Brain Nitric Oxide Synthase Activity in Normal, Hypertensive, and Stroke-Prone Rats

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Background and Purpose Nitric oxide (NO) has been implicated in the mechanism of cerebral injury from both permanent and transient focal ischemia. During ischemia there is an increase in the extracellular concentration of excitatory amino acids, which presumably results in increased intracellular calcium and stimulation of the enzyme NO synthase, which catalyzes the conversion of L-arginine to citrulline and NO. Once formed, NO is believed to react with other metabolites (eg, superoxide anion) to produce toxic byproducts. The relation of the NO-mediated mechanism with spontaneous stroke has not been previously evaluated.

Endothelium-dependent NO-mediated cerebral vasodilation is impaired in spontaneously hypertensive stroke-prone rats (SHRSP). In addition, NO inhibits platelet adhesion and aggregation, and the activation of platelets and monocytes appears closely related to the occurrence of cerebrovascular occlusions in SHRSP. With high activity of NO synthase found within brain, we hypothesized that there may be a genetic defect of brain nitric oxide synthase, the enzyme responsible for nitric oxide production. To test the hypothesis that brain nitric oxide synthase activity is altered in spontaneously hypertensive stroke-prone rats compared with spontaneously hypertensive or Wistar-Kyoto rats.

Methods A colony of spontaneously hypertensive stroke-prone rats was bred, in which the rate of neurological events under salt load was assessed. In a separate cohort of animals brain nitric oxide synthase activity was measured in spontaneously hypertensive stroke-prone rats (n=6) and in spontaneously hypertensive (n=6) and genetically related Wistar-Kyoto rats (n=6). Calcium dependency of nitric oxide synthase was also assessed in cortical brain samples from the three rat strains to determine if altered calcium-dependent activation of nitric oxide synthase was present.

Results Brain nitric oxide synthase activity was highest in the cerebellum (eg, spontaneously hypertensive stroke-prone rats: cerebral cortex, 10.6±0.9; cerebellum, 50.1±12.0; brain stem, 14.7±10.3 pmol/mg protein per minute); however, there was no difference among the three rat strains in any region (eg, cerebral cortex: spontaneously hypertensive stroke-prone, 10.6±0.9; spontaneously hypertensive, 10.8±0.5; Wistar-Kyoto, 10.9±0.7 pmol/mg protein per minute) or at any calcium concentration tested.

Conclusions A genetic defect of brain nitric oxide synthase is unlikely to be the cause of stroke predisposition in spontaneously hypertensive stroke-prone rats. (Stroke. 1994;25:1674-1678)

Key Words cerebral ischemia, focal genetics nitric oxide rats

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Materials and Methods A colony of SHRSP was bred in our institution from three pairs of male and female rats obtained from the National Institutes of Health (Laboratory Sciences Section, Veterinary Resources Program, National Center for Research Resources, Bethesda, Md). For each SHRSP, age-matched SHR and WKY rats were obtained (Charles River, Kingston, NY) at the time of weaning (3 to 4 weeks of age). All rats were kept in the same virus-free environment and maintained on regular diet (NIH 31, Zeigler Bros, Inc) with water ad libitum.

Genetic integrity in the three strains was assessed by testing isozyme phenotypes (HBB, PEP-3, AKP-1, ES-1, ES-3, ES-14) in two rats from each strain. The result of isozyme testing conformed to the accepted genetic profile for these strains. Because of the strong influence of environmental factors on...
stroke rate in SHRSP.\textsuperscript{12} We also assessed in our institution the incidence of neurological events or premature death under chronic salt loading in the three strains. To this end a group of 60 age-matched male rats (n=20 in each strain) was given 0.9% saline as drinking water from the age of 4 weeks while being maintained on a regular diet, and the incidence of motor deficit, seizures, or death was assessed for approximately 1 hour, twice a day, over a period of 9 weeks. Motor deficit was assessed by observing each animal’s gait. The animal was determined to have seizures if it exhibited myoclonic movements at any time during the observation period.

For determination of NO synthase activity (whole brain tissue), we used a separate group of 48 male age-matched SHRSP, SHR, and WKY rats, aged 2 to 3 months, fed a regular diet and tap water. NO synthase activity was determined with modification of the techniques described by Dwyer et al\textsuperscript{13} and Bredt and Snyder\textsuperscript{14} before and after administration of \textsuperscript{L}-nitro-\textsuperscript{L}-arginine methyl ester (L-NAME). We compared NO synthase activity among the three rat strains in samples obtained from brain cortex, cerebellum, and brain stem (n=6 rats in each strain). In each experiment and for each tissue studied, samples from age-matched rats of each strain were always processed in parallel. Samples were assayed in vitro for NO synthase activity with modifications of previously described techniques,\textsuperscript{14} by determining the conversion of \textsuperscript{[14C]}L-arginine to \textsuperscript{14C}\textsuperscript{L}-citrulline, the formation of which is stoichiometric with NO synthesis.

Briefly, after decapitation, brain samples were quickly obtained, rinsed in ice-cold buffer [50 mmol/L Tris(hydroxymethyl)aminomethane HCl, pH 7.4, with 2 mmol/L EDTA] and placed on ice for processing. Samples were sonicated in 20 vol (wt/vol) of buffer and centrifuged at 10 000g for 15 minutes at 4°C, and the supernatant was assayed in duplicate. The reaction, conducted at 20°C, was initiated by adding 25 \mu L of brain supernatant to 100 \mu L of reaction mixture containing 1 \mu mol/L \textsuperscript{[14C]}L-arginine, 1 mmol/L NADPH, and 1 mmol/L CaCl\textsubscript{2}. The reaction was terminated after 30 minutes by adding 2 mL of stop buffer (30 mmol/L \textsuperscript{2}-hydroxyethylpiperazine-N\textsuperscript{-2}-ethanesulfonic acid [HEPES], pH 7.2, and 3 mmol/L EDTA). \textsuperscript{[14C]}L-citrulline was eluted on chromatography columns (resin AG-50W-X8, Na\textsuperscript{+} form, pH 7.0) and quantified by liquid scintillation spectroscopy (Beckman LS 1800). Total citrulline recovered was calculated from specific activity of the \textsuperscript{[14C]}L-arginine, correcting for counting efficiency, and was expressed as picomoles per minute per milligram protein. To assess that citrulline production was due to NO synthase activity, parallel samples were processed in the presence of 100 \mu mol/L L-NAME, demonstrating complete (>99%) inhibition of \textsuperscript{L}-arginine conversion. Protein concentration was measured by the method of Bradford.\textsuperscript{15}

Because dietary calcium supplementation offers partial protection from salt-induced stroke in SHRSP,\textsuperscript{10} we also compared cerebral cortical NO synthase activity calcium dependency (n=6 rats in each strain) by varying the calcium concentration in the reaction medium for Wistar-Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and stroke-prone SHR (SHRSP) (mean±SEM, n=6 in each strain). NO activity was detected from cerebral cortex than in cortex and brain stem in each strain, but there was no difference among the three strains in any of the three regions.

**Discussion**

Predisposition to stroke in SHRSP bred in our institution was well preserved in that 20 of 20 animals either died or demonstrated significant neurological impair-
ment after 9 weeks of chronic salt loading, whereas in the genetically related SHR and normotensive (WKY) strains, only one rat died and none demonstrated neurological impairment. In all rats tested, high levels of brain NO synthase activity were demonstrated, with the highest levels occurring in the cerebellum, in agreement with previous anatomic observations. However, SHRSP as well as SHR showed no difference in regional brain NO synthase activity compared with their normotensive controls (WKY rats). These results do not support the hypothesis that the genetic predisposition to stroke in SHRSP is due to an altered ability of brain NO synthase to produce NO.

In brain, impairment of endothelium-dependent vasodilation has been demonstrated in the SHR strain as well as in the SHRSP strain compared with their normotensive controls (WKY rats). In addition, accumulation of monocytes and platelets has been described in cerebral perforating arteries, which may be related to an alteration in NO-mediated control of blood cell interactions with cerebral vessel walls. However, the lack of a difference in brain NO synthase activity between the genetically related hypertensive strains SHR and SHRSP and their normotensive control (WKY rats) suggests that an enzymatic defect in brain NO synthase does not account for the alteration in the NO-mediated cerebral vascular control. These findings are consistent with the conclusion of Mayhan that the mechanism for altered endothelium-dependent vasodilation during chronic hypertension is due to activation of the prostaglandin H₂/thromboxane A₂ receptor rather than decreased release of an endothelium-dependent relaxation factor.

Our study cannot rule out a specific alteration in endothelial NO production with normal neuronal NO synthase activity because the major fraction of NO synthase measured in our samples comes from neurons and glia rather than endothelium. It is not possible to determine the percentage of neuronal versus nonneuronal NO synthase in our brain samples. The techniques used for measurement of NO synthase activity in this study evaluated only the amount of constitutive calcium/calmodulin-dependent NO synthase and not inducible NO synthase. Constitutive NO synthase is present in both neurons and endothelium but not in glia. Therefore, our study cannot rule out a specific alteration in endothelial NO synthase activity with normal neuronal NO synthase activity because the major component of brain is neurons (approximately 70% of rat brain volume) rather than endothelium. In favor of the hypothesis of a specific alteration in endothelium NO synthase is the finding of morphological alterations of cerebral vessel wall, including endothelium, in SHRSP.

Alternatively, in vivo brain NO synthase activity may be impaired despite normal in vitro findings if the availability of any of the cofactors necessary for brain NO synthase activity is limited in the in vivo environment. For example, brain NO synthase activity is calcium dependent, as previously demonstrated and confirmed by this study. Our data do not suggest a different calcium requirement for brain NO synthase activity among the three rat strains. However, alterations in intracellular calcium concentration and activation of calcium channel currents have been described in SHR vascular smooth muscle, blood cell, and brain synaptosomes. An abnormal movement of calcium may thus occur in SHRSP brain, which could be responsible for an altered in vivo NO synthase activity. Similarly, a lack of endogenous L-arginine, the natural substrate for NO synthase, has been described in some experimental models of hypertension. Yet dietary L-arginine supplementation does not reduce the stroke rate in SHRSP or the hypertension in SHR. Finally, endogenous inhibitors may be present in vivo, counteracting the effect of an otherwise normally functioning NO synthase. Consistent with this are the observation of the accumulation of an endogenous NO synthase inhibitor in chronic renal failure and the observation that renal function impairment occurs in parallel with stroke in SHRSP.

We did not evaluate histological evidence of infarction or hemorrhage in the cohort of animals presented in this study. Some authors have found that infarction was the predominant histological finding in SHRSP, whereas others demonstrated that hemorrhage was the predominant histological finding in SHRSP fed a Japanese-style diet. A possible explanation for this apparent discrepancy between these two studies is that in the later study histology was not done immediately, which allowed for hemorrhage into the original area of infarction.

The finding of similar basal NO synthase activity between strains does not fully address the possibility of differences between strains in stimulated NO synthase activity. To address this question we also compared the effect of increasing concentrations of calcium, a factor that increases NO synthase activity (Fig 2), on brain NO synthase activity in vitro. These data demonstrate no difference in stimulated NO synthase between strains. Nonetheless, it is still possible that there may be differences between strains in stimulated NO synthase activity in vivo. Evaluation of NO synthase activity in vivo is more difficult and must take into consideration possible confounding effects of anesthetics on NO synthase activity.

In conclusion, we found that a genetic defect in maximally stimulated brain NO synthase activity is unlikely to account for the predisposition to stroke in the SHRSP strain because brain NO synthase activity is not different from the non–stroke-prone hypertensive strain (SHR) or from the normotensive strain (WKY rat). However, our data cannot rule out a specific defect in endothelium NO synthase activity or in the sensitivity of brain or endothelium NO synthase to specific stimulators or inhibitors in vivo.

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References

Nitric oxide (NO) and L-citrulline are formed from the substrate L-arginine by the group of enzymes known as NO synthases. Because NO itself is very labile and relatively difficult to measure, activity of NO synthase is often assayed by determining the rate of production of L-citrulline. Two major classes of NO synthase, constitutive and inducible, are known to exist. Activity of the constitutive isoforms is calcium- and calmodulin-dependent and is normally present in neurons, endothelium, and probably astrocytes. The inducible isoform of NO synthase is probably not normally present in brain but is normally present in neurons, endothelium, and probably astrocytes. Activity of the isolated enzyme preparation was calcium independent. Activity of inducible NO synthase is generally calcium independent.

Although the findings have not been consistent, a number of studies support the concept that overproduction of NO during ischemia and/or reperfusion may contribute to brain injury. The authors examined the hypothesis that activity of brain NO synthase may be altered in spontaneously hypertensive rats, which are predisposed to develop stroke. NO synthase was isolated from several major brain regions, and the preparation was used to measure enzyme activity (determined by measuring production of L-citrulline) in normotensive and spontaneously hypertensive rats. No difference was observed in regional activity of NO synthase in chronically hypertensive rats. No difference was observed in regional activity of NO synthase in chronically hypertensive rats.

Editorial Comment

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Production of NO by constitutive NO synthase is dependent on the presence of L-arginine, calcium, calmodulin, NADPH, and several enzyme cofactors. Activity of NO synthase is also pH dependent. The findings of the present study suggest that predisposition to stroke in SHRSP is not a result of major alterations of brain NO synthase activity. As the authors acknowled-
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