Endothelium-Derived Nitric Oxide Synthase Inhibition

Effects on Cerebral Blood Flow, Pial Artery Diameter, and Vascular Morphology in Rats

Ricardo Prado, MD; Brant D. Watson, PhD; John Kuluz, MD; and W. Dalton Dietrich, PhD

Background and Purpose: We determined the effects of inhibiting the production of cerebral endothelium-derived nitric oxide on pial artery diameter, cortical blood flow, and vascular morphology.

Methods: An inhibitor of endothelium-derived nitric oxide synthesis, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), or an equivalent volume of 0.9% saline was infused into rats intra-arterially in a retrograde fashion via the right external carotid artery at a rate of 3 mg/kg/min to a total dose of 190 mg/kg or intravenously at 1 mg/kg/min to a total dose of 15 mg/kg. Large pial arteries were continuously visualized through an operating microscope, and cortical cerebral blood flow was monitored by laser-Doppler flowmetry. To localize areas of morphological interest, the protein tracer horseradish peroxidase was injected 15 minutes before termination of the L-NAME infusion and the rats were perfusion-fixed 15 minutes later for light and electron microscopic analysis.

Results: Infusion of L-NAME significantly raised arterial blood pressure at both doses (for 190 mg/kg, from 103.2±3.4 to 135±3.4 mm Hg; for 15 mg/kg, from 125±2.8 to 144.4±4.0 mm Hg). Pial arteries constricted within 10 minutes after the start of the intracarotid infusion to 40% of the preinfusion diameter, while cortical cerebral blood flow decreased to an average of 72.5% of that at baseline. Morphological abnormalities in the experimental rats included microvascular stasis and focal areas of blood–brain barrier disruption to protein. Ultrastructural examination of cortical leaky sites revealed constricted arterioles with many endothelial pinocytotic vesicles and microvilli.

Conclusions: These observations suggest that inhibition of endothelium-derived nitric oxide synthesis affects the relation between cerebral arterial diameter and cerebral blood flow and can lead to subtle cerebral vascular pathological changes consistent with focal brain ischemia. (Stroke 1992;23:1118–1124)

KEY WORDS • blood–brain barrier • cerebral blood flow • endothelium-derived relaxing factor • rats

The discovery that endothelium-dependent mechanisms underlie the effects of many vasoactive substances has generated widespread interest. For example, endothelium-dependent vascular smooth muscle relaxation is now known to be mediated by endothelium-derived nitric oxide (NO), a local humoral substance synthesized from the amino acid L-arginine and released from endothelial cells under basal and stimulated conditions. The biological actions of NO have been attributed to its subcellular utilization, although one or several thiol adducts of NO may be intrinsic products of vascular endothelium. The NADPH-dependent enzyme nitric oxide synthase is required for the formation of NO from L-arginine.

In particular, the L-arginine/NO/guanosine monophosphate pathway appears crucial in regulating cerebral vascular homeostasis by means of maintaining vascular tone. For example, sustained vasoconstriction of the rat internal carotid artery in vivo and elevation of arterial blood pressure were observed following intravenous administration of the nitric oxide synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA) and L-iminoethyl-L-ornithine. These effects were reversed by L-arginine administration. In addition, direct and indirect evidence indicates that humans and animals with atherosclerosis or chronic hypertension exhibit abnormal NO production/release, as evidenced by inappropriate responses to various endothelium-dependent vasorelaxant agents. These studies suggest that NO synthesis is vital in regulating the hemodynamics of the normal circulation.

We surmised that inhibition of NO synthesis would alter cerebral vascular function in particular and, if sus-
tained, would initiate local or systemic deterioration of vascular integrity. The aims of this study were therefore to determine whether inhibition of NO production in brain endothelium would lead to alterations in the tone of large cortical pial arteries (in terms of changes in pial artery diameter), contribute to the regulation of cortical cerebral blood flow (cCBF) (determined by laser-Doppler flowmetry), and induce morphological changes in the ultrastructure of vascular endothelial cells.

Materials and Methods

Twenty-seven food-deprived male Wistar rats weighing 250–350 g were anesthetized with 4% halothane and maintained on a 70%/30% mixture of N₂O/O₂ and 0.5% halothane delivered via an endotracheal tube (PE-240). Femoral venous and arterial catheters (PE-50) were inserted for fluid administration, blood gas determination, and blood pressure monitoring. The rats were mechanically ventilated with a Stoelting small-animal respirator (Wood Dale, Ill.) after muscle paralysis with 0.35 mg/kg i.v. pancuronium bromide. Retrograde right external carotid artery catheterization (PE-50) was carried out for the infusion of a 0.9% saline solution of L-NAME (Sigma Chemical Co., St. Louis, Mo.) at a rate of 3 mg/kg/min (5 μl/min) for a total dose of 190 mg/kg; L-NAME was administered in other rats as a single intravenous dose of 15 mg/kg (1 mg/kg/min for 15 minutes). The pH of the L-NAME solution was adjusted to 6.0 by the addition of 4N NaOH, and the osmolality was measured at 1,270 mosm/L. Before the initial incision of the external carotid artery, 50 units of heparin sodium was administered intra-arterially to prevent thrombosis at the catheter tip.

In 17 rats, a 3-mm² burr hole was made over the right frontoparietal cortex approximately 1.3 mm posterior and 5.5 mm lateral to the bregma. Under constant irrigation with saline the bone was drilled with a diamond burr to a thin layer, and areas of interest were selected before incision and removal of this remaining layer of bone. The pial vasculature was visualized through the intact dura mater, which was kept moist with saline. The rats were divided into two groups. In one group (n = 4), infused with L-NAME at 190 mg/kg, the diameter of a single large pial artery selected from each rat was measured through a Zeiss operating microscope (×40) (Thornwood, N.Y.) equipped with a reticle in one eyepiece. In the second group, infused with either 190 mg/kg L-NAME intra-arterially (n = 7) or 15 mg/kg L-NAME intravenously (n = 6), cCBF was measured by means of a 0.8-mm-diameter fiber-optic probe coupled to a BPM-403a laser-Doppler blood perfusion monitor (Vasamedics, Inc., St. Paul, Minn.) and lowered via a micromanipulator under optical guidance onto the surface of the intact dura mater.

In a separate group of rats (n = 5) the morphological characteristics of an intracarotid infusion of 3 mg/kg/min L-NAME for 60 min (total dose 190 mg/kg) were documented. Control rats (n = 5) received an equivalent volume of 0.9% saline via the right external carotid artery (5 μl/min for 60 minutes). Fifteen minutes before the end of the L-NAME infusion, 30 mg horseradish peroxidase (HRP, type II, Sigma) dissolved in 1 ml saline was injected intravenously over 2 minutes. Before HRP administration, 0.5 mg/100 g diphendyhydramine was administered intramuscularly to prevent HRP-induced hypotension. Fifteen minutes after HRP administration, the rats were perfusion-fixed1920 transcardially at 120 mm Hg after a 2-minute rinse with normal saline. The brains were removed immediately from the calvariae and placed in chilled fixative. The brains were then sectioned coronally at 75–100 μm with an Oxford Vibratome and reacted with 3,3'-diaminobenzidine for the demonstration of HRP extravasation. To obtain morphological evidence for microvascular changes following L-NAME infusion, ipsilateral cortical areas demonstrating extravasated HRP were dissected, osmicated, dehydrated, and embedded in plastic.1920 One-micrometer sections were cut, stained with toluidine blue, and examined using a light microscope. Thin sections were collected on uncoated grids, stained with uranyl acetate and lead citrate, and examined using a Zeiss EM-10C electron microscope. Other Vibratome sections were mounted and air-dried on glass slides, dehydrated, cleared, and coverslipped.

Laser-Doppler measurements of cCBF are expressed graphically as mean±SEM percentages of baseline values. Differences between baseline mean arterial blood pressure (MABP), pial artery diameter, and cCBF in rats infused with either L-NAME or saline were analyzed with SYSTAT employing the Friedman nonparametric analysis of variance, with p<0.05 being considered significant.21 Baseline measurements of pial artery diameter and cCBF were recorded over 1 hour during the intracarotid (5 μl/min for 60 minutes, 300 μl) or after the intravenous (500 μl over 15 minutes) infusion of saline and averaged. Following a 1-hour equilibration period L-NAME was administered in a retrograde fashion into the right external carotid artery, and pial artery diameter was measured 10, 30, and 60 minutes after the infusion was begun. The cCBF readings were commenced 5 minutes after the intracarotid or intravenous infusion of L-NAME and were subsequently recorded at 15, 30, and 60 minutes.

Results

Physiological Variables

In all rats after L-NAME infusion MABP was significantly elevated from baseline values (see below). In animals infused with L-NAME arterial pH was 7.388±0.04, PacO₂ was 36.5±2.68 mm Hg, and PacO₂ was 156.7±23.7 mm Hg. Control animals displayed an arterial pH of 7.452±0.02, a PacO₂ of 37.3±1.8 mm Hg, and a PacO₂ of 145.7±6.9 mm Hg. Thus, physiological parameters (except MABP) after the infusion of L-NAME in all groups were within normal limits and demonstrated no significant change during the experiments.

Pial Artery Diameter

In each rat equipped with a dural window, the selected large pial artery constricted during the intracarotid infusion of L-NAME (Figure 1); the average pial artery diameter of 128.0±3.2 μm decreased significantly to 41.8±3.3 μm at baseline at 10 minutes, 39.7±4.4 μm at 30 minutes, and 38.2±7.1% at 60 minutes of infusion (p<0.05 in each case). At baseline MABP for this group was 90.6±4.4 mm Hg; MABP increased to 133.6±9.7 mm Hg by 10 minutes, 131±10.1 mm Hg by 30 minutes, and 132.5±9.5 mm Hg by 60 minutes of L-NAME infusion (p<0.05 in each case, Figure 1).
Cortical Cerebral Blood Flow

The intracarotid infusion of L-NAME at 3 mg/kg/min did not decrease cCBF significantly until 15 minutes of infusion, when cCBF decreased to 79.8±11.2% of baseline (p<0.05); at 5 minutes cCBF was not reduced significantly (Figure 2). The cCBF remained below baseline for the remainder of the experiment. MABP increased to 140.0±4.2 mm Hg at 60 minutes of infusion from a baseline level of 101.7±3.4 mm Hg (p<0.05). However, at 5 minutes of infusion MABP was not significantly elevated from baseline (Figure 2).

The intravenous administration of L-NAME at 15 mg/kg significantly decreased cCBF to 73.0±10.1% of baseline at 5 minutes; cCBF was further reduced to 67.6±8.1% of baseline 15 minutes after L-NAME administration (Figure 3). MABP increased (p<0.05) from a baseline value of 125±2.8 mm Hg to 144.4±4.0 mm Hg at 60 minutes following L-NAME administration (Figure 3). Baseline levels of cCBF were not altered by either the intracarotid or the intravenous administration of saline (data not shown).

Morphological Analysis

In the five control rats MABP was 102.2±6.8 (range, 90–120) mm Hg. In contrast, L-NAME infusion significantly increased MABP from 106.8±5.5 (range, 98–127.5) mm Hg to 135.0±3.5 (range, 125–145) mm Hg (p<0.05). The rise in MABP was gradual and plateaued at 6.04±0.6 minutes.

Cleared Vibratome sections from the control rats did not demonstrate consistent vascular abnormalities. Microvessels appeared well perfused, and red blood cell stasis was not seen. Light microscopic analysis of thick plastic sections stained with toluidine blue demonstrated well-perfused blood vessels that appeared unremarkable except for mild perivascular astrocytic swelling. In contrast, vascular abnormalities were observed in all rats infused with L-NAME (Figure 4). Light microscopic examination of cleared Vibratome sections demonstrated focal sites of vascular stasis within cortical and subcortical regions both ipsilateral and contralateral to the carotid infusion. Single columns of red blood cells indicating capillary congestion were also detected throughout both hemispheres. Longitudinally oriented vessels frequently displayed irregular contours and appeared constricted. In addition, areas of focal HRP extravasation were detected bilaterally within the cortex and striatum. Although HRP leakage was occasionally associated with congested vessels, extravasated HRP was most commonly seen in vessels with clear lumens.

At both the light and electron microscopic levels, pial and cortical arteries and arterioles ipsilateral to the carotid infusion appeared moderately constricted (Figures 4A and 4B). The endothelial lining of leaky vessels was irregular in contour, with many endothelial micro-
FIGURE 4. Transmission electron micrographs of cortical vessels from rats infused with 3 mg/kg/min NO-nitro-L-arginine methyl ester hydrochloride. a: Arteriole exhibits numerous endothelial microvilli projecting into lumen (arrowheads). Endothelial cytoplasmic vacuoles are also apparent. E, endothelial cell nucleus. ×12,460. b: This endothelial cell appears irregular in contour, with luminal folds (arrowheads). ×12,460. c: Extravasated horseradish peroxidase (HRP) is seen in endothelial (E) and smooth muscle (SM) basal lamina of this arteriole. Endothelial cell also contains many cytoplasmic vesicles. ×15,575. d: Pinocytotic vesicles (arrowheads), some containing HRP, are present in this otherwise normal-appearing endothelial cell. Extravasated HRP is apparent within endothelial and smooth muscle (SM) basal laminae. ×24,920.

villi projecting into the vessel lumen (Figure 4A). Compared with sections from control rats, the perivascular spaces of many arterioles also appeared enlarged. Transmission electron microscopic analysis of leaky sites identified arterioles with HRP reaction product within endothelial pinocytotic vesicles, smooth muscle basal laminae, and surrounding extracellular spaces (Figures 4C and 4D). Smooth muscle cells appeared unremarkable. Confirmation of red blood cell stasis was also obtained by ultrastructural examination (not shown).

Discussion
The choice of L-NAME as an in vivo inhibitor of nitric oxide synthase was dictated by its solubility to the
required concentration in saline, its ready availability, and its previous characterization as an NO synthesis inhibitor both in vitro and in vivo in a range of doses encompassing those we used. We were concerned with establishing an infusion rate of inhibitor that would reduce pial artery diameter but not elevate MABP beyond the upper limit of autoregulation. These conditions were satisfied by several infusion rates, including our maximal rate of 3 mg/kg/min. Further, the vasoconstriction and blood pressure elevation seen after 1 hour of infusion were completely reversed within 10 minutes by infusion of 33 mg/kg/min L-arginine hydrochloride (5 μl/min) (unpublished observations). These observations, which are consistent with the operational requirements for an in vivo inhibitor of endothelial nitric oxide synthase, duplicate the previously observed blood pressure increases seen with intravenous L-NAME infusion in rats and the reversal of the increase with L-arginine hydrochloride.10

This study demonstrates that slow retrograde infusion of L-NAME into an external carotid artery or a vein produces significant reductions in cCBF (23% and 27%, respectively) while inducing gradual and modest increases in MABP. Significant decreases in cCBF were noted previously by Beckman and colleagues when nitric oxide synthase was inhibited by nitroarginine hydrochloride, as formulated by that group and administered intravenously. In that study a 50% decrease in cCBF was recorded during intravenous infusion at 30 mg/kg/hr, while blood pressure increased by approximately 25%. After 60 minutes of infusion, cCBF remained reduced for 24 hours (J.S. Beckman, personal communication). Importantly, this increase in blood pressure could not be reversed by L-arginine, implying that NO inhibition by their nitroarginine hydrochloride is different in character from that induced by L-NAME. On the other hand, 5 mg/kg nitroarginine hydrochloride decreased cCBF without elevating systemic blood pressure. Macrae and colleagues likewise showed a significant decrease in cCBF following nitric oxide synthase inhibition with 30 mg/kg L-NAME; cCBF was reduced from a preinfusion value of 102±4 ml/min/100 g to 63±4 ml/min/100 g 15 minutes after drug infusion. Taken together, these data demonstrate that NO influences the regulation of cCBF.

The rise in MABP observed during the infusion of L-NAME was gradual and did not exceed the upper limit (150–160 mm Hg) of cerebral autoregulation. In normotensive subjects, sudden and extreme rises in systemic blood pressure beyond the upper limit of autoregulation can lead to an increase in cerebral blood flow as a result of passive cerebral vasodilation. In the present study with nitric oxide synthase inhibition therefore contrast with those seen following acute increases in systemic arterial blood pressure. Arterial vasoconstriction and significantly reduced cCBF in the setting of elevated MABP were recorded. The fact that cCBF was reduced during MABP elevations indicates that cerebral vascular resistance is increased during nitric oxide synthase inhibition.

Our direct measurements on pial arteries demonstrated a 60% reduction of lumen diameter following L-NAME infusion. In addition, morphological analysis of parenchymal vessels showed evidence for mild to moderate vasoconstriction of arterioles. Enlarged perivascular spaces and irregularly contoured endothelial cells were consistent with increased vascular tone. In a pial window preparation, Rosenblum et al demonstrated a dose-dependent vasoconstriction of mouse pial arterioles following topical application of L-NMMA. Pial vasoconstriction was dependent on an intact endothelium and was not associated with increased blood pressure. Faraci demonstrated in rats that the topical application of L-NMMA in the range 10^-3 to 10^-4 M induced constriction that was dependent on the initial diameter of the blood vessel. Furthermore, the basilar artery was 10-fold more sensitive than pial arterioles to L-NMMA. The degree of vascular constriction observed in our study contrasts with the minimal and modest changes in pial vascular diameter reported by those authors. These contrasting results are likely due to differences in the route of administration (superfused versus infused), the effective intravascular concentration of inhibitor, the time of observation, the species, and the vessel (arteries or arterioles) considered.

In rats infused with L-NAME, evidence for red blood cell stasis was observed in cortical and subcortical brain regions. This finding implies that ischemia has developed secondary to L-NAME treatment. Our hemodynamic data, showing moderate cCBF reductions, do not exclude the existence of microregions experiencing zero flow. The existence of ischemic microregions may be evidenced, in principle, as a decrease in the blood "volume" (in reality, the volume density of moving red blood cells) as monitored by laser-Doppler flowmetry. This was not observed because we were unable to monitor blood volume simultaneously with cCBF. We presume, however, that histological evidence of stasis is more sensitive to (i.e., precedes) possible changes in blood volume reported electronically because static red blood cells can readily be detected in tissue sections regardless of the actual tissue volume.

In addition to arterial constriction and red blood cell stasis, our experimental rats displayed endothelial abnormalities that may also have contributed to the hemodynamic and vascular alterations reported. Cortical arterioles displayed many endothelial pinocytotic vesicles and microvilli 1 hour after the start of drug infusion. Similar endothelial alterations have been documented in animal models of brain ischemia, as well as of acute hypertension and trauma. Pinocytotic transport of substances across the endothelial barrier is considered a possible mechanism for increased vascular permeability and may have participated in the permeability alterations seen in this study. Since vasoactive substances are present in the blood, the transendothelial conductance of water- or blood-borne vasoactive agents could influence luminal aperture and vascular tone. In addition, luminal projections such as endothelial microvilli might be expected to increase vascular resistance and consequently decrease cCBF. In this regard, formation of endothelial microvilli has previously been suggested to contribute to postischemic hypoperfusion following global forebrain ischemia.
Taken together, these morphological findings demonstrate that the inhibition of NO synthesis leads to subtle alterations in cerebral blood vessels that may have resulted from secondary brain ischemia rather than as a direct consequence of such inhibition. In this latter connection, we remark that no truly valid control exists for these experiments; because the arginine uptake system of endothelium is t-specific, it does not respond to D-NAME, the inactive enantiomer of L-NNAME (D. Rees and R. Palmer, personal communications). There is as yet no L-NNAME analogue that can pass the endothelial barrier and yet serve as an inactive inhibitor analogue at the nitric oxide synthase level of specificity. At best, D-NAME can serve as a nonspecific control for the hydrochloride salt moiety, but we have obviated this use by controlling the solution pH and thereby largely neutralizing its acidity. In light of these considerations, we nonetheless postulate that an interlocking cascade of events (e.g., varying degrees of vasoconstriction) begun by nitric oxide synthase inhibition affects the distribution of microvascular blood flow by generating a broad range of microvascular conductances. Under our conditions the distribution of conductances likely includes the zero flow state, consistent with induction of secondary cerebral ischemia in those particular microregions.

In conclusion, our findings indicate that the structure and function of the cerebrovasculature are altered as a consequence of artificially inhibiting NO synthesis. A fundamental role is therefore indicated for the continuing synthesis of NO in maintaining the proper delivery of nutrients to the adjacent parenchymal tissues. In clinical conditions that are associated with cerebrovascular injury (i.e., chronic hypertension, ischemia, trauma), altered NO synthesis and subsequent vascular dysregulation may be important pathophysiological events. Thus, pharmacological treatments directed at preserving or increasing NO production in the vascular space may prove to be beneficial in the prevention and treatment of cerebrovascular disease and stroke.

Acknowledgments

We wish to thank Marcilia Halley for expert technical assistance and Helen Valkowitz for typing. We also thank Dr. Myron D. Ginsberg for his help in completing this study by making the laser-Doppler flowmeter available to us.

References


R Prado, B D Watson, J Kuluz and W D Dietrich

*Stroke*. 1992;23:1118-1123
doi: 10.1161/01.STR.23.8.1118

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/23/8/1118

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org/subscriptions/