# Increases in local cerebral blood flow associated with somatosensory activation are not mediated by NO

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**Adachi, Keiji, Shinichi Takahashi, Peter Melzer, Kenneth L. Campos, Thomas Nelson, Charles Kennedy, and Louis Sokoloff.** Increases in local cerebral blood flow associated with somatosensory activation are not mediated by NO. *Am. J. Physiol.* 267 (*Heart Circ. Physiol.* 36): H2155–H2162, 1994.—Effects of inhibition of nitric oxide (NO) synthase by $N^G$-nitro-L-arginine methyl ester (L-NAME) on the increases in local cerebral blood flow (LCBF) produced in the whisker-to-barrel sensory pathway by vibrissal stimulation were studied in conscious rats with the autoradiographic iodo[$^{14}$C]antipyrine method. Unilateral whisker stroking increased LCBF in the ipsilateral trigeminal spinal and principal sensory nuclei, contralateral ventral posteromedial thalamic nucleus, and contralateral somatosensory barrel cortex. Intravenous L-NAME (30 mg/kg) lowered baseline LCBF without altering the percent increases due to stimulation. Intracisternal infusions of L-NAME in doses about 10 times the molar content of free arginine in brain inhibited brain NO synthase activity by 88%, but the percent augmentations of LCBF by stimulation remained unchanged. Chronic treatment with L-NAME (50 mg/kg ip twice daily for 4 days) inhibited NO synthase activity in brain by 84% but also failed to reduce the percent increases in LCBF due to stimulation. These results indicate that NO does not mediate the increases in LCBF associated with functional activation.

$N^G$-nitro-L-arginine methyl ester; whisker barrels; nitric oxide; nitric oxide synthase; functional activation; brain; iodo[$^{14}$C]antipyrine

CEREBRAL ENERGY METABOLISM and blood flow are both increased by functional activation of the neural tissues and are decreased when functional activity is reduced (12, 37). Energy metabolism produces $CO_2$ and $H^+$ and consumes $O_2$, and increased $P_{CO_2}$ and $H^+$ concentrations and decreased $P_{O_2}$ in blood and/or tissue dilate cerebral vessels and raise cerebral blood flow (CBF); changes in the opposite direction constrict the cerebral vessels and reduce CBF (22). Chemical factors related to energy metabolism, therefore, have been long suspected as mediators of the adjustment of CBF to the changing metabolic demands of altered neuronal activity (23, 36). Despite many efforts, however, changes in $P_{CO_2}$, pH, and $P_{O_2}$ have not been found adequate to account fully for the apparent coupling of blood flow to functional activity, and other potential chemical mediators are still being sought. Adenosine, adenine nucleotides, $K^+$, prostaglandins, etc. may be released into the extracellular space during neuronal activation. All can dilate cerebral vessels, but none has been shown to satisfy all the requirements (23).

Relaxation of arterial smooth muscle by acetylcholine has been found to be dependent on the formation of an endothelial factor, endothelium-derived relaxing factor (9). Endothelium-derived relaxing factor has been identified as nitric oxide (NO), and NO mediates the vasodilator actions of a number of pharmacological agents in a variety of organs (19, 28). NO is derived from the oxidation of L-arginine catalyzed by the enzyme NO synthase, which is found in vascular endothelium but is also widely distributed in various cell types of the brain and peripheral tissues (2, 3). There are at least two, possibly more, isoforms of the enzyme that are either constitutive or inducible. The constitutive form is $Ca^{2+}$/calmodulin dependent and is found in endothelium and neurons (2, 3, 28). Inducible forms are found in macrophages (28), glia (10) and probably in other cell types as well. Because of the $Ca^{2+}$ dependence the NO synthase can be activated to produce NO by various chemical signals that allow $Ca^{2+}$ influx into cells. This property and the cellular and tissue distributions of the $Ca^{2+}$-dependent activity have led to speculation that NO mediates the coupling of blood flow to neuronal functional and metabolic activities (11, 17). Several studies have examined the effects of inhibitors of NO synthase on cerebrovascular responses to functional activation in neural pathways (1, 4, 14, 16, 21, 31, 33, 41). In general, the results have been contradictory for reasons that are not obvious (18). Differences in the use of anesthetized or conscious animals, methods for estimating CBF, identity and mode of administration of the inhibitors, and degrees of enzyme inhibition undoubtedly have played a role.

In a preliminary study we examined the acute effects of $N^G$-monomethyl-L-arginine and $N^G$-nitro-L-arginine methyl ester (L-NAME), inhibitors of NO synthase, in conscious rats (38). The $N^G$-monomethyl-L-arginine was administered intravenously; the L-NAME was either injected intravenously or infused intracisternally to bypass the blood-brain barrier, which might possibly limit access of the drug to the intracerebral NO synthase. When given intravenously, both inhibitors raised blood pressure, increased cerebral vascular resistance, and lowered CBF but had little effect on the percent increases in local CBF normally produced by whisker stroking in the whisker-to-barrel sensory pathway. Intracisternal L-NAME had no effects on baseline CBF or on the functional activation of blood flow. The effectiveness of NO synthase activity inhibition, however, was not determined in that study. In the present study we extended the experiments with L-NAME and included enzyme assays to evaluate the effectiveness of the enzyme inhibition in brain. We also examined the effects of twice-daily intraperitoneal injections of 50 mg/kg of L-NAME for 4 days and confirmed that this regimen

inhibits brain NO synthase activity almost completely (6). It fails, however, to reduce stimulus-induced increases in local CBF in the vibrissal sensory pathway.

## MATERIALS AND METHODS

### *Chemicals and Animals*

4-Iodo-[*N*-methyl-$^{14}$C]antipyrine ([$^{14}$C]IAP; specific activity 54 mCi/mmol) and L-[*U*-$^{14}$C]arginine (specific activity 339 mCi/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA); $N^G$-nitro-L-arginine methyl ester hydrochloride, NADPH, calmodulin, and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO); catalase was purchased from Boehringer Mannheim (Indianapolis, IN); 6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydrobiopterin was obtained from B. Schircks Laboratories (Jona, Switzerland).

Normal, adult, male Sprague-Dawley rats weighing 300–450 g were obtained from Taconic Farms (Germantown, NY). The animals were maintained on Purina Laboratory Chow or its equivalent and allowed water ad libitum until the day of the experiment.

### *Preparation of Animals*

All procedures in animals were in strict accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [DHHS Publication (NIH) 86–23, Revised 1985, Animal Resources Program, DRR/NIH, Bethesda, MD 20205] and were approved by the local animal care and use committee. Under light halothane/nitrous oxide anesthesia PE-50 polyethylene catheters (Clay-Adams, Parsippany, NJ) were inserted in a femoral artery and vein. In rats that were to receive infusions of L-NAME intracisternally, a midline incision was made in the upper cervical region, and the soft tissues were dissected to expose the atlantooccipital membrane. A PE-10 polyethylene catheter (Clay-Adams) then was inserted into the cisterna magna through a small incision to a depth of ~2 mm and fixed in place with acrylic cement (Super Bonder, Loctite Corporation, Newington, CT). Lidocaine cream (5%) was applied to all surgical wounds before closure. The vibrissae on the right side of the face were then cut close to the skin to minimize spurious stimulation on the unstimulated control side. Then, a loose-fitting bivalved plaster cast was applied to the lower abdomen, pelvis, and hind legs to prevent locomotion, and the animals were allowed 3–4 h for recovery from the anesthesia.

### *Measurement of Physiological Variables*

Several physiological variables were measured repeatedly after the 3-h period of recovery from anesthesia to assess the physiological status of the animals. Mean arterial blood pressure was measured with a Micro-Med Blood Pressure Analyzer (Micro-Med, Louisville, KY) that had been calibrated with an air-damped mercury manometer. Arterial blood $P_{CO_2}$, $P_{O_2}$, and pH were determined with a pH/blood-gas analyzer (model 170, Ciba Corning Diagnostics, Medfield, MA). Arterial blood hematocrit was determined from blood samples centrifuged in a Beckman Microfuge B (Beckman Instruments, Fullerton, CA). Body temperature was maintained at 37°C by a servo-controlled infrared heating lamp connected to a rectal thermometer.

### *Assay of NO Synthase Activity*

NO synthase activity was measured in brain homogenates by assay of the rate of conversion of [$^{14}$C]arginine to [$^{14}$C]citrulline according to a modification (13) of the procedure of Bredt and Snyder (3). Whole rat brain was homogenized in three volumes of ice-cold 50 mM tris(hydroxymethyl)aminomethane·HCl, pH 7.4, containing 1 mM EDTA and (in mg/l) 10 antipain, 10 leupeptin, 10 soybean trypsin inhibitor, 10 pepstatin, 10 chymostatin, and 100 phenylmethylsulfonyl fluoride. The homogenate then was centrifuged at 20,000 *g* for 15 min, and NO synthase activity was assayed in 1–2 μl of the supernatant fraction. The reaction mixture contained 0.06–0.2 mg protein/ml added with the supernatant fluid, 1.72 μM L-[U-$^{14}$C]arginine (0.59 μCi/ml); 33 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4; 0.6 mM EDTA, pH 7.4; 0.67 mM NADPH; 0.83 mM $CaCl_2$; 1 mg/ml of bovine serum albumin; 8.7 U/μl of catalase; 0.5 mM dithiothreitol; 0.88 μM calmodulin; and 60 μM tetrahydrobiopterin in a final volume of 150 μl. The mixture was incubated at 25°C for 15 min, and the product, [$^{14}$C]citrulline, was separated by chromatography on AG 50W-X8, 200–400 mesh, $Na^+$ form (Bio-Rad Laboratories, Melville, NY) and assayed by liquid scintillation counting with external standardization.

Enzyme assays were performed on the brains of two rats representative of each of the four conditions in which local CBF was measured: *1*) normal controls receiving vehicle, *2*) acute intravenous injection of L-NAME, *3*) intracisternal infusion of L-NAME, and *4*) intraperitoneal injections of L-NAME twice daily for 4 days. The details of each condition are described below.

### *Experimental Conditions Under Which Local CBF was Measured*

Unilateral stroking of whiskers in rats produces selective unilateral increases in local CBF in the whisker-to-barrel cortex sensory pathway (12). To establish a baseline for comparison with inhibitor-treated animals and to identify specific regions in which CBF was altered by whisker stimulation, local CBF was measured in a group of control rats that received only normal saline intravenously (Fig. 1). The effects of unilateral whisker stroking on local CBF were examined in rats treated with three different regimens for L-NAME-induced inhibition of NO synthase activity. Each of these conditions was examined in parallel with control rats, which were treated similarly but with administration of vehicle instead of L-NAME.

*Acute intravenous L-NAME administration.* A dose of 30 mg/kg of L-NAME dissolved in 1.2–1.5 ml of normal saline was infused intravenously over a 1- to 1.5-min period. The control rats received similar infusions of normal saline alone. Ten minutes after completion of the infusion, when the mean arterial blood pressure had stabilized at an elevated level, the procedure for measuring local CBF flow was initiated. Throughout the period of measurement of CBF the vibrissae on the left side of the face were manually stroked forward and backward with a small paint brush at a rate of 2–4 strokes/s.

*Intracisternal infusion of L-NAME.* L-NAME, 15 μg/μl in artificial cerebrospinal fluid (CSF) adjusted to pH 7.25, was infused intracisternally at a rate of 2 μl/min for 20 min. The total dose of L-NAME was ~600 μg (i.e., 2.2 μmol or about 10 times the total estimated content of L-arginine in rat brain). The control rats received infusions of equivalent amounts (40 μl) of artificial CSF alone. Unilateral vibrissal stimulation as described above and measurement of local CBF were initiated immediately after completion of the infusion.

*Chronic intraperitoneal L-NAME administration.* Rats were injected intraperitoneally twice daily with 50 mg/kg of L-NAME in 1.2–1.5 ml of normal saline for 4 days; control rats were similarly injected with normal saline alone. Measurement of local CBF during unilateral vibrissal stimulation was

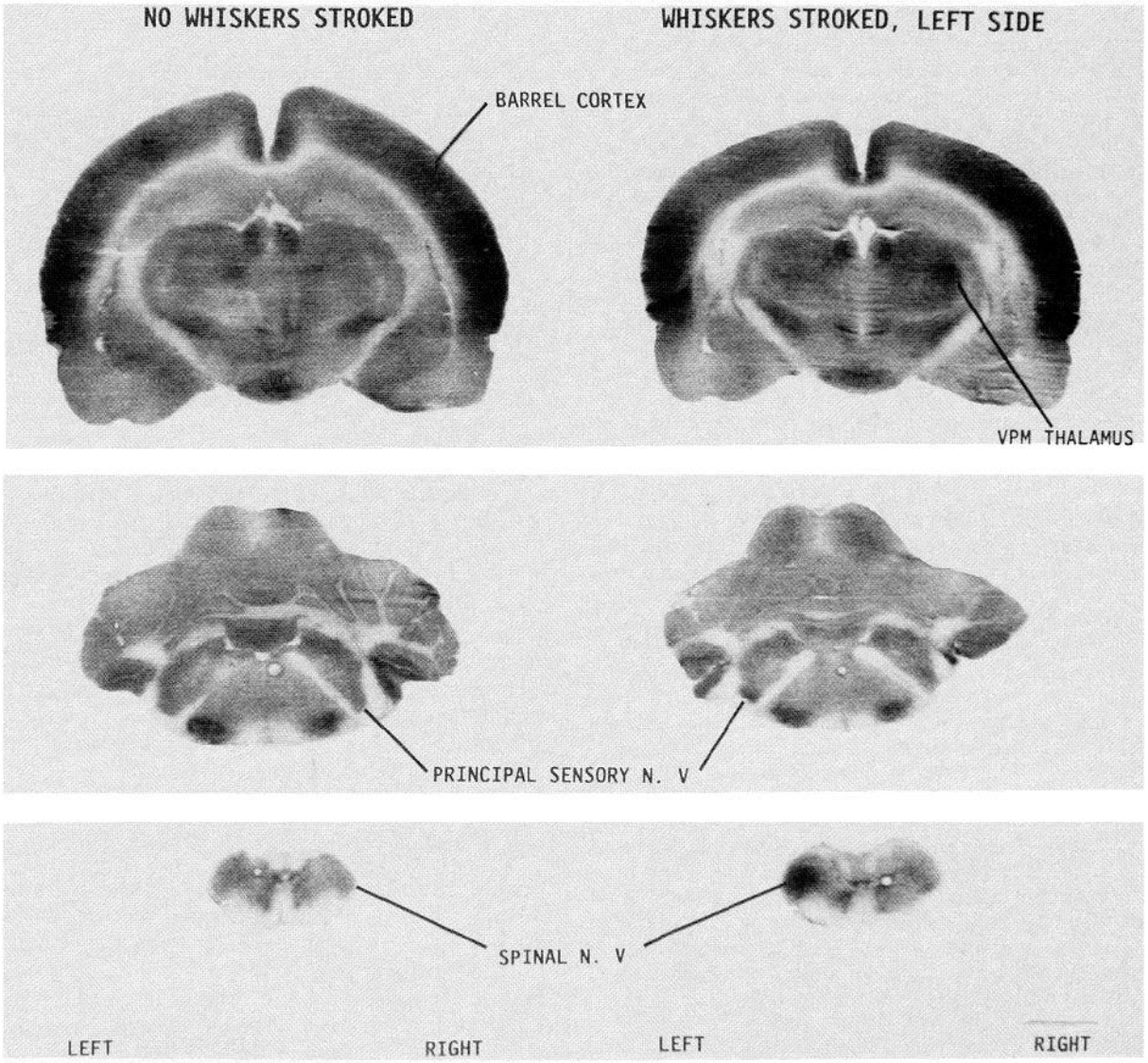


Fig. 1. Iodo[$^{14}$C]antipyrine autoradiographs of coronal sections of rat brain illustrating effects of whisker stroking on rates of blood flow in stations of the whisker-to-barrel pathway; the darker the region, the higher its rate of blood flow. Sections are at levels of barrel cortex and ventral posteromedial nucleus (VPM) of thalamus (*top*), trigeminal principal sensory nucleus (*middle*), and trigeminal spinal subnucleus caudalis (*bottom*). *Left*: autoradiographs representative of those obtained from control rats in which whiskers were not stimulated during iodo[$^{14}$C]antipyrine infusion. *Right*: autoradiographs representative of those from rats with left unilateral whisker stroking.

performed as described in *Intracisternal infusion of L-NAME* 12–14 h after the last injection.

*Measurement of Local CBF*

*Procedure.* Local CBF was measured by the quantitative autoradiographic [$^{14}$C]IAP technique of Sakurada et al. (34). In brief, 40 μCi of [$^{14}$C]IAP contained in ~0.8 ml of normal saline were infused intravenously by a pump driven by a computer program designed to produce a linear rise in arterial [$^{14}$C]IAP concentration. Throughout the infusion timed arterial blood samples were collected at ~3-s intervals on preweighed filter paper disks in small plastic beakers. The beakers were immediately sealed and reweighed to determine the mass of the blood samples, and the volumes of the samples were calculated from the sample weights based on an assumed blood density of 1.05 g/ml. The filter paper disks were then placed in scintillation vials, and the hemoglobin and plasma proteins were precipitated on the filter paper by the addition of 10 ml of AQUASOL (Du Pont-New England Nuclear). One milliliter of water was added, and the vials were shaken and kept for 24 h to extract the [$^{14}$C]IAP from the precipitated blood into the phosphor solution. The [$^{14}$C]IAP in the sample was then assayed by liquid scintillation counting (Packard Instruments, Downers Grove, IL) with external standardization. At a precisely recorded time, ~1 min after the start of the infusion, the rat was decapitated, and the brain was removed as quickly as possible and frozen in isopentane chilled to −45°C with dry ice.

*Measurement of local $^{14}$C concentrations in brain tissue.* The frozen brains were cut into 20-μm-thick coronal sections in a cryostat maintained at −24°C. The sections were thaw-mounted on glass coverslips, mounted on cardboard, and apposed to Kodak EMC-1 film (Kodak, Rochester, NY) in cassettes together with a set of calibrated $^{14}$C-labeled methylmethacrylate standards. Sets of four contiguous sections were taken every 200 μm throughout the entire rostrocaudal span of the rat brain for autoradiography. Local tissue concentra-

tions of $^{14}C$ were determined from the autoradiograms by quantitative densitometry with an adaptation (G. Mies, MPI, Cologne, Germany) of a MacIntosh-based imaging program (W. Rasband, Image 1.08, National Institute of Mental Health, Bethesda, MD).

*Calculation of local CBF.* Local CBF was calculated from the local tissue concentrations of $^{14}C$ and the time course of the arterial $^{14}C$ concentration by the operational equation of the method (34) with correction for delay and washout of the arterial catheter sampling system as described by Freygang and Sokoloff (8). The arterial catheter was kept relatively short (16 cm) to ensure that the flow-to-volume ratio in the catheter was at least 40 or above to minimize the magnitude of these corrections.

*Data analyses.* Four structures in the vibrissal sensory pathway were selected for determination of local CBF. These were the trigeminal spinal subnucleus caudalis, the trigeminal principal sensory nucleus, the ventral posteromedial nucleus (VPM) of the thalamus, and the barrel cortex (5, 35, 42). Vibrissal stroking has previously been shown to result in increased glucose utilization in these stations of the pathway (12, 35) and also increased blood flow in the barrel field of the somatosensory cortex (12). In addition, four other regions not directly involved in this sensory pathway (i.e., vestibular nucleus, lateral thalamus, superficial layers of the superior colliculus, and visual cortex) were examined for comparison. All of these regions could be readily identified in the digitized images reconstructed from the autoradiograms from the patterns of optical density when compared with the stained sections of the brains after autoradiography and an atlas of rat brain (32) (Fig. 1). For each of these structures values for blood flow were determined separately for the stimulated and unstimulated sides of the brain and compared statistically by Student's *t*-test for paired comparisons. The percent increases in blood flow due to the vibrissal stimulation were calculated as the percent difference between the rates in the stimulated and unstimulated sides. The nonparametric Wilcoxon-Mann-Whitney test was applied to determine the statistical significance of the differences in stimulus-induced percent increases in blood flow in controls and L-NAME–treated rats.

## RESULTS

### *Effects of L-NAME Administration on NO Synthase Activity in Brain*

Acute intravenous administration of L-NAME was found to reduce NO synthase activity in brain to 47% of control levels 10 min after the injection, the time at which local CBF was measured (Table 1). When infused intracisternally or administered chronically (50 mg/kg ip injected twice daily for 4 days), brain NO synthase (NOS) activity was reduced to 12 and 16% of control levels, respectively (Table 1).

### *Systemic Effects of L-NAME*

Intravenously administered L-NAME (30 mg/kg) raised mean arterial blood pressure in every animal from a mean of 130 ± 2 to 151 ± 2 (SE) mmHg ($n = 4$; $P < 0.0001$, paired *t*-test). The blood pressure at the time of the CBF measurement in the L-NAME-treated rats was ~20% above that of the saline controls. Arterial pH and $P_{CO_2}$ were unaffected by intravenous L-NAME injections.

Intracisternal infusions of L-NAME had no effect on arterial blood pressure but resulted in a fall in arterial $P_{CO_2}$ from 37 ± 0.8 to 33 ± 0.3 (SE) mmHg ($n = 7$; $P < 0.05$) and rise in pH from 7.44 ± 0.01 to 7.48 ± 0.01 (SE) ($n = 7$; $P < 0.05$ by paired *t*-test).

Table 1. *Effects of various treatments with L-NAME on NO synthase activity in rat brain*

| Treatment | Relative Activity |
|---|---|
| Controls | 100 |
| L-NAME | |
| Acute intravenous injection (30 mg/kg) | 47 |
| Intracisternal infusion over 20 min (600 μg) | 12 |
| Intraperitoneal injection 2 times/day × 4 days (50 mg/kg) | 16 |

Values are means of values obtained when NO synthase activity was assayed in parallel in individual whole brain homogenates of 2 rats for each listed condition (see MATERIALS AND METHODS for assay conditions). Enzyme activity was determined as nmol citrulline produced · mg protein$^{-1}$ · min$^{-1}$. Rates in the appropriate vehicle-treated controls (e.g., saline or artificial cerebrospinal fluid) were set at 100. L-NAME, $N^G$-nitro-L-arginine methyl ester.

Chronic intraperitoneal administration of L-NAME was without effects on arterial pH and $P_{CO_2}$ but produced a sustained significant increase in arterial blood pressure to levels approximating those after the single intravenous dose. Mean arterial blood pressure in these animals was 154 ± 3 ($n = 6$) compared with 125 ± 5 (SE) mmHg ($n = 4$) in the saline-treated controls ($P < 0.0001$, *t*-test for group comparisons).

### *Effects of L-NAME on Stimulus-Induced Increases in Local Blood Flow*

Unilateral stroking of whiskers had no significant effects on blood flow in the four structures outside the whisker-to-barrel pathway (i.e., vestibular nucleus, lateral thalamus, superficial layers of superior colliculus, and visual cortex) that were examined (data not shown). Stroking the vibrissae on the left side resulted in distinct increases in blood flow in the left trigeminal subnucleus caudalis and principal sensory nucleus, right VPM of the thalamus, and right barrel cortex compared with the values in the homologous structures on the contralateral side or in the unstimulated, saline-treated control rats (Figs. 2–4). The greatest responses were found in the subnucleus caudalis, but the increases in the trigeminal principal sensory nucleus, VPM of the thalamus, and barrel cortex were also quite substantial (Figs. 2–4).

Although intravenous administration of L-NAME raised arterial blood pressure, it significantly reduced blood flow in all brain structures below the values in corresponding structures of saline-treated rats, including the stations of the whisker-to-barrel pathway on both the stimulated and unstimulated sides (Fig. 2). Despite the ~53% inhibition of brain NO synthase activity (Table 1), unilateral whisker stroking increased CBF unilaterally in the stations of the whisker-to-barrel pathway. The absolute increments due to stimulation were less than those in the saline-treated control rats, but the baseline values in the contralateral unstimulated structures were lower, and the percent increases, therefore, were not statistically significantly below those in the saline treated-controls (Fig. 2).

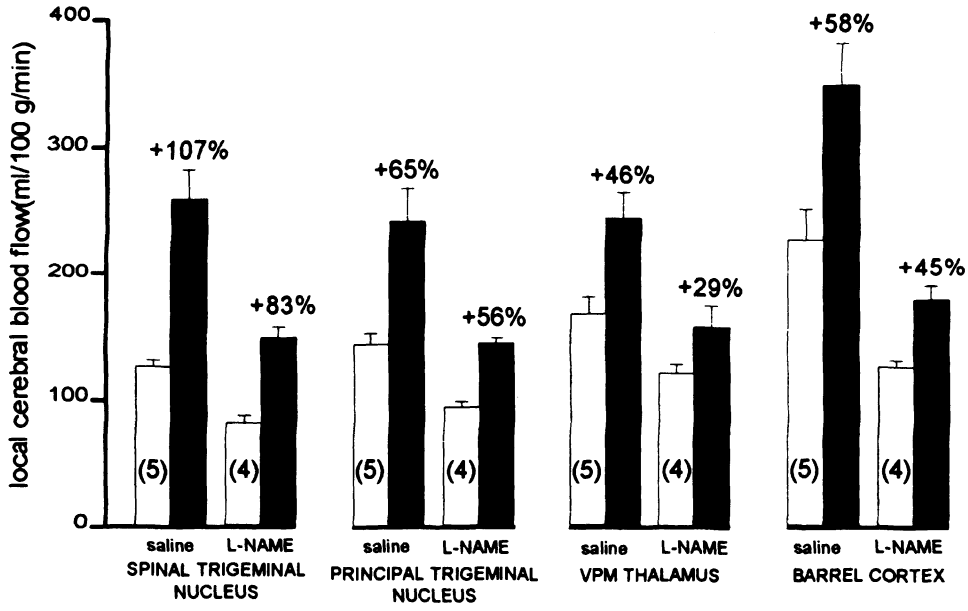


Fig. 2. Effects of doses (30 mg/kg iv) of $N^G$-nitro-L-arginine methyl ester (L-NAME) on baseline rates of local cerebral blood flow and on increases in blood flow induced by unilateral whisker stroking in 4 stations of the whisker-to-barrel pathway. Data are mean rates of local cerebral blood flow ± SE obtained from the no. of rats shown in parentheses. Solid bars, stimulated side; open bars, control side.

When L-NAME was infused intracisternally, thus bypassing the blood-brain barrier, there was also a slight tendency toward lower rates of blood flow throughout the brain, but the percent increases in CBF elicited by functional activation of the stations of the whisker-to-barrel pathway were essentially the same as those in control rats infused with artificial CSF (Fig. 3), despite the 88% inhibition of the brain NO synthase activity in the brain (Table 1).

Inhibition of rat brain NO synthase activity by L-NAME has been reported to be a relatively slow process but to be almost complete after intraperitoneal injections of 50 mg/kg of L-NAME twice daily for 4 days (6). In the present study this treatment inhibited total NOS activity, including both parenchymal and vascular enzyme activities, by 84% (Table 1). Nevertheless, despite this degree of NO synthase inhibition, the whisker stroking resulted in percent increases in blood flow in all the stations of the whisker-to-barrel pathway that were as great as those in the saline-treated control rats (Fig. 4).

## DISCUSSION

In agreement with a number of previous studies in which quantitative measurements of CBF were made (27, 38, 39, 41), the results of the present study demonstrate that inhibition of NO synthase activity by a single intravenous dose of L-NAME reduces CBF while raising systemic arterial blood pressure, evidence of an increase in cerebral vascular resistance (Fig. 2). The increased blood pressure is presumably due to a widespread vasoconstriction; the increased cerebral vascular resistance indicates that the cerebral vessels were also affected. These changes indicate that NO synthase activity was effectively inhibited in at least the vascular

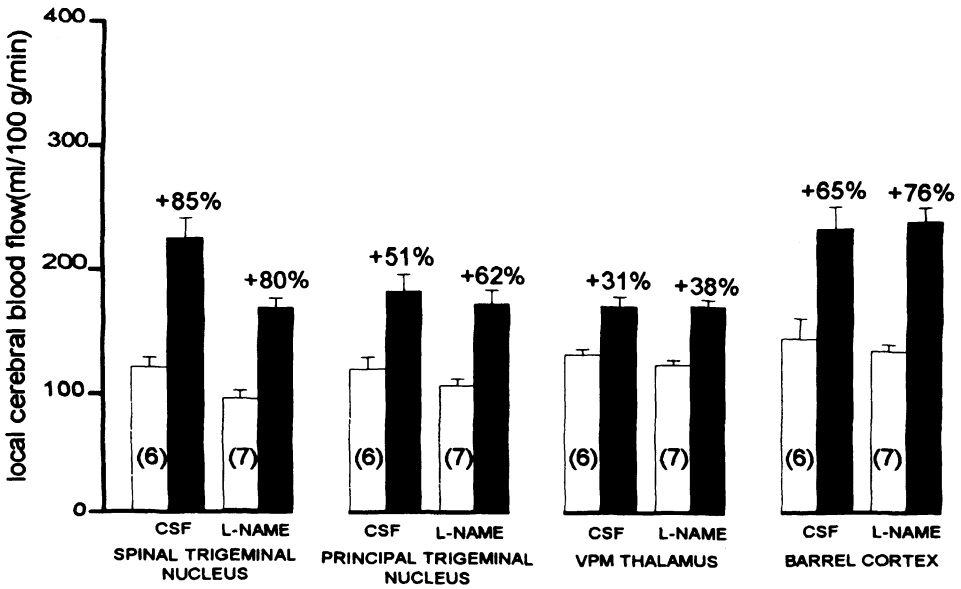


Fig. 3. Effects of intracisternally infused artificial cerebrospinal fluid (CSF) or L-NAME on resting local cerebral blood flow and on increases in blood flow produced by unilateral whisker stroking in 4 stations of the whisker-to-barrel pathway. Data are means ± SE of rates of blood flow obtained in the no. of rats shown in parentheses. Means of %differences between stimulated (solid bars) and unstimulated (open bars) sides obtained in individual rats are indicated above bars.

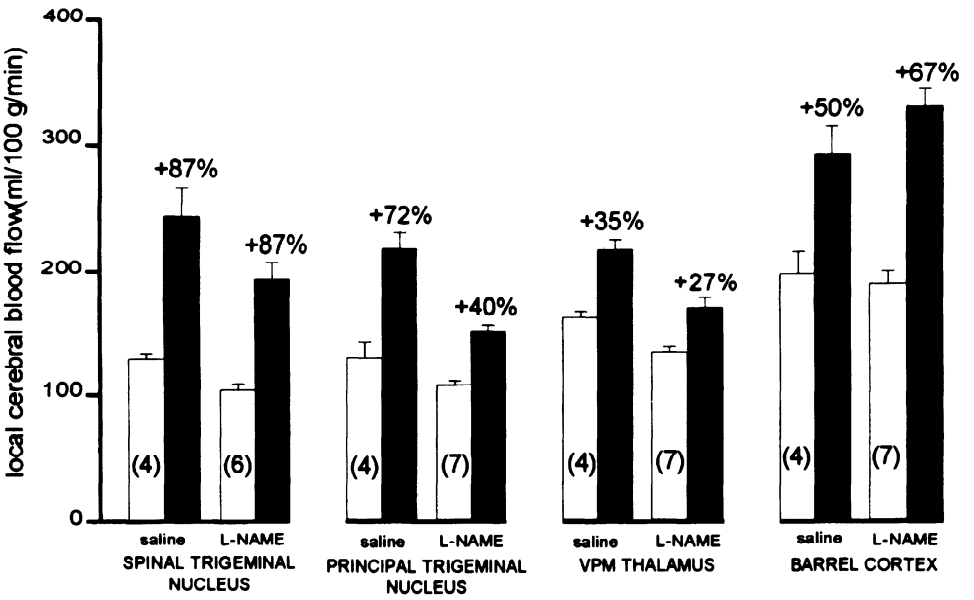


Fig. 4. Effects of twice-daily injections (50 mg/kg ip) of L-NAME for 4 days on local cerebral blood flow and on increases in blood flow produced by unilateral whisker stroking in the whisker-to-barrel pathway. Data are means ± SE of values for local blood flow obtained in the no. of rats shown in parentheses. Means of %differences between stimulated (solid bars) and unstimulated (open bars) sides found in individual rats are indicated above bars.

endothelium and that it normally plays a role in the regulation of systemic as well as cerebrovascular tone. Irikura et al. (21) pointed out the importance of ensuring effective inhibition of the NO synthase activity in assessing the effects of inhibitors on the responses of the cerebral circulation to neural functional activation. Enzymatic assay in vitro of NOS activity in the homogenized brain after the acute intravenous injection of L-NAME showed only ~53% inhibition of the total brain activity (Table 1). It was uncertain, however, how this inhibition was distributed between the endothelial and the parenchymal enzymes. It was possible that the blood-brain barrier limited the access of L-NAME to the parenchymal enzyme, which conceivably could have been the enzyme activity most involved in the coupling of blood flow to functional activity. Therefore, to bypass the blood-brain barrier, L-NAME was infused directly into the cisterna magna and was indeed found to inhibit brain NOS activity by 88% (Table 1). Despite the greater degree of NOS inhibition with intracisternal administration of L-NAME, the effects on resting CBF and vascular resistance, though qualitatively similar, were far fewer than was found with intravenous administration of the inhibitor, and again there were no significant reductions in the percent increases in blood flow due to stimulation in the stations of the whisker-to-barrel pathway (Fig. 3). The comparative effects of the two modes of administration indicate that NO does have a role in the normal regulation of cerebral vascular resistance and that it is the vascular endothelial NO synthase activity which contributes more to this function than the brain enzyme but that neither is required for the enhancement of blood flow by increased neural functional activity.

Dwyer et al. (6) reported that the inhibition of NO synthase activity by L-NAME is not immediate but occurs only after a delay. Therefore, the possibility was considered that inhibition of NOS activity in the brain in vivo after the intravenous or intracisternal administration of L-NAME had not yet been established during the 10- to 20-min period before the measurement of CBF and that the inhibition observed in the in vitro assays of enzyme activity had occurred only after homogenization of the brain or during the assay itself. For this reason we also applied the procedure used by Dwyer et al. (6) for almost complete inhibition of NOS activity in rat brain in vivo, i.e., twice-daily intraperitoneal injections of 50 mg/kg of L-NAME for 4 days and found the brain enzyme activity to be inhibited by 84%. Presumably this procedure would have resulted in inhibition of both the vascular endothelial and parenchymal enzymes in the brain, and, in fact, baseline blood flow was reduced throughout the brain by approximately the same degree as that after the acute intravenous injection (Fig. 4). Nevertheless, there were no significant reductions in the percent enhancement of blood flow by stimulation in any of the stations of the whisker-to-barrel pathway (Fig. 4), further evidence for the lack of a role for NOS activity in the coupling of local CBF to functional activity.

The results of these studies are at variance with several reports that inhibitors of NO synthase reduce or block increases in blood flow that normally accompany functional activation of neural pathways (4, 14, 16, 18, 21, 31, 33). These studies examined vascular responses in pathways activated by a variety of means, such as electrical stimulation of afferents, spreading depression induced by direct mechanical stimulation, topical application of *N*-methyl-D-aspartate, and in some cases physiological activation of the barrel cortex by vibrissal stroking, as in the present study. With one exception (31), these studies did not measure tissue perfusion rates quantitatively but relied on indirect means to estimate changes in blood flow, such as laser-Doppler recordings (4, 14, 16, 33) or observations of pial and superficial cortical blood vessels through cranial windows (10, 21). These techniques may detect vascular responses that may not accurately reflect changes in true tissue perfusion rates. For example, laser-Doppler signals depend on red cell velocities in the field of view, and pial vessel diameters mainly reflect blood volume.

Inasmuch as NO synthase inhibitors alter vascular tone, they could alter the relationships of red cell velocities and pial diameters to true tissue perfusion rates. Madsen et al. (26), for example, noted a discrepancy between the results of transcranial Doppler sonography of middle cerebral arterial blood velocity and true measurement of CBF during exercise. Northington et al. (31) measured blood flow quantitatively by the hydrogen clearance technique in the sensory cortex in rats during sciatic nerve stimulation and in which 1 mM L-NAME or artificial CSF was administered directly into the sensorymotor cortex by microdialysis. We have also attempted to examine the effects of NO inhibitors administered locally by microdialysis on the changes in blood flow accompanying seizures induced by topical application of penicillin but found the results to be variable and ambiguous because the autoradiographs revealed heterogeneous mixtures of regions with high and low blood flow surrounding the dialysis probe (Nelson, unpublished data).

There has been one other report that the increases in blood flow in the barrel cortex in response to vibrissal stimulation are relatively unaffected by intravenous administration of L-NAME (41). Interestingly, in that study, like in ours, local CBF was measured quantitatively with the [$^{14}$C]IAP method in conscious, unanesthetized rats. In all of the other studies the animals were under general anesthesia. Virtually all anesthetic agents alter CBF (24, 36). Some, most notably halothane and related volatile compounds, attenuate endothelium-dependent relaxation (29, 40), possibly by interference with NO activation of guanylylcyclase (15), a part of the mechanism by which NO relaxes smooth muscle. Halothane itself has been reported to relax and to increase guanosine 3′,5′-cyclic monophosphate levels in vascular smooth muscle independently of NO (30). NO can be reduced to NO•, oxidized to nitrite and secondarily in the presence of oxyhemoglobin or oxymyoglobin to nitrate, or slowly form nitrosothiols, and all of these products may have different biologic actions from each other and from NO (20, 25). Anesthetic agents may alter the rates of formation and disposition of NO and of its various products and thus modify the neuronal and vascular responses to increases in neuronal activity. Whatever the explanation for the discrepancy between the results of the experiments in conscious and anesthetized rats, it is clear that nearly complete inhibition of NO synthase in brain and its vascular endothelium, confirmed by direct enzyme assay, does not diminish the enhancement of blood flow in functionally activated cerebral tissues. Based on these results, it is difficult to conclude that NO itself is the mediator of the coupling of blood flow to functional and/or metabolic activities in the brain. It is possible that it plays some role in concert with other factors that cannot be observed unless the other mechanisms were blocked.

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