisms (Ginsberg and Busto, 1989). However, a number of investigators reported inconsistent degrees of cerebral ischemia using this technique, even when the initially suggested Wistar strain of rat is used (Ginsberg and Busto, 1989). One possibility for this variability is inconsistent coagulation of the vertebral

Table 1
Comparison of resting physiological parameters before and after pre-treatment: cerebral blood flow (cBF) expressed as % of initial control levels, mean systemic arterial blood pressure (MSAP; mmHg) and heart rate (HR; beats min^{-1})

Treatment	*n*	cBF	MSAP		HR	
		% Control	Before	After	Before	After
Saline	8	93 ± 9	96 ± 4	97 ± 5	442 ± 13	442 ± 14
D-NAME (10 mg kg^{-1})	7	95 ± 13	97 ± 7	100 ± 6	467 ± 11	467 ± 11
L-NAME (2 mg kg^{-1})	7	106 ± 11	99 ± 9	124 ± 11[a]	427 ± 9	388 ± 16
L-NAME (10 mg kg^{-1})	8	94 ± 4[a]	121 ± 3	138 ± 5[b]	462 ± 13	424 ± 10[a]
L-NAME (50 mg kg^{-1})	6	74 ± 12[a]	89 ± 12	114 ± 7[a]	446 ± 26	444 ± 15
L-ARG (300 mg kg^{-1})	6	94 ± 11	103 ± 5	76 ± 7[b]	451 ± 12	422 ± 10
L-Arginine (300 mg kg^{-1}) + L-NAME (10 mg kg^{-1})	7	88 ± 7	94 ± 6	134 ± 7[b]	428 ± 12	406 ± 14
7NI vehicle (DMSO)	7	99 ± 8	97 ± 7	100 ± 8	422 ± 15	417 ± 17
7NI (30 mg kg^{-1})	7	65 ± 7[a]	109 ± 5	99 ± 7	428 ± 11	412 ± 11

Values represent means ± S.E.M. All measurements taken before and 20–30 min after drug or vehicle administration.
[a] $P < 0.05$.
[b] $P < 0.01$.

arteries. This speculation is supported by the almost 100% success rate achieved by Todd et al. (1986) using adult Sprague–Dawley rats where the vertebral arteries were divided under direct visualization.

Pre-treatment with L-NAME produced a dose-related attenuation of post-occlusive reactive hyperemia. Our results are most consistent with those reported in abstract form by Maynard et al. (1993) using bilateral carotid occlusion plus hypotension for 10 min. With this two-vessel model, chronic L-NAME (50 mg day^{-1} for 4 days, i.p.) reduced the peak hyperemia from $440 \pm 201\%$ to $243 \pm 144\%$ of control levels in 14 anesthetized Wistar rats. As in the present study, L-arginine largely prevented the action of L-NAME. The other report most similar to the present study is by Greenberg et al. (1995) using intracranial pressure elevation (15 min) to produce global ischemia in anesthetized piglets. They also found L-NAME (10 and 50 mg kg^{-1}, i.v.) to produce a dose-dependent blockade of the approximately 300% increase of blood flow seen 8 min after reperfusion (Greenberg et al., 1995). However, use of microsphere blood flow measurements in the above study, somewhat limits appreciation of the time course of the full reactive hyperemic response.

Although not measured in the present study, it is not likely that drug-induced alterations of blood chemistry contributed to the observed blockade of reactive cerebral hyperemia by L-NAME. In studies by other investigators, also in rats, no significant changes in pH, pCO_2, or pO_2 were seen following systemic treatment with comparable doses of L-NAME (Kumura et al., 1994; Fouyas et al., 1997), L-arginine (Dalkara et al., 1994; Sadoshima et al., 1997), or 7-nitroindazole (Wang et al., 1995; Fouyas et al., 1997).

Prado et al. (1993) found L-NAME (30 mg kg^{-1}, i.v.) to inhibit normalization of cerebral blood flow levels after even a 10-min bilateral common carotid artery occlusion (two-vessel model) in anesthetized Wistar rats. They reported an immediate hyperemic response (lasting 1–2 min) in 5 of 6 control and 3 of 8 L-NAME treated rats. It is of interest that the peak hyperemic response, when observed, was attenuated by about 50% in the L-NAME group (Prado et al., 1993). Finally, a recent study using anesthetized spontaneously hypertensive rats, also utilizing bilateral carotid artery occlusion, was undertaken to investigate NO involvement in ischemia-reperfusion (Sadoshima et al., 1997). With this model, carotid occlusion for 30 min produced an almost total cerebral ischemia which was followed by a modest hyperemia of less than 25% after release of the occlusion. After nitric oxide synthase inhibition with N^G-nitro-L-arginine (5 mg kg^{-1}, i.v.), a 22% hyperemic response was still observed (Sadoshima et al., 1997). Also, unlike other investigations with carotid occlusion (Prado et al., 1993), supposed inhibition of NO did not prevent normalization of cerebral blood flow during the reperfusion period (Sadoshima et al., 1997). Although the reasons for such divergent results with a similar experimental protocol are not clear, they may be due to the different dose levels used. For example, in the present study, normalization of cerebral blood flow was seen at 10 mg kg^{-1} of L-NAME but not at the 50 mg kg^{-1} dose.

Experiments with D-NAME and L-arginine demonstrate that L-NAME is most likely acting by inhibition of nitric oxide synthase and not by some other non-specific pharmacological mechanism. Consistent with most studies, L-arginine (300 mg kg^{-1}, i.v.) had no significant effect on basal cerebral blood flow (Faraci and Brian, 1994). This finding suggests that the amount of substrate is not the limiting factor for NO production under resting conditions. L-Arginine pre-treatment prevented the L-NAME attenuation of reactive hyperemia and also produced a significant elevation in the magnitude of the hyperemic response. This later observation is consistent with the report by Sadoshima et al. (1997) where the same dose of L-arginine also potentiated the post-occlusive hyperemia. In contrast to the present study, these investigators found L-arginine to significantly increase resting cerebral blood flow. L-Arginine increases resting blood flow in the ischemic penumbra after middle cerebral artery occlusion, also in the spontaneously hypertensive rat (Dalkara et al., 1994).

There are two well-characterized isoforms of constitutive nitric oxide synthase, endothelial and neuronal, both of which could contribute to post-ischemic vasodilator responses (Moncada et al., 1991; Bredt and Snyder, 1994). For example, it has been suggested that elevations of intraneuronal calcium concentrations in response to depolarization activates NO production which could then produce cerebral vasodilation (Gally et al., 1990; Iadecola, 1993). Most previous studies of NO effects on cerebral blood flow have used non-specific nitric oxide synthase inhibitors such as L-NAME (Iadecola et al., 1994) although, more recently, selective inhibitors of nitric oxide synthase have been identified (Moore et al., 1993).

In line with other studies in normotensive rats, both L-NAME and 7-nitroindazole reduced resting cerebral blood flow. However, only L-NAME produced a concomitant elevation of arterial blood pressure (Tanaka et al., 1991; Iadecola et al., 1994; Wang et al., 1995; Fouyas et al., 1997). These results demonstrate that 7-nitroindazole does not inhibit endothelial nitric oxide synthase and suggest that nitric oxide released tonically from neurons may play an important role in maintenance of basal cerebral blood flow.

It is of interest, that even though both L-NAME and 7-nitroindazole decreased basal cerebral blood flow to a similar extent, only L-NAME attenuated post-ischemic hyperemia. These results suggest that NO contributing to hyperemic cerebral vasodilation is derived from vascular endothelium and not from activation of neuronal elements. In contrast, numerous studies have shown mediation of local cerebral vasodilator responses to a variety of stimuli provoking increases in neuronal activity (for reviews see Faraci and Brian, 1994; Iadecola, 1997).

It is clear that, in rats, cerebral reperfusion after either focal or global ischemia results in an enhanced production and release of NO (Tominaga et al., 1994; Kumura et al., 1994; Shibata et al., 1996). However, neither the origin nor the pathophysiological role of this released NO is known. Indeed, the role played by NO in response to ischemia is complex with evidence for both protective and cytotoxic involvement (Iadecola et al., 1994; Faraci and Brian, 1994; Pelligrino et al., 1996). It is likely that early production of NO plays a beneficial role in protecting the brain from early ischemic damage due to its ability to increase cerebral blood flow by vasodilation and by its capacity to inhibit platelet aggregation by acute upregulation of cell adhesion molecules (Zhang et al., 1995; Lefer and Lefer, 1996).

In conclusion, we have provided evidence for a causal role for NO in mediation of the cerebral hyperemia seen during reperfusion after a 20-min global ischemic challenge in anesthetized rats. L-NAME produced dose-related attenuation of the hyperemic response and this effect was countered by pre-treatment with L-arginine. The observation that the neuronal nitric oxide synthase inhibitor, 7-nitroindazole, was inactive, suggests that the NO is released from the vascular endothelium and not from neurons.

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