Maturation alters cerebral NOS kinetics in the spontaneously hypertensive rat

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Maturation alters cerebral NOS kinetics in the spontaneously hypertensive rat. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1367–R1373, 1997.—Using 14C-labeled arginine to 14C-labeled citrulline conversion assays in brain homogenates from 14- to 18-day-old and adult spontaneously hypertensive rats, we tested the hypotheses that maturation increases neuronal nitric oxide synthase (nNOS) activity and that this increase involves changes in cofactor availability and/or nNOS kinetics. nNOS activity (in pmol·mg−1·min−1) was 46% higher in adults (19 ± 2) than in pups (13 ± 1). The addition of 264 µM calmodulin (CaM), 3 µM FAD, 3 µM flavin adenine mononucleotide (FMN), and 10 µM tetrahydrobiopterin (BH4) increased NOS activity by 3.46, 4.5, and 98% in pups and by 19, 40, and 102% in adults, respectively. All cofactor effects were significant except for CaM in the pup homogenates. Cofactor effects were not significantly different between pup and adult homogenates, except for BH4, which increased absolute NOS activity more in adults than in pups. Values of maximal enzyme velocity (Vmax) for nNOS in the absence of added cofactors were greater in adults than in pups (104 ± 5 vs. 53 ± 3, P < 0.05). Addition of 3 µM FAD or 3 µM FMN increased pup Vmax values to 68 ± 2 and 99 ± 5, respectively, but had no effect in adults. BH4 did not affect Vmax in either group. Control values of the Michaelis-Menten constant (Km) for L-arginine were greater (P < 0.05) in pups (5.7 ± 0.4 µM) than in adults (4.3 ± 0.2 µM) and were significantly reduced by 10 µM BH4 to 3.8 ± 0.2 and 2.9 ± 0.1 µM, respectively. Neither FAD nor FMN affected Km values in either group. The results indicate that endogenous nNOS cofactor levels are not saturating in either pups or adults, changes in cofactor levels differentially affect NOS kinetics in pups and adults, and age-related differences in NOS activity result from fundamental differences in NOS kinetics. These findings support the general hypothesis that the increased vulnerability to ischemic stroke associated with maturation is due in part to corresponding increases in the capacity for nitric oxide synthesis.

arginine, calmodulin; flavin adenine dinucleotide; flavin mononucleotide; nitro-L-arginine methyl ester; newborn; nitric oxide; spontaneously hypertensive rat; tetrahydrobiopterin

MATERIALS AND METHODS

Measurement of cerebral NOS activity. NOS activity was determined as the conversion of 14C-labeled L-arginine (DuPont, Boston, MA) to 14C-labeled citrulline, modified from the method of Bredt and Snyder (3). Each assay sample contained 25 µl of supernatant from a cerebral homogenate, 25 µl (45 pmol) of L-14C-arginine, 75 µl of reaction buffer [(in mM) 50 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1 EDTA, 1 CaCl2, and 1 β-NADPH at pH 7.4], and 25 µl of reaction buffer containing varying concentrations of unlabeled L-arginine and/or NOS cofactors or inhibitors (see Methods of age and cofactor addition on NOS kinetics). After a 10-min incubation at 37°C, the reaction was terminated by addition of 2.0 ml of an ice-cold stop solution containing 20 mM HEPES and 2 mM EDTA at pH 5.5. The combined volume was then applied to Poly-Prep chromatography columns preloaded with 1.0 ml AS 50W-X8 resin (Bio-Rad, Richmond, CA) and rinsed with 2.0 ml distilled water. This preparation was then applied to a high-performance liquid chromatography column containing a 14C-labeled L-arginine and <7% citrulline capture. The eluted volume containing 14C-citrulline was measured by liquid scintillation counting. A separate standard curve was run with each assay to correct for interassay variations in quench and background.
counting efficiency. Samples and standards were run in duplicate, and NOS activity was calculated as picomoles per milligram protein per minute.

Preparation of wholebrain homogenates. Adult SHR brains were homogenized in 50 mM HEPES with 1 mM EDTA at pH 7.4 and then centrifuged at 5,400 g for 1 h at 4°C. As determined in extensive preliminary studies, this centrifugation speed was the lowest that reproducibly yielded a clear supernatant, which is essential for reliable protein measurements. This minimum centrifugal force was chosen in an effort to minimize loss of soluble large-molecular-weight components of possible importance for the NOS assay. The supernatants were analyzed for protein content with biocytochomic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard and were then frozen at −80°C for subsequent measurement of NOS activity. The ratio of tissue wet weight to homogenization buffer volume was routinely adjusted for each preparation to yield an average protein concentration of −60 µg in each 25-µl aliquot of supernatant. Brains from litters of 14- to 18-day-old pups were similarly treated with the exception that all brains from a single litter were pooled before centrifugation. Brains from litters of 14- to 18-day-old pups were homogenized in 50 mM HEPES with 1 mM EDTA at pH 7.4 and then centrifuged at 5,400 g for 1 h at 4°C. As determined in extensive preliminary studies, this centrifugation speed was the lowest that reproducibly yielded a clear supernatant, which is essential for reliable protein measurements. This minimum centrifugal force was chosen in an effort to minimize loss of soluble large-molecular-weight components of possible importance for the NOS assay. The supernatants were analyzed for protein content with biocytochomic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard and were then frozen at −80°C for subsequent measurement of NOS activity. The ratio of tissue wet weight to homogenization buffer volume was routinely adjusted for each preparation to yield an average protein concentration of −60 µg in each 25-µl aliquot of supernatant. Brains from litters of 14- to 18-day-old pups were similarly treated with the exception that all brains from a single litter were pooled before centrifugation.

Validation experiments. Four series of validation experiments were performed. The first series tested the ability of 100 µM nitro-L-arginine methyl ester (L-NAME) (Sigma, St. Louis, MO), a competitive analog of L-arginine, to inhibit NOS activity in pup and adult homogenates. A second series of validation experiments verified that the NOS activity assayed in our preparations was calcium dependent. In these experiments, NOS activity was measured in pup and adult homogenates in the presence and absence of 1 mM EGTA. A third series of validation experiments addressed the possibility that homogenate protease activity degraded significant amounts of NOS in our preparations. In these experiments, NOS activity was determined in pup and adult homogenates in the presence and absence of a mixture of inhibitors that included 75 mM aprotinin, 1 mM leupeptin, 0.25 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin A, 1 mM benzamidine, and 1 mM iodoaceticamide. Some but not all previous studies of NOS activity in the brain have reported significant effects of protease inhibition (19, 22). If the extent of proteolytic degradation of NOS activity were greater in neonates than adults, this difference would appear as an age-related difference in NOS activity in our preparation.

Because some investigators have reported that citrulline can be converted back into L-arginine in certain preparations (31), a fourth validation series addressed the extent to which this reaction occurred in our preparation. Clearly, developmental differences in the magnitude of this reaction could contribute to apparent age-related differences in NOS activity measured with our assay system. In this series of experiments, the activity assay was conducted as described above with the exception that [14C]citrulline was used instead of [14C]L-arginine and activity was measured as the rate of disappearance of [14C]citrulline.

Effects of age and cofactor addition on basal NOS activity. If age-related differences in NOS activity are due mainly to differences in cofactor availability, then addition of saturating concentrations of the various NOS cofactors should eliminate age-related differences in NOS activity. To test this idea, we ran six parallel determinations of NOS activity from each homogenate with one of each of the following additions: 1) none (control), 2) 264 µM calmodulin (CaM), 3) 3 µM FAD, 4) 3 µM flavin mononucleotide (FMN), 5) 10 µM tetrahydrobiopterin (BH4), and 6) all four cofactors at the indicated concentrations. Pup and adult homogenates were simultaneously analyzed in each assay run to minimize interassay contributions to possible age-related differences in NOS kinetics. All other aspects of the assay were as described above.

To calculate Km and tissue Vmax values, rates of [14C]citrulline production were plotted against their corresponding concentrations of unlabeled L-arginine and were fitted via nonlinear regression to the competition equation

\[ V = \frac{V_{\text{max}} [S]_{i}}{K_{m} + [S]_{i}} \]

where V is the rate of [14C]citrulline production, Vmax is the tissue maximum rate of [14C]citrulline production, [S]i is the concentration of “hot” labeled substrate (0.3 µM in these experiments), Km is the NOS Michaelis-Menten constant, and [S]j is the concentration of “cold” unlabeled substrate.

To examine the general kinetic mechanism by which cofactor additions increased NOS activity, determinations of NOS Km and tissue Vmax were also performed in the presence of the following NOS cofactors (in µM): 1) 264 CaM, 2) 3 FAD, 3) 3 FMN, or 4) 10 BH4. To minimize the contributions of interassay variability to our measurements, these determinations were run in parallel along with a control set (no cofactor addition) for each homogenate tested. Thus each assay consisted of a series of six tubes with varying unlabeled L-arginine concentrations for each of five different treatments for a total of 30 tubes per age group per assay.

Because the optimum maximum concentration of added BH4 could conceivably vary with age because of possible differences in either endogenous BH4 levels or NOS affinity for BH4, we performed one additional series of kinetic measurements to examine the relation between BH4 concentration and NOS kinetics. For these measurements, each assay consisted of a series of six tubes with varying unlabeled L-arginine concentrations as described above for each of the following six concentrations of BH4: 0.01, 0.03, 0.1, 0.3, 1.0, and 10 µM. The values of NOS Km and Vmax produced by these measurements were plotted against their corresponding BH4 concentrations and were fitted with rectangular hyperbolas with the use of nonlinear regression to estimate the BH4 concentrations that produced one-half-maximal and maximal values of Vmax and one-half-minimal and minimal values of Km.

Data analysis and statistics. All nonlinear regressions were performed using a least-squares error routine implemented in the SOLVER subroutine of Microsoft Excel, version 5.0. Pair t-tests were used to analyze the effects of L-NAME, EGTA, and protease inhibitors on basal NOS activity. All other values of NOS activity were analyzed using a two-way
RESULTS

A total of 24 litters of SHR rat pups and 48 adult SHR rats were used to produce the homogenates used in these measurements. From these homogenates, we made a total of 114 NOS activity measurements using pup homogenates and another 116 measurement using adult homogenates. The protein concentrations in the pup and adult homogenates averaged 396 ± 17 and 419 ± 39 µg/ml, respectively. These values were not significantly different.

Validation results. In the first series of validation experiments, addition of 100 µM l-NAME inhibited basal NOS activities >99.8% in both age groups. In the second validation series, addition of 1 mM EGTA also inhibited basal NOS activities >99% in both pup and adult homogenates. In the third validation series, basal NOS activity was not significantly increased in the presence of protease inhibitors, indicating that proteolysis of NOS was not significant during the short incubation periods used in our assays. In the fourth validation series, the counts per minute of [14C]citrulline converted from [14C]arginine from control and treated values. Across all treatments, adult values were significantly greater than pup values [by analysis of variance (ANOVA)].

Fig. 1. Effects of age and cofactor addition on basal nitric oxide synthase (NOS) activity. A: absolute values of NOS activity under control conditions and in presence of (in µM) 264 calmodulin, 3 FAD, 3 flavin mononucleotide (FMN), 10 tetrahydrobiopterin (BH₄), and all cofactors simultaneously at these concentrations in both pup and adult homogenates. Values are means, and vertical error bars indicate SE for no. of homogenates in parentheses. *Significant age-related differences (P < 0.05) between corresponding pup and adult values. †Significant within-age differences between control and treated values. Across all treatments, adult values were significantly greater than pup values [by analysis of variance (ANOVA)]. B: same data as in A, expressed as percentages of control values. For percent values, there were no significant differences between pup and adult values for any single treatment or across all treatments combined.

analysis, only the differences in the control and BH₄ groups were significant. Interestingly, when the absolute increases in NOS activity were calculated for each cofactor, their sum was not significantly different than the increase produced by the addition of all cofactors simultaneously; the individual effects of each cofactor appeared to be fully additive in both pup and adult homogenates.

On a percentage basis (Fig. 1B), addition of (in µM) 264 CaM, 3 FAD, 3 FMN, 10 BH₄, and all cofactors simultaneously increased basal NOS activity in the homogenates by 3 ± 3, 46 ± 17, 45 ± 4, 88 ± 9, and 329 ± 6%, respectively. As indicated by our post hoc analysis (Fisher’s PLSD), all these increases except that for CaM were significant. Corresponding values in adult homogenates averaged 19 ± 6, 40 ± 24, 36 ± 17, 102 ± 13, and 255 ± 49%, respectively, and all of these increases were significant (Fig. 1). When pup and adult percent values were compared by ANOVA, adult values were not significantly greater than pup values across all treatments. In addition, the sums of the individual percent increases for each cofactor were not significantly different from the percent increase produced by
the addition of all cofactors simultaneously. Again, the individual effects of each cofactor appeared to be fully additive in both pup and adult homogenates.

NOS kinetics: effects of age and cofactor addition. Under basal conditions, the NOS $K_m$ for L-arginine averaged 5.72 ± 0.40 µM in the pup homogenates ($n = 15$), which was significantly greater than that in the adult homogenates ($n = 16$), which averaged 4.32 ± 0.24 µM (Fig. 2A). Although addition of 3 µM FAD or 3 µM FMN had no significant effect on $K_m$ in either pup or adult homogenates, addition of 10 µM BH$_4$ reduced $K_m$ values to 3.75 ± 0.17 and 2.89 ± 0.11 µM in pup and adult homogenates, respectively. When the $K_m$ values were analyzed by ANOVA, adult values were significantly less than pup values within each treatment group. Across all treatments, adult values were also significantly less than pup values within each treatment group.

Values for $V_{max}$ under basal conditions averaged 53 ± 3 pmol·mg$^{-1}$·min$^{-1}$ in the pup homogenates ($n = 15$), which was significantly less than that in the adult homogenates ($n = 16$), which averaged 104 ± 5 pmol·mg$^{-1}$·min$^{-1}$ (Fig. 2B). Addition of 3 µM FAD or 3 µM FMN had no effect on $V_{max}$ in either pup or adult homogenates. The added concentration of BH$_4$ necessary to produce one-half the observed decrease in $K_m$ values was 3.51 ± 0.17 µM observed in adult homogenates. Added concentration of BH$_4$ necessary to attain half-maximal change in $K_m$ averaged 200 and 340 nM in pup and adult homogenates, respectively. The minimum value of this relation averaged 4.46 ± 0.14 µM in pup homogenates, which was significantly less than average value of 3.51 ± 0.17 µM observed in adult homogenates. Added concentration of BH$_4$ necessary to attain half-maximal change in $K_m$ averaged 200 and 340 nM in pup and adult homogenates, respectively. When the $K_m$ values were analyzed by ANOVA, adult values were significantly less than pup values across all treatments, and post hoc analysis indicated that adult values were also significantly less than pup values within each treatment group.

Values for $V_{max}$ in both pup and adult homogenates. Clearly, 10 µM BH$_4$ produced a maximal decrease in $K_m$ and adult homogenates (Fig. 3A). The minimum $K_m$ values attained in pup homogenates averaged 4.46 ± 0.14 µM, which was significantly greater than the minimum $K_m$ value of 3.51 ± 0.17 µM observed in adult homogenates. The added concentration of BH$_4$ necessary to produce one-half the observed decrease in $K_m$ averaged 200 nM in the pup and 340 nM in the adult homogenates. Increasing BH$_4$ concentrations from 0.01 to 10 µM produced hyperbolic decreases in $K_m$ values in both pup and adult homogenates (Fig. 3A). The minimum $K_m$ value attained in pup homogenates averaged 4.46 ± 0.14 µM, which was significantly greater than the minimum $K_m$ value of 3.51 ± 0.17 µM observed in adult homogenates. The added concentration of BH$_4$ necessary to produce one-half the observed decrease in $K_m$ averaged 200 nM in the pup and 340 nM in the adult homogenates. Clearly, 10 µM BH$_4$ produced a maximal decrease in $K_m$ in both pup and adult homogenates. Increasing BH$_4$ concentrations from 0.01 to 10 µM, however, had no significant effect on $V_{max}$ in either pup or adult homogenates (Fig. 3B).
DISCUSSION

Intensive recent efforts have led to the cloning and biochemical characterization of NOS in multiple species, including humans. The majority of this research, however, has focused on the role of nitric oxide in adult tissues, despite growing evidence that NOS changes with age and plays a critical role in brain development (4, 19). It also remains possible that age-related differences in resistance to ischemia (28) may derive from corresponding differences in cerebral NOS activity, owing to the importance of nitric oxide as a mediator of ischemic cerebral damage (6, 8, 16). The present studies address this hypothesis by determining if and how cerebral NOS activity increases with age.

Characterization of NOS activity. The validation experiments suggested that NOS-independent mechanisms of nitric oxide production contributed negligibly to our measurements of NOS activity. Because these measurements quantified citrulline production, they were independent of any nonenzymatic production of nitric oxide via nitrite reduction (34). Back conversion of citrulline to arginine, which is significant in some tissues (27), was also negligible in our preparations. Consistent with previous findings (22), addition of protease inhibitors did not increase apparent NOS activity in samples from either age group, suggesting that differential rates of NOS proteolysis could not explain the age-related differences in NOS activity observed. In addition, administration of the nonspecific NOS inhibitor L-NAME completely inhibited all citrulline production. Together these findings indicate that all differences in NOS activity measured in the present experiments resulted from differences in active enzyme concentration and/or enzyme specific activity.

In all NOS assays, a key determinant of the enzyme activity measured is the predominant NOS isoform in the preparation. Because the present studies utilized only cleared homogenate supernatants, the endothelial nitric oxide synthase (eNOS) isoform probably contributed little to the measured activity, given that most eNOS is membrane associated and sediments with the particulate fraction as shown in many preparations (26). Soluble cytosolic nNOS has been reported, although the size of this fraction is generally quite small and attributable to precursor or intermediate forms of eNOS before acylation and incorporation into the plasmalemma (26) or to agonist-induced transient translocation from the membrane-bound fraction to the cytosolic fraction (21). If we assume low basal levels of endothelial stimulation in the brains used to prepare our NOS homogenates and that the abundance of endothelial cells was low relative to that of neurons in the brain tissues we homogenized, then the levels of eNOS in our preparations were probably minimal.

Regarding nNOS, the present measurements may underestimate total NOS activity because of a loss of some particulate nNOS activity associated with endoplasmic reticulum membranes (14, 19). However, because relatively high g forces are required to sediment this fraction of NOS activity (14), we purposely used the lowest g force and duration necessary to clear the supernates of particulate matter (only 5,400 g for 60 min) to minimize loss of particulate nNOS. This approach, combined with recent findings that purified nNOS may exist predominantly in a soluble form (25), suggests that the great majority of “particulate nNOS” activity demonstrated in other studies was present in our assays.

Given that basal inducible nitric oxide synthase (iNOS) activity in brain homogenates is generally low (12), it is doubtful that iNOS contributed significantly to the observed NOS activities. Nonetheless, in light of evidence that iNOS content may be higher in immature than in mature rat brain tissues (12), we examined the effects of calcium chelation with EDTA on the observed NOS activities. This treatment, which preferentially inhibits constitutive NOS activity (10), completely inhibited all NOS activity in our preparations, indicating that iNOS content in our homogenates was negligible. Together these findings strongly suggest that soluble cytosolic nNOS was the predominant isoform responsible for the NOS activities measured in the present experiments.

Maturational differences in NOS activity. Under the conditions of the present experiments, NOS activity was significantly greater in homogenates from adults than from pups under all conditions examined. Because these measurements were performed at normal body temperature, at equivalent protein concentrations, and in supernatants spun as slowly as possible to clear particulate matter but maximize retention of large soluble cytosolic components, these findings are relevant to the in vivo situation and suggest that nNOS activity in the intact brain increases as a consequence of maturation. To confirm that the observed differences did not result from differences in substrate or cofactor availability, we determined tissue V_max values at cofactor concentrations reported to be saturating in numerous rat brain preparations (18, 22, Fig. 3). The adult V_max values obtained (−104 pmol·mg⁻¹·min⁻¹) agreed well with previously published values for rat brain nNOS that ranged from 42 (18) to 100 (22) pmol·mg⁻¹·min⁻¹ but were also significantly greater than the V_max values observed in pup homogenates (−53 pmol·mg⁻¹·min⁻¹). Together these findings suggest that maturation is associated with an increase in either the concentration or the specific activity of rat brain nNOS.

The concept that cerebral nNOS concentrations may be modulated during maturation is supported by a variety of indirect observations. Many hormones that typically change during pregnancy, development, and early postnatal life have been shown to have potent effects on nNOS expression in rat brain (11). This diversity of influences emphasizes that regulation of nNOS expression is highly complex and involves multiple gene promoters (11). Clearly, many additional experiments will be required to identify which if any of these factors are involved in mediating the effects of maturation on cerebral nNOS activity.
As for $V_{\text{max}}$, both basal and minimum $K_m$ values for arginine also differed significantly with age. In adult homogenates, basal values of $K_m$ were within the range previously published for rat brain [2.0 µM (24) to 8.4 µM (18)]. Unlike the observed age-related differences in $V_{\text{max}}$ values, maturational differences in $K_m$ cannot be explained by differences in enzyme concentration alone. Instead, age-related $K_m$ differences suggest that maturation may somehow alter the NOS isoform profile in rat brain. One possible mechanism for this alteration could be through alternative splicing of the nNOS mRNA (29), as has already been demonstrated in several species.

Another important finding of the present study was that the apparent affinity for BH$_4$ differed in homogenates from pups and adults (see Fig. 3). Large differences are typical of previous studies of NOS affinity for BH$_4$, in which reported values have varied widely from 39 nM in rat cerebellar NOS (30) to 250 nM in purified pig brain NOS (17). Given that increased substrate concentrations can enhance NOS affinity for BH$_4$ (17), the observed age-related differences in apparent affinity for BH$_4$ may be a consequence of age-related differences in endogenous arginine concentration. Alternatively, these differences may reflect age-related NOS isoform differences. Because the endogenous BH$_4$ concentrations in our homogenates were unknown, the observed differences in affinity for BH$_4$ may also simply reflect differences in initial BH$_4$ concentration. This latter possibility is supported by numerous reports that BH$_4$ availability is tightly regulated, rate limiting for NOS activity in many tissues (13), not saturating for brain NOS at endogenous concentrations (20), and changes with age (15).

Aside from age-related changes in the nNOS isoform, maturational differences in nNOS activity and affinity for BH$_4$ might also be explained by posttranslational alterations. For example, age-related differences in nNOS phosphorylation could be involved (23) as could differential activity of endogenous nNOS inhibitors (7). Maturational differences in the mechanisms mediating feedback inhibition of nitric oxide on nNOS could also play some role (20). Evidence that cerebral nNOS may be modulated by superoxide (20) further suggests that age-related changes in free radical metabolism could also play a role in age-related differences in rat brain nNOS activity. Without a doubt, many additional experiments will be required to clarify which if any of these mechanisms is involved.

Perspectives

Whereas basal NOS activity was 46% greater in adult than in pup homogenates in absolute terms, basal NOS velocities were ~19% of their corresponding $V_{\text{max}}$ values in both age groups, indicating that both pups and adults have equivalent capacities to increase basal nitric oxide production approximately fivefold. Although the increases in arginine availability typical of maturation in many tissues (9) may recruit much of this reserve, increased cofactor availability alone may also significantly enhance basal NOS activity. Increased FAD and FMN augmented basal NOS activity by 46 and 45% in pup homogenates, and by 40 and 36% in adult homogenates, respectively, indicating that basal flavenoid concentrations were not saturating for NOS. CaM increased basal NOS activity relatively little when added alone (pup 3%, adult 19%), but raised NOS activity dramatically by 88 (pup) and 102% (adult) in the presence of saturating concentrations of BH$_4$. Together these findings reinforce the view that NOS cofactor concentrations are not saturating under basal conditions in either the mature or immature brain. Because the effects of each cofactor on basal NOS activity were independent of but additive to the effects of each other cofactor, increases in NOS cofactor availability, through changes in either endogenous metabolism or increased dietary intake, can significantly modulate nitric oxide production. These findings further suggest that NOS activity and its potential roles in cerebrovascular regulation and infarct formation may be amenable to pharmacologic or dietary manipulation of cofactor metabolism.

In view of nitric oxide’s role in coupling between cerebral metabolic and blood flow, the observed differences in NOS activity could help explain age-related differences in metabolic and hypercapnic cerebrovascular regulation (16, 32). Maturational differences in cerebral NOS activity may also help explain the well-described but poorly understood age-related differences in vulnerability to ischemic cerebral insults (28). Although the general applicability of the present results obtained in SHR rats remains to be determined but seems probable (5, 33), the results suggest that further studies of the mechanisms responsible for age-related changes in NOS activity will be a fruitful area for future investigation.

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