Nitric Oxide Deficiency Contributes to Large Cerebral Infarct Size


Abstract—The purpose of this study was to examine the role played by a deficit in nitric oxide (NO) in contributing to the large cerebral infaracts seen in hypertension. Cerebral infarction was produced in rats by occlusion of the middle cerebral artery (MCA). Studies were performed in Sprague-Dawley (SD) rats subjected to NO synthase blockade (N^G^-nitro-L-arginine [L-NNA], 20 mg · kg^-1 · d^-1 in drinking water) and in spontaneously hypertensive stroke-prone rats (SHRSP). NO released in the brain in response to MCA occlusion was monitored with a porphyrinic microsensor in Wistar-Kyoto rats. The increment in NO released with MCA occlusion was 1.31 ± 0.05 μmol/L in L-NNA–treated rats, 1.25 ± 0.04 μmol/L in SHRSP, 2.24 ± 0.07 μmol/L in control SD rats, and 2.25 ± 0.06 μmol/L in Wistar-Kyoto rats (P < 0.0001 for control versus the other groups). Infarct sizes in the L-NNA–treated and control SD rats were 8.50 ± 0.7% and 5.22 ± 0.7% of the brain weights, respectively (P < 0.05). The basilar arterial wall was significantly thicker in L-NNA–treated rats compared with their controls. We conclude that both the deficit in NO and the greater wall thickness contribute to the larger infarct size resulting from MCA occlusion in SHRSP and in L-NNA–treated rats compared with their respective controls. (Hypertension. 2000;35:1111-1118.)

Key Words: nitric oxide ■ nitric oxide synthase ■ infarct ■ stroke ■ infarct size ■ hypertension, NOS-deficiency

The stroke-prone spontaneously hypertensive rat (SHRSP) has been used extensively in studies of the pathophysiological and genetic factors responsible for stroke. A commonly used strategy for the study of stroke with this model has involved quantifying the size of the infarcted area that results from obstruction of the middle cerebral artery (MCA). Whereas SHRSP always develop a large infarct in the region of the hemisphere supplied by the MCA, the only remaining source of blood flow for this region results from obstruction of the MCA, the only remaining source of blood flow for this region. The characteristic difference in these vessels is an increase in the wall-to-lumen ratio. Arteries and resistance vessels from SHRSP have both an increase in wall thickness and a decrease in lumen diameter. These characteristics cause not only a direct increase in vascular resistance but also an increase in vascular reactivity to vasoconstrictor agents. The possible contribution of a deficit in NO to the enlarged cerebral infarct in hypertension has been studied extensively, but its involvement has received conflicting support. Many observations of alterations in infarct size resulting from experimental manipulations of NO have been published. These observations have been interpreted as indicating that NO may either decrease or increase in- or decrease in- or increase in- or increase in- or increase in infarct size. Among related observations suggesting that a deficiency in NO may be involved in the failure of collateral dilatation and the resultant enlarged infarct in SHRSP are the studies of Malinski and colleagues, who observed an abrupt increase in NO concentration in the region of the brain made ischemic by occlusion of the MCA. This observation suggests that the resultant vasodilatation is a physiological compensatory mechanism for ischemia. Faraci has reported that NO is a potent vasodilator of the cerebral vasculature, and Toda and Okamura have observed that these vessels are richly supplied with nitroxeenic nerves. Finally, Cabrera et al have demonstrated a deficit in NO production in the brain of SHRSP.
Relevant insight into the mechanisms by which NO has this dual effect on infarct size has been provided by Huang and colleagues. In one study, they performed MCA occlusion on mice that had had their endothelial NO synthase (NOS) gene disrupted (eNOS knockout mice). In these mice, the resulting cerebral infarct was enlarged, yet when these mice were treated with nitro-L-arginine, eliminating residual NOS production of NO, the infarct size was reduced. From another study, these authors reported that in mice in which the gene for neural NOS had been disrupted (nNOS knockout mice), the infarct size resulting from MCA occlusion was smaller than that in the wild-type mice. When nNOS knockout mice were treated with an NOS inhibitor, the infarct size was larger.

From these studies, Huang et al conclude “that NO possesses a dual role in focal cerebral ischemia.” Depending on its source, NO may either decrease or increase infarct size. If its source is endothelial NOS, the NO causes vasodilatation and a decrease in infarct size. If the NO is from neuronal NOS, it increases the infarct size because of its neurotoxic action. Yoshida et al found support for this interpretation in their observation that in mice treated with 7-nitroindazole, a specific inhibitor of neuronal NOS, the infarct size was reduced.

The present study was performed to evaluate the possible contributions of deficient NO release and arterial wall hypertrophy to the enlarged infarct developed after MCA occlusion in SHRSP. Two types of studies were performed in this evaluation: (1) NO release in the brain after MCA occlusion was measured in vivo with a porphyrinic microsensor. The evaluation: (2) In the second approach, we studied the effect of the standard reduction in NOS activity used to produce hypertension in genetically normal rats. Results compared. (2) In the second approach, we studied the effect on infarct size of the standard reduction in NOS activity used to produce hypertension in genetically normal rats. Results indicated that less NO was released in response to MCA occlusion in SHRSP than in control rats and that when production of NO is blocked in normal rats, they develop larger infarcts.

**Methods**

**Production of Infarcts**

Male Sprague-Dawley (SD) rats (250 to 300 g, Harlan, Indianapolis, Ind) were treated with the NOS inhibitor L-NNA in drinking water at a concentration of 120 mg/L, giving a dose of ~20 mg/kg per day for 14 days. Control SD rats were given only tap water. All rats were on a 12-hour light/dark cycle and had free access to food and water until the day of the experiment. The rats were then anesthetized with ketamine hydrochloride (Ketalor, 150 mg/kg body wt IM, Parke-Davis) The MCA in both groups (n = 6 for each group) was occluded by cautery as described by Tamura et al. The location of the occlusion was just distal to the origin of the striate branch as described by Coyle. MCA occlusion by thread ligation was performed on additional SD rats (n = 4 for each group) by use of methods described by Coyle. All animals were allowed to recover after surgery with free access to tap water and rat chow.

**Quantification of Infarct Size**

Fifty-eight hours after occlusion of the MCA, the rats were euthanized with an overdose of pentobarbital sodium and decapitated, and their brains were carefully removed. The brains were weighed, and 1-mm coronal slices were stained with a 4% 2,3,5-triphenyltetrazolium chloride solution for 30 minutes at 37°C Any excess fluid was blotted from the slices with filter paper. The infarcted (non-stained) area was then dissected from the slices and weighed, and its size was expressed as a percentage of the whole brain weight. The staining and weighing were performed by a technician who did not know the source of the brain.

**Measurement of NO Release**

Other groups of male SD rats treated with L-NNA (n = 6) plus controls (n = 5) and male SHRSP (n = 6) plus WKY (n = 6) were used in the study of NO release. The latter inbred strains of rats have been raised in our laboratory for 15 years. All rats were prepared for MCA occlusion by catherization as described previously. In addition, a porphyrinic microsensor was positioned with a micromanipulator so that its tip was 1 mm distal along the MCA from the point of occlusion and was 1 mm deep in the parietal lobe of the brain. This position was chosen because it was in the known site of the infant. Measurements of NO release were made continuously before, during, and after the occlusion. The maximum sustained concentration of NO after the occlusion was quantified as the level to which the concentration rose above baseline.

**Morphometric Comparisons of Basilar Arteries**

Male SD rats were again divided into L-NNA–treated (n = 6) and control (n = 6) groups. Animals were anesthetized with pentobarbital sodium (50 mg/kg body wt IP) and injected with papaverine (30 mg/kg IV) for maximum vasodilatation. After papaverine had circulated for 30 seconds, the animals were intravenously administered 1 mL heparin (100 U/mL). A 16-gauge needle was then inserted into the aorta via the left ventricle, and the right atrium was opened to allow drainage. The animal was first flushed with 120 mL PBS at 100 mm Hg pressure before fixation. After the flush with PBS, the animal was perfused with 180 mL 4% paraformaldehyde at the same pressure. Brains were carefully removed and prepared for paraffin embedding. After paraffin embedding, 4-μm cross-sectional slices were made of the basilar arteries and placed on slides. After staining with hematoxylin and eosin, slides were examined under a microscope (Leitz orthoplan) at ×25. Images were taken with a digital camera (Sony DKC 5000), and cross-sectional areas were analyzed by use of NIH Image. This application was chosen because of the ability to quantify cross-sectional areas of images. The outer circumference of the vessel wall was traced as well as the internal circumference (lumen). The measurements were taken 3 times to increase accuracy. These measurements were then automatically converted to square microns by use of NIH Image. The inner area (area of the lumen) was subtracted from the area of the entire vessel (outside circumference) to give the area of the vessel wall.

**Results**

Systolic pressures (tail-cuff method) of the control SD rats averaged 128±7 mm Hg; those of the L-NNA–treated SD rats averaged 159±7 mm Hg. Systolic pressures of the WKY averaged 116±7 mm Hg; those of the SHRSP averaged 200±6 mm Hg.

**Infarct Size**

Representative brain slices from which infarct sizes were determined in L-NNA–treated and in control rats are depicted in Figure 1A. The mean infarct weights of control and treated rats were compared separately for animals that had undergone MCA occlusion by catherization (n = 6, each group; Figure 1B) and by ligation (n = 4, each group). The mean infarct weights after MCA catherization were 6.76% of brain weight for the control rats and 8.92% for the treated rats (P < 0.05). The mean infarct weights after MCA ligation were 2.92% for
the control rats and 7.88% for the treated rats (P<0.05). Infarct sizes resulting from cauterizing the MCA were larger than those resulting from ligating the artery. This is especially evident in the control rats (Figure 1B) and may be attributed to the broader region of the artery affected by the cauterization.

After MCA occlusion, the treated rats evidenced more neurological signs than did the control rats. These signs included circling to the contralateral side and paralysis. Of the treated rats, only 10 of 19 MCA-occluded rats survived 48 hours for infarct size quantification, whereas all 10 control rats survived.

Measurement of NO Release

NO release in the region of the infarct was recorded continuously before, during, and after MCA occlusion. Figure 2A shows representative tracings of these recordings from an L-NNA–treated and a control SD rat and also from SHRSP and WKY. The mean increment in maximum NO concentration (Figure 2B) after occlusion in six L-NNA–treated rats was 1.31±0.05 μmol/L. This increment in NO concentration was significantly less than that in 5 control rats in which the increment was 2.24±0.07 μmol/L (P<0.001). The increment in NO concentration after occlusion in SHRSP rats was 1.25±0.05 μmol/L; that after occlusion in WKY was 2.25±0.06 μmol/L (P<0.001).

Arterial Wall Thickness

Figure 3A depicts representative cross sections of basilar arteries from both L-NNA–treated and control rats. When the area of the arterial wall is expressed as a percentage of the area of the entire artery, the value for the 6 treated rats was significantly greater than that for the 6 control rats (25.9±0.6% versus 22.2±0.5%, P<0.05; Figure 3B).

Discussion

This is the first comparison to be reported of directly measured NO release in a cerebral infarct of hypertensive versus normotensive rats. Two observations in the present study support the possibility that a deficit in NO release in the brain contributes to the greater infarct size that results from MCA occlusion in SHRSP compared with normotensive control rats: (1) The measured increase in NO concentration in the brain in response to this occlusion in SHRSP was almost identical to the increase in NO concentration observed in L-NNA–treated (NO-deficient) rats. These increases were 60% of the increases in NO concentration observed in control WKY and SD rats. (2) The size of the infarct resulting from MCA occlusion was 63% greater in SD rats treated with L-NNA than that in untreated (control) SD rats.

Table 1 summarizes data from published reports comparing infarct sizes of genetically hypertensive rats with those of their normotensive controls. Because the absolute values varied with the units used in measuring the infarct (ie, area, volume, or percent of the whole brain), the relevant data are the percentages comparing infarct size in the hypertensive rat with that in its control. In each report, the same unit was used to determine the infarct size in hypertensive and control rats. In these reports, the infarcts of the genetically hyperten-
sive rats ranged from 51% to 180% larger than the infarcts of the normotensive control rats. In the present study, the infarcts of the L-NNA–treated rats were in the lower part of this range: 63% larger than those of the controls.

The functional effects of MCA occlusions in genetically hypertensive rats and in L-NNA–treated rats were similar. Both had more neurological symptoms after MCA occlusion than did their control rats, and survival rate for the postocclusion period was poor for both the NOS-blocked (present study) and the genetically hypertensive rats. Not a single rat of the control group of either study died as a result of MCA occlusion.

In the present study, the systolic pressure of the L-NNA–treated group of rats was 159 ± 7 mm Hg; that of the control group was 128 ± 7 mm Hg. The blood pressure difference was similar to the above for our genetically hypertensive rats.

Figure 2. NO concentration measured in vivo in response to MCA occlusion. The porphyrinic NO microsensor was positioned in the site of the infarction in the parietal lobe of the brain. A, Representative tracings of NO release before and during occlusion of MCA from a control SD rat (a), an L-NNA–treated SD rat (b), WKY (c), and SHRSP (d). Note that the same scale is used for the ordinates of the control and hypertensive rats. B, Comparisons of NO release after MCA occlusion in control (n = 5) and L-NNA–treated (n = 6) rats and in WKY (n = 6) and SHRSP (n = 6). **P < 0.0001.
versus their controls, whereas pressure was 200±6 mm Hg for SHRSP and 116±7 mm Hg for WKY. Although in these observations there is an association between an elevated blood pressure and an enlarged infarct, there are compelling arguments that the elevated blood pressure, per se, does not make the infarct larger. Prior studies3,4 have reported that enlarged infarcts occur in young SHRSP before the elevated blood pressure is established. As is evident in Table 2, normalizing blood pressure in genetically hypertensive rats by antihypertensive treatment results in only a small correction in infarct size: 29.7% (average of the 3 studies39–41 involving treated rats in Table 2) compared with 122.7% (average of 5 studies3,35–38 in Table 1) enlargement of the infarct size in these untreated hypertensive rats with reference to the value in control rats. Also, rats made equivalently hypertensive by mineralocorticoid administration do not develop infarcts as large as those developed by SHRSP.42 The most convincing evidence for the lack of correlation between blood pressure and infarct size is provided by a recent study by Carswell et al.36 They reported on infarct size in the F1 hybrids of a cross between SHRSP and WKY. In these rats, they observed a statistically significant inverse correlation between mean arterial blood pressure and infarct size.

Survival of the brain tissue that had received its blood supply from the MCA before occlusion depends on blood coming from the anterior and posterior cerebral arteries via collateral arteries. It follows that the adequacy of this collateral supply determines the condition of tissue supplied by the MCA, and on the basis of the present observation, this supply is inadequate when the production of NO has been deficient.

The important physiological roles played by NO in cerebrovascular dilatation are well established. Not only has it been established that endothelial NO release is the mechanism for the vasodilatation produced by acetylcholine,23 but it has also been reported that the classical vasodilatation resulting from kainate43 or N-methyl-D-aspartate44 is mediated by NO. Neurogenically induced cerebrovasodilation is mediated by nitroxidergic nerves.24

An interesting and relevant role of NO in the brain is its release in response to ischemia. This NO release serves the compensatory function of decreasing cerebrovascular resistance, thus increasing blood flow to the ischemic area. Kumura et al45 have reported that nitrate (the stable product of NO metabolism) in the jugular vein rose from 36±9 to 53±8 µmol/L 2 hours after MCA occlusion in the rat. After 4 hours, it had returned to 42±9 µmol/L. Interestingly, after 30 minutes of reperfusion, it had risen to 72±7 µmol/L.

Studies from Malinski’s laboratory21,22 used the porphyrinic microsensor to measure NO concentration in the region of the brain made ischemic by MCA occlusion. Baseline concentration of NO was ≈10 nmol/L. Concentration of NO during MCA occlusion increased >200-fold, to 2.2 µmol/L. In the present study, MCA occlusion caused an elevation of NO concentration of 2.25 µmol/L in control rats, of 1.31 µmol/L in L-NNA–treated rats, of 2.52 µmol/L in WKY, and of 1.25 µmol/L in SHRSP.

The importance of a normal production of NO to cerebral blood flow is demonstrated by the observation that this blood flow is reduced by 25% to 30% after treatment of the rat with...
Nitro-l-arginine methyl ester (L-NAME). This represents an even greater increase in cerebrovascular resistance, because the treatment caused a 30 mm Hg elevation in blood pressure. When this observation is applied to our present study, it seems reasonable to conclude that such an increase in resistance to blood flow through collateral vessels supplying the ischemic area that had been supplied by the MCA would contribute to the 63% greater infarct size in the L-NNA–treated rat than in the control rat.

Evidence suggests that a similar deficit in NO release may contribute to the large infarct that develops in SHRSP. Inhibition of NOS in the central nervous system causes a pressor response. This observation indicates that NO is normally produced in the central nervous system, where it has a tonic blood pressure–lowering effect. We recently reported that a deficient NO production in the central nervous system of SHRSP may contribute to the elevation of arterial pressure in these rats. We reported the following observations, which indicate that there is a deficient central NO production in the SHRSP: Stimulation of NOS with an intracerebroventricular injection of calcium caused less of a depressor response in SHRSP than in WKY. Inhibition of NOS with an intracerebroventricular injection of L-NAME caused less of a pressor response in SHRSP than in WKY. Likewise, blockade of the action of cGMP (a mediator of the action of NO) caused less of a pressor response in SHRSP than in WKY. Finally, the depressor response resulting from the central injection of an NO donor was much greater in SHRSP than in WKY. We interpreted this observation as reflecting a deficit in the negative-feedback action of endogenous NO in SHRSP. Our present observations of a lesser increment in NO concentration in SHRSP than in WKY resulting from MCA occlusion are in accord with these earlier blood pressure studies.

For these reasons, it appears that a deficient concentration of NO could contribute to the impaired collateral blood flow and enlarged infarcts in L-NNA–treated rats and in SHRSP. Another condition shared by these 2 groups of rats must be considered as contributory to these deleterious effects of MCA occlusion. This other condition is the structure of the collateral vessels supplying the territory of the occluded MCA. Coyle and Heistad studied the anastomoses between the anterior cerebral artery and the MCA 1 month after MCA occlusion. There were the same number of anastomotic vessels in SHRSP and WKY, 24 to 29 in each. However, the mean luminal diameter (papaverine-dilated and latex-filled) in SHRSP was 32 ± 2 mm, whereas that in WKY was 55 ± 3 mm. Nordborg and Johansson have reported a significantly greater media thickness/radius ratio of cerebral vessels in 15- and 200-day-old SHR compared with age-matched WKY. They offer this observation as an explanation for the greater cerebrovascular resistance during maximum vasodilatation in SHR.

Arribas et al reported a similarly greater wall/lumen ratio in rats after treatment with L-NAME. This structural change in the vessel wall would be expected to contribute to the greater cerebrovascular resistance reported by Tanaka et al in rats after treatment with N′-monomethyl-l-arginine. In the

| Table 1: Infarct Size Resulting From MCA Occlusion in Genetically HT Rats Compared With Normotensive Controls |
|---|---|---|---|---|---|---|
| Reference | Type of Rat | Rats (HT/Control), n | Duration of Occlusion, d | Method of Measuring Infarct | Absolute Measurements (HT/Control) | Percentage Larger Than Control* |
| 35 | SHR/Wistar | 8/7 | 1 | Volume | 204.6/135.6 | 51% |
| 36 | SHRSP/WKY | 15/17 | 1 | Percentage of brain area | 36.6/14.2 | 161% |
| 37 | SHR/WKY | 8/8 | 2 | Percentage of brain area | 39/15 | 160% |
| 38 | SHRSP/WKY | 8/12 | 1 | Percentage of brain area | 27.8/11.0 | 153% |
| 3 | SHRSP/WKY | 9 wk | Male | 7/5 | 1 | Percentage of brain area | 18.5/9.4 | 97% |
| | | | Female | 8/5 | 1 | Percentage of brain area | 19.3/7.9 | 144% |
| | | 24 wk | Male | 5/4 | 1 | Percentage of brain area | 20.5/10.5 | 95% |
| | | | Female | 5/5 | 1 | Percentage of brain area | 18.9/11.6 | 63% |

HT indicates hypertensive. Absolute measurements refer to column to the immediate left.

*Calculated as infarct measurements of [(HT−control)/control]×100.

| Table 2: Effects of Antihypertensive Treatment on Infarct Size in Genetically HT Rats |
|---|---|---|---|---|---|---|
| Reference | Type of Rat | Rats (HT/Control), n | Duration of Occlusion, d | Method of Measuring Infarct | Absolute Measurements (HT/Control) | Percentage Larger Than Control* |
| 39 | SHR/hydralazine-treated SHR | 17/18 | 1 | Area | 230/189 | 22 |
| 40 | SHR/cilazapril-treated SHR | 13/15 | 3 | Area | 178/117 | 52 |
| 41 | SHR/hydralazine-treated SHR | 6/5 | 2 | Area | 138/120 | 15 |

*Calculated as infarct measurements of [(HT−control)/control]×100.
present study, we have found that compared with controls, the L-NNA–treated rats have greater wall thickness of the cerebral artery. A similar structural difference in the anastomotic arteries would contribute to a deficit in collateral blood flow to the infarcted area.

We conclude that in SHRSP and in L-NNA–treated rats, there is a deficit in NO and a greater vascular wall thickness that could contribute to the impaired collateral blood flow that is responsible for the greater infarct size resulting from MCA occlusion.

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References

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