Inhibition of Brain GTP Cyclohydrolase I and Tetrahydrobiopterin Attenuates Cerebral Infarction via Reducing Inducible NO Synthase and Peroxynitrite in Ischemic Stroke

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Background—Inducible NO synthase (NOS)–derived peroxynitrite (ONOO−) during ischemia/reperfusion contributes to ischemic brain injury. However, inducible NOS (iNOS) regulation in ischemic stroke remains unknown. Tetrahydrobiopterin (BH4) is an essential cofactor for NOS activity. The present study tested the hypothesis that inhibition of endogenous BH4 rate-limiting enzyme GTP cyclohydrolase I (GTPCH I), and thus BH4 synthesis, reduces cerebral infarction via inhibiting iNOS and ONOO− in transient focal ischemia.

Methods—Focal ischemia (2 hours) was created in adult male Sprague-Dawley rats (250 to 300 g) by middle cerebral artery occlusion (MCAO). Rats were treated 12 hours before MCAO with vehicle or diamino-6-hydroxypyrimidine (DAHP; 0.5 g/kg IP), a selective GTPCH I inhibitor. Brains were harvested 24 hours after reperfusion for assays of infarct volume, blood–brain barrier (BBB) permeability, GTPCH I activity, BH4 levels, GTPCH I and NOS mRNA, protein expression, and superoxide anion (O2−·) and ONOO− levels.

Results—Endogenous GTPCH I activity, BH4 levels, iNOS activity, and (O2−· and ONOO− levels were all augmented after ischemia/reperfusion. DAHP treatment significantly reduced GTPCH I activity, resulting in decreased BH4 levels, iNOS activity, and ONOO− levels. Consequently, DAHP treatment significantly reduced the infarct size compared with the nontreated group (22.3±5.6 versus 38.3±7.4%; n=6; P<0.05). Similarly, BBB permeability was significantly reduced after DAHP pretreatment compared with the control group (4.11±0.22 versus 7.78±0.44 µg/g tissue; n=5; P<0.05).

Conclusion—These results demonstrate that blockade of endogenous brain BH4 synthesis attenuates cerebral infarction via inhibiting iNOS and ONOO−, which may provide a mechanistic basis of novel therapeutic strategies for ischemic stroke.

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Key Words: GTP cyclohydrolase ■ nitric oxide synthase ■ stroke, ischemic ■ tetrahydrobiopterin

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middle cerebral artery occlusion (MCAO), indicating a crucial role of GTPCH I and BH4 on iNOS regulation in ischemic stroke.

Materials and Methods

Animal Preparation

Adult male Sprague-Dawley rats weighing 250 to 300 g were studied in 4 groups: (1) normal control, (2) MCAO, (3) MCAO with DAHP treatment (0.5 g/kg IP; dissolved in 0.5 mL dimethyl sulfoxide [DMSO]) 12 hours before ischemia; and (4) MCAO with vehicle (0.5 mL DMSO IP) 12 hours before ischemia. In a published report7 and in our preliminary studies, it has been found that ≥8 hours are required to observe a significant reduction of brain BH4 levels after DAHP administration.

MCAO Occlusion, Infarct Size, and BBB Permeability

Rats were anesthetized with halothane (4% for induction, 1.5% for maintenance). Body temperature was monitored by a rectal probe and maintained at 37°C to 37.5°C by an automatic homeothermic blanket control unit (Harvard Apparatus). All other physical parameters of rats included in the study were within normal limits. MCAO model was established by proximal occlusion of the left MCA with the use of a nylon monofilament as described previously.8 After a 2-hour occlusion, reperfusion was accomplished by careful withdrawal of the monofilament. The common carotid artery was ligated distal to the incision. After 24-hour reperfusion, brains were harvested for the various measurements. Infarct volume was evaluated by 2,3,5-triphenyltetrazolium chloride staining as described.9 BBB permeability was assessed by measuring Evans blue extravasations as described.10

Physiological Measurements

Blood pressure and heart rate were measured continuously with a standard recorder (model Quad Bridge ML118; AD Instruments) and a standard monitor (Perimed). Briefly, a small incision was made at the temporalis muscle was retracted, and the underlying fascia was cleared. A small area of skull ~1 mm posterior and 5 mm lateral to the bregma was thinned to allow placement of the laser Doppler perfusion monitor (Perimed). Briefly, a small incision was made at the midpoint between the left orbit and the external auditory canal under anesthesia. The temporalis muscle was retracted, and the underlying fascia was cleared. A small area of skull ~1 mm posterior and 5 mm lateral to the bregma was thinned to allow placement of the laser Doppler perfusion monitor. CBF was monitored and compared before and after the MCAO procedures in the animals treated with DAHP or saline.

Measurement of BH4 Levels, GTPPC I Activity, and NOS Activity

BH4 levels and GTPCH I activity were assayed by high-performance liquid chromatography (HPLC) analysis as we described.11 Total biopterins (BH4 plus dihydopterin [BH2] plus oxidized biopterin) were determined by acid oxidation, whereas BH4 and oxidized biopterin were determined by alkaline oxidation. BH4 content was calculated by subtracting BH2 plus oxidized biopterin from total biopterins. GTPCH I activity was assayed according to the HPLC method with measurement of neopterin, which was released from dihydroneopterin triphosphate after oxidation and phosphatase treatment assay. NOS activity assays were performed by measuring L-[3H]-arginine to L-[3H]-citrulline conversion as described previously.12 To evaluate the effect of endogenous BH4 on NOS activity, no exogenous BH4 was added to the reaction mixture. Calcium-independent NOS activity was used as an index for iNOS activity in which calcium was chelated by an extracellular chelator EGTA (1 mMmolar/L) and an intracellular chelator 1,2-bis[2-aminophenoxy]ethane N,N,N’,N’-tetraacetic acid acetoxymethyl ester (20 μmol/L). Calcium-dependent NOS activity was used as an index for endothelial NOS (eNOS) plus neuronal NOS (nNOS) activity designated as constitutive NOS (cNOS) in which no chelation took place.

Western Blot Analyses of GTPCH I and NOS Protein and ONOO−

Brain samples were homogenized and subjected to electrophoresis on SDS-PAGE as we described.11 The blotted polyvinylidene difluoride membrane was incubated respectively with anti-iNOS (1:1000), anti-eNOS (1:500), anti-nNOS (1:1000; all from Transduction Laboratories), anti-rat GTPCH I (1:1000; a kind gift from Dr Christian Hesslinger, Pharmazentrum Frankfurt, J.W. Goethe-University, Frankfurt, Germany) and anti-nitrotyrosine antibody (1:1000; Upstate Biotechnology) for 2 hours and then incubated with a horseradish peroxidase–linked antibody (1:10000; Santa Cruz Biotechnology) for 40 minutes. The positive band was revealed using enhanced chemiluminescence Western blotting detection reagents and autoradiography film.

RT-PCR for GTPCH I and NOS

Total RNA isolation, reverse transcription, and competitive PCR were performed according to standard techniques.11,12 PCR was performed using the primer sequences as listed in the supplemental Table. GAPDH was used as a reference standard for eNOS and nNOS, whereas β-actin was used for iNOS and GTPCH I because of the fact that the GAPDH band was too close in proximity to the eNOS and GTPCH I bands, whereas β-actin band was too close to the eNOS and nNOS bands, respectively. NOS isoforms, GTPCH I, GAPDH, and β-actin were amplified as described.12

Measurement of Brain Superoxide

Measurement of superoxide was performed by lucigenin (5 μmol/L) enhanced chemiluminescence as well as in situ detection by superoxide-selective oxidative fluorescent dye dihydroethidium (DHE) as we described.11 DHE is freely permeable to cell membranes and fluoresces red when oxidized to ethidium bromide by superoxide. Unfixed frozen brain sections (30 μm) were placed on glass slides and submerged in 10−6 mol/L DHE (Sigma) in PBS buffer, pH 7.4, and incubated at 37°C for 30 minutes in a dark humidified container. Fluorescence in brain sections was then detected by a Zeiss 210 confocal microscope with a 590-nm long-pass filter. The intensity of the fluorescence was analyzed and quantified by densitometry (NIH Image software).

Statistics

Data were expressed as mean±SEM. Repeated-measures ANOVA was used for comparison of multiple values obtained from the same subject, whereas factorial ANOVA was used for comparison of data obtained from 2 independent samples of subjects. Bonferroni’s procedure was used to control type I error. Values of P<0.05 were considered statistically significant.

Results

Physiological Parameters

As shown in the Table, blood pressure and heart rate, as monitored from 15 minutes before MCAO procedure to 15 minutes after the procedure, were found to be not significantly different for the entire procedure. Regional CBF was reduced to ≈15% of the baseline levels (ie, 100%) as measured before MCAO.

Inhibition of GTPCH I Reduces Infarct Volume and BBB Permeability

MCAO caused reproducible infarcts involving the cerebral cortex and the striatum after 2 hours of ischemia and 24 hours of reperfusion as shown by the white-colored areas in Figure 1A. As shown in Figure 1B, DAHP pretreatment 12 hours
Before MCAO significantly decreased the infarct size compared with the nontreated group (22.3 ± 5.6 vs. 38.3 ± 7.4%; n = 6; P < 0.05). Vehicle (DMSO) treatment failed to decrease the infarct size (36.5 ± 6.9% vs. control group; n = 6; P > 0.05). Similarly, BBB permeability by Evans blue extravasations, as shown in Figure 2, was significantly reduced after DAHP pretreatment compared with either the control group (4.11 ± 0.22 vs. 7.78 ± 0.44 μg/g tissue; n = 5; P < 0.05) or the vehicle-treated group (7.28 ± 0.87 μg/g tissue vs. control group; n = 5; P > 0.05).

GTPCH Activity and BH₄ Levels Are Increased 24 Hours After Ischemic Reperfusion

GTPCH I mRNA expression in the ischemic region was significantly increased compared with the control side of the same brain (Figure 3A; n = 4 to 5; P < 0.05). DAHP or vehicle treatment had no effects on GTPCH I mRNA levels. Likewise, GTPCH I protein expression in the ischemic area was augmented significantly compared with the controls (Figure 3B; n = 4 to 5; P < 0.05), and DAHP or vehicle treatment had no effect on GTPCH I protein levels. In contrast, DAHP but not vehicle treatment significantly decreased GTPCH I enzymatic activity (Figure 3C) and BH₄ levels (Figure 3D) in normal brain tissues and ischemic brain regions (n = 4 to 5; P < 0.05). BH₄ levels in the ischemic area were significantly increased compared with the control (n = 4 to 5; P < 0.05). GTPCH I activity and BH₄ levels in DAHP-treated ischemic brains were not different compared with those of normal brains (n = 4 to 5; P > 0.05).

Inhibition of GTPCH I Reduces Increased Brain iNOS Activity and ONOO⁻ Levels

As shown in Figure 4A, ischemic reperfusion induced a substantial increase in iNOS activity, which was significantly attenuated by DAHP but not vehicle treatment (Figure 4A; n = 4 to 5; P < 0.01). In contrast to iNOS activity, total eNOS activities (eNOS plus nNOS activities) were significantly reduced 24 hours after ischemic reperfusion (Figure 4B; n = 4 to 5; P < 0.05) and were not affected by DAHP or vehicle treatment. However, the markedly increased levels of ONOO⁻, the product of NO and superoxide, were significantly inhibited by DAHP but not vehicle treatment in the ischemic tissues compared with the controls (Figure 4C; n = 4 to 5; P < 0.05).

eNOS, nNOS, and iNOS Expression and Superoxide Levels

Meanwhile, neither eNOS nor nNOS mRNA and protein expression in the infarct regions was altered compared with the controls, and neither DAHP nor vehicle treatment affected them (supplemental Figure IA, IB, ID, and IE; available online at http://stroke.ahajournals.org; n = 4 to 5; P > 0.05). In contrast, iNOS mRNA and protein expression were significantly increased in the ischemic area compared with the controls (supplemental Figure IC and IF; n = 4 to 5; P < 0.05), although neither DAHP nor vehicle treatment influenced such alterations. Although superoxide levels in ischemic regions were significantly higher compared with the controls as shown by DHE fluorescent confocal microscopy (supplemen-
Figure 2. A, Representative images of coronal brain sections with Evans blue extravasations. The blue-colored area represents the Evans blue extravasations. Bar=0.5 cm. B, Summary of BBB permeability as assessed by quantifying the amount of Evans blue in the brain. Results were expressed as micrograms per gram brain tissue. n=5 in each group; *P<0.05 vs other groups.

Discussion

The major new findings of the present study are: (1) GTPCH I activity, BH4 levels, iNOS activity, and ONOO\(^-\) levels were all increased 24 hours after cerebral ischemic reperfusion; and (2) in vivo inhibition of GTPCH I activity decreased its product BH4 levels, with a concomitant reduction of iNOS activity and ONOO\(^-\) levels, resulting in reduced infarct volume and cerebral edema. These results indicate that regulation of GTPCH I activity and BH4 synthesis is critically involved in iNOS activation during focal cerebral ischemia, which may provide a mechanistic basis of novel therapeutic strategies for ischemic stroke.

NOS Isoforms, Oxidative Stress, and Ischemic Stroke

All 3 NOS isoforms are expressed in the brain and require BH4 as an essential cofactor.\(^2,13\) These isoforms of NOS have been reported to play important but opposite roles in focal cerebral ischemia.\(^13\) Activation of iNOS or nNOS contributes to neuronal death attributable to oxidation of structural neuronal proteins during ischemic reperfusion.\(^1,2\) In contrast, NO produced by eNOS maintains the homeostasis of the cerebrovascular endothelium and preserves CBF to ischemic regions.\(^2,13\) Consistent with these principles, mice that are deficient of iNOS or nNOS genes develop smaller infarcts induced by MCAO, whereas eNOS knockout mice produce larger cerebral infarcts compared with their wild-type littermates.\(^13\) However, important differences exist regarding the time course of nNOS and iNOS activation in ischemic stroke. nNOS is acutely activated (ie, within a few hours) but short-lived after ischemic reperfusion, whereas the late iNOS activation peaks around 24 hours after reperfusion, when glial and inflammatory cells enter the infarct zone.\(^14,15\) In the present study, we found that nNOS and eNOS mRNA and protein expression are not different in the control and ischemic areas 24 hours after reperfusion, although their enzymatic activities were reduced in the ischemic region 24 hours after reperfusion. These data are consistent with previous studies in which the reduced cNOS (nNOS and eNOS) activity was thought to reflect the death of nNOS-containing neurons and eNOS-containing microvascular endothelium.\(^14,15\) In addition, the unaltered eNOS mRNA and protein expression profiles 24 hours after reperfusion were similar to previous studies.\(^16\) In contrast to cNOS activity, we found that there was a substantial increase of iNOS activity in the ischemic area, accompanied by increased iNOS mRNA and protein expression. Because it has been shown that: (1) iNOS activity is significantly enhanced at the 24-hour point, which starts to decline at the 48-hour point in rodent MCAO models; (2) the early but not late oxidative stress matches the process of infarct maturation,\(^14,15\) we chose the 24-hour time point to access the effect of GTPCH inhibition to ascertain that the observed protection is not artificial.

In addition to iNOS activation, production of reactive oxygen species such as the (O2\(^-\)) is also increased on ischemic reperfusion.\(^1,2\) Superoxide has been found to be generated after ischemic reperfusion, which is known to interact with NO to form ONOO\(^-\), resulting in neuronal loss during cerebral ischemia.\(^1,2\) Consistently, we found that (O2\(^-\)) and ONOO\(^-\) levels were significantly augmented in the infarct region.

BH4, iNOS, and Ischemic Stroke

BH4 is an essential cofactor for all 3 NOS isozymes, and a deficiency of BH4 results in decreased NOS enzymatic activity.\(^4,5\) Biosynthesis of BH4 is tightly regulated by its
rate-limiting enzyme GTPCH I. Although BH4 regulation of iNOS activity in vascular cells and peripheral tissues has been reported, little is known regarding the functional influence of BH4 on brain iNOS activity. The present study investigated the relative roles of GTPCH I and BH4 on iNOS regulation and cerebral infarction in a well-established model of focal cerebral ischemia. Our findings demonstrate, for the first time, that GTPCH I enzymatic activity and BH4 levels were significantly elevated 24 hours after ischemic reperfusion, which correlated to the increased iNOS enzymatic activity that has been reported to reach its peak within the same time frame. Furthermore, in vivo inhibition of GTPCH I by its selective inhibitor DAHP resulted in reduced BH4 levels and a marked suppression of iNOS enzymatic activity. Previous studies established the selectivity of DAPPH on GTPCH I activity because of its ability to compete for binding with substrate GTP as well as to augment GTPCH I feedback regulatory protein. Consistently, the present study revealed that DAHP treatment did not alter GTPCH I mRNA and protein expressions, although both were increased in the ischemic brain. Because DAHP pretreatment 12 hours before MCAO did not reduce the elevated iNOS mRNA and protein expressions, its inhibition of iNOS enzymatic activity is most likely attributable to the blockade of de novo BH4 biosynthesis. Most important, the findings of the present study demonstrate that in vivo GTPCH I inhibition attenuates cerebral injury manifested in reduced infarct size and cerebral edema. Previous studies have shown that GTPCH I and iNOS are expressed in neural and glial tissues. In the present study, our findings indicate that the expression of both enzymes is enhanced concomitantly on cerebral ischemia, suggesting that increased GTPCH I is required to coordinate the need of excessive iNOS activity. Together, these experimental observations strongly suggest that adequate BH4 synthesis from its de novo pathway governed by GTPCH I is required for iNOS activation in focal cerebral ischemia.
Because GTPCH I is the rate-limiting enzyme for BH4 synthesis, which is required for cNOS and iNOS activity, inhibition of GTPCH I may inevitably affect both as well. However, our findings showed that focal ischemia already reduced cNOS activity significantly (Figure 4B), which was consistent with previous findings. These data suggest that the single-dose regimen of intraperitoneal DAHP used did not further worsen the situation on cNOS. Because DAHP treatment significantly reduced the infarct volume, the approach targeting the focal ischemic, but not normal tissue, may still be valid aimed at the overwhelming late iNOS activation and the resultant oxidative stress–induced neuronal injury (eg, from ONOO⁻). Finally, a single intraperitoneal DAHP treatment did not significantly change the mean arterial blood pressure (Table).

**Possible Mechanism of Augmented GTPCH I Activity and BH₄ Levels in Ischemic Stroke**

As the committing and rate-limiting enzyme for BH₄ synthesis, GTPCH I is known to be subjected to direct regulation at transcriptional and post-transcriptional levels. Proinflammatory cytokines such as interleukin-1β (IL-1β), interferon-γ, and tumor necrosis factor-α (TNF-α) have been reported to increase GTPCH I transcription, enzyme activity, and BH₄ levels in various cell types including neuronal cells. Available evidence also indicates that focal ischemia activates IL-1β and TNF-α gene expression that contribute to iNOS activation via necrosis factor κB, resulting in postischemic intracerebral inflammatory response and neurological damage. Based on the above experimental observations, we speculate that the observed GTPCH I activation and resultant de novo BH₄ synthesis in cerebral ischemia may represent a common link through which proinflammatory cytokines activate iNOS for massive NO production (Figure 5). Future studies are warranted to ascertain a direct causative relationship between proinflammatory cytokine signaling and GTPCH I upregulation in ischemic brain injury.

In conclusion, the present study demonstrates that GTPCH I activity, BH₄ levels, iNOS activity, and ONOO⁻ levels are increased 24 hours after cerebral ischemic reperfusion. In vivo inhibition of GTPCH I activity by DAHP decreases BH₄ levels, iNOS activity, and ONOO⁻ levels, resulting in reduced neuronal damage. We speculate that the observed GTPCH I activation and resultant de novo BH₄ synthesis in cerebral ischemia may represent a common link through which proinflammatory cytokines activate iNOS for massive NO production in ischemic stroke.
increased 24 hours after cerebral ischemic reperfusion, and in vivo inhibition of GTPCH I activity decreased BH$_4$ levels, iNOS activity, and ONOO$^-$ levels, resulting in decreased cerebral infarct and cerebral edema. These findings may provide a mechanistic basis for new therapeutic strategies aimed at regulating GTPCH I expression and BH$_4$ synthesis for preventing against iNOS-induced oxidative stress in ischemic stroke.

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