Neuroprotection and P450 2C11 Upregulation After Experimental Transient Ischemic Attack

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Background and Purpose—Transient ischemic attack (TIA) is a risk factor for stroke. However, TIA may also serve as a preconditioning stimulus, reducing damage from subsequent stroke. We tested the hypothesis that experimental TIA induces expression of P450 2C11, an arachidonic acid epoxygenase that produces vasodilator epoxyeicosatrienoic acids, leading to increased tissue perfusion and reduced stroke damage.

Methods—Wistar rats underwent three 10-minute middle cerebral artery occlusions (TIA) or sham surgery. Three days later, animals were subjected to 2-hour middle cerebral artery occlusion and 24 hours of reperfusion. Brains were stained with 2,3,5-triphenyltetrazolium chloride for infarct size measurement or processed for quantification of P450 2C11 mRNA and protein with the use of RNase protection assay and Western blotting. Regional cerebral blood flow (CBF) at the end of 2-hour ischemia was measured in separate groups of rats with iodoantipyrine autoradiography.

Results—Cerebral infarct was reduced by >50% in TIA- versus sham-preconditioned animals. 2C11 mRNA and protein were increased in ipsilateral hemisphere by 3 days after TIA but not sham surgery. Induction of 2C11 by TIA was also evident in ipsilateral hemisphere at 24 hours after 2-hour middle cerebral artery occlusion and 24 hours of reperfusion. End-ischemic regional CBF was not different between TIA- and sham-preconditioned groups.

Conclusions—We conclude that experimental TIA induces ischemic tolerance by a mechanism temporally linked to upregulation of P450 2C11. Enzyme induction does not attenuate ischemic severity by amplifying end-ischemic CBF.

(Stroke. 2002;33:1677-1684.)

Key Words: cerebral ischemia, transient ■ gene expression ■ neuroprotection ■ stroke, experimental ■ rats

Transient ischemic attack (TIA) has long been identified as a risk factor for stroke. However, recent clinical evidence suggests that TIA may improve stroke outcome1,2 by serving as a preconditioning stimulus and triggering neuroprotective mechanisms.3,4 Distinction between the role of TIA as risk factor or protectant is difficult in humans because TIA is a clinical diagnosis of a range of cerebrovascular events that vary in duration, location, severity, and number.5,6 TIA frequently precedes severe ischemia, the impact of which may mask any defense mechanisms induced by prior events. Therefore, vascular or neuronal mechanisms of TIA-induced protection remain illusive. Numerous animal models have been described in which brief episodes of global or focal reduction of cerebral blood flow (CBF) are applied, followed by a more severe ischemic stress designed to kill neurons. Two phases of protection have been documented in experimental models of TIA: an early phase occurring within minutes7 and a delayed phase8–11 occurring within days after preconditioning ischemia, or TIA. The early phase of preconditioning is attributed to flow-mediated mechanisms or changes in cellular metabolism.7 Acquisition of delayed tolerance to ischemia represents an adaptive response to stress12 and a shift in brain to a more protected phenotype.13 Late-phase preconditioning has been the subject of intense investigations, but its precise mechanisms remain unknown. It is now recognized that late-phase preconditioning is a multistep process with one set of mechanisms triggered by the preconditioning stimulus to initiate the phenotypic changes (initiators) and other mechanisms mediating the protective phenotype (effectors).13

In this study we examine the expression profile of P450 2C11 arachidonic acid (AA) epoxygenase after focal ischemic preconditioning and its relation to acquired tolerance to cerebral ischemia. We used an animal model to simulate the clinical situation whereby brief transient focal ischemia, such as occurs during TIA, precedes a more severe ischemic stroke. We examined whether TIA is neuroprotective and how the protection relates to the expression of 1 member of the cytochrome P450 gene family, P450 2C11, which has recently been implicated as an important signaling mechanism in brain and cerebral blood vessels.14–16 Cytochrome P450 2C11 metabolizes AA to epoxyeicosatrienoic acids.
Experimental TIA

Transient focal cerebral ischemia was achieved by modifying a previously described technique for intraluminal MCA occlusion.\textsuperscript{5,23,24} Briefly, rats were anesthetized with halothane and instrumented with a femoral artery catheter and a small probe for monitoring laser-Doppler perfusion (LDP) over ipsilateral parietal cortex.\textsuperscript{23,24} A 4.0 nylon surgical suture was inserted through the external carotid artery and advanced into the internal carotid artery until LDP dropped. At the end of each of three 10-minute occlusions, the filament was retracted from the internal carotid artery, and flow was restored through the common carotid artery, which was associated with rapid restoration of LDP. At the end of the first and second 10-minute occlusions, the rat remained under anesthesia for 45 minutes. At the end of the third occlusion, the filament was withdrawn, and the rat recovered and was observed for 1, 2, or 3 days. Sham-operated animals underwent the same surgical procedure, anesthesia, and instrumentation as preconditioned animals. The filament was inserted and advanced for a distance of 5 mm beyond the internal carotid artery/external carotid artery bifurcation. The filament was then fixed in place, and LDP, temperature, blood pressure, and gases were monitored for 2 hours. Naïve, control animals were cohoused but received no intervention.

Experimental Stroke Procedure

Three days after TIA or sham procedure, some animals received 2-hour MCA occlusion, as previously described.\textsuperscript{23,24} Animals were reanesthetized and instrumented for LDP and physiological monitoring as described above. The right common carotid artery was reexposed, and an occluding filament was inserted through a small incision into the internal carotid artery until LDP dropped. The filament was then fixed in place and was withdrawn after 2 hours to initiate reperfusion. After 22 hours of reperfusion, the rat was decapitated under deep halothane, and the brain was removed and sliced into seven 2-mm-thick coronal sections for TTC staining. Infarcted areas within cortex and striatum were measured in slices and integrated across the hemisphere to calculate infarct volume.

RNase Protection Assay

P450 2C11 mRNA was measured with the use of both indirect and direct RPA. Indirect RPA was performed on RNA samples isolated from whole hemispheres at 22 hours of reperfusion (poststroke expression), as previously described.\textsuperscript{25} Direct RPA was performed on tissue samples microdissected from 8 distinct brain regions (dorsomedial and dorsolateral somatosensory cortex and dorsolateral and ventromedial striatum of ipsilateral and contralateral hemispheres), as previously described.\textsuperscript{25} A rat 2C11 cDNA fragment (296 bp long, between 873 and 1169 bp of 2C11 cDNA sequence)\textsuperscript{14} was amplified by polymerase chain reaction with the use of the following primers: 5'GAAA GCTT GGTG GCTA CTG-3' (sense) and 5'-CTTG GGAA TGAA GTA-3' (antisense). The T7 phage polymerase promoter sequence (5'TAAT ACGA CTCA CTAT AGGG-3') was added to the 5' end of the antisense primer to allow for in vitro transcription of 2C11 antisense RNA from the polymerase chain reaction product. Radiolabeled 2C11 antisense riboprobe was transcribed to a specific activity of approximately 10\textsuperscript{8} cpm/μg. Total RNA for indirect RPA was prepared separately from ischemic and contralateral hemispheres of individual animals with the use of TRIzol (Gibco BRL). Micropunches representing 1 region were pooled from 3 to 4 animals, homogenized in lysis solution for direct RPA (Ambion), and incubated with excess bcl-2 and β-actin probes overnight. Samples were digested with RNase and resolved via electrophoresis on a denaturing polyacrylamide gel. Protected fragments were visualized on a PhosphorImager, and bands were quantified with the use of ImageQuant software (Molecular Dynamics). Optical density of 2C11 and β-actin bands was sampled with the use of a rectangle with predetermined dimensions based on the band with highest intensity. Mean 2C11/β-actin ratio of sham animals was considered 100%, and ratios of all other animals were expressed as a percentage of sham.

Western Blotting

Microsomal proteins were prepared from whole hemispheres or from middle coronal slices (at the level of caudate between 0 and −2 mm relative to bregma), as previously described.\textsuperscript{14,15} Brain tissue was homogenized in 250 mmol/L sucrose, 10 mmol/L KPO\textsubscript{4}, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride (pH 7.7) in the presence of protease inhibitors (2 mmol/L EDTA, 10 mmol/L KPO\textsubscript{4}, 1 mmol/L EDTA, 0.1 mmol/L dithiothreitol, and 30% glycerol (pH 7.25). The homogenate was centrifuged at 10 000 g for 15 minutes at 4°C, and the supernatant was further centrifuged at 100 000 g for 60 minutes at 4°C. The protein concentration was measured by BCA protein assay (Pierce). Samples of 100 μg protein were then boiled in Laemmli buffer for 5 minutes, electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels, and blotted onto polyvinylidene fluoride membrane (Bio-Rad). Blots were blocked with 5% nonfat dry milk in 0.05 mol/L Tris-buffered saline/0.05% Tween for 2 hours at room temperature, then incubated with rabbit anti-rat 2C11 polyclonal antibody\textsuperscript{14} (1:2500, overnight at 4°C), followed by incubation with biotinylated goat anti-rabbit IgG.
and horseradish peroxidase (1:5000, 1 hour at room temperature). Blots were incubated with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham) for 1 minute, and signals were visualized on Hyperfilm ECL after exposure for 0.5 to 3.0 minutes. Films were photographed with a digital camera, and optical density of 2C11 bands was measured with the use of a rectangular sampling tool (Inquiry, Loats) based on the size of the band with highest intensity. The same rectangle was then used to measure background density in adjacent areas within the same lane, which was subsequently subtracted from corresponding 2C11 bands and expressed as a percentage of sham animals.

**CBF Measurement Using IAP Autoradiography**

Regional CBF was measured at the end of 2-hour MCA occlusion in TIA- and sham-preconditioned rats with the use of quantitative [14C]IAP autoradiography, as described previously. Twenty microcuries of [14C]IAP (New England Nuclear) was infused intravenously, and 20-μL samples of arterial blood were collected over 45 seconds. The rat was decapitated, and the brain was quickly frozen and cryosectioned into 20-μm-thick coronal sections. Sections were exposed to film for 1 week with 14C standard, and the concentration of 14C in blood samples was measured by liquid scintillation spectroscopy. Images representing coronal levels 0, −2, and −4 mm relative to bregma were digitized, and regional CBF in cortex and striatum was determined with the use of image analysis software (Inquiry, Loats), as previously described. Additionally, cumulative distribution of CBF rates within the ischemic hemisphere was determined in TIA- versus sham-pretreated groups, as previously described.

**Statistical Analysis**

Direct RPA values represent 3 to 4 pooled samples. All other values are mean±SEM. Physiological variables, LDP, and time course of 2C11 protein induction were subjected to 2-way ANOVA. Differences in infarct size, regional CBF, and 2C11 expression after stroke were determined by 1-way ANOVA. Post hoc comparisons were made with Newman-Keuls test. The criterion for statistical significance is \( P<0.05 \).

**Results**

Physiological variables measured before and during 2-hour stroke did not differ among groups (Table). Figure 1 traces LDP changes during TIA or sham surgery. LDP was reduced by 70% during TIA but remained within 97±12% of baseline during sham surgery. During 2-hour stroke procedure, LDP was equivalently reduced in sham- and TIA-pretreated rats to 34±5% and 34±4% and returned on reperfusion to 109±6% and 92±10% of baseline, respectively.

**Effect of TIA on Infarct Size**

Cortical and striatal infarcts were reduced by 50% to 60% in TIA-pretreated compared with sham-pretreated rats (Figure 2; \( P<0.05 \)). There were no significant differences in infarct size.
Effect of TIA on P450 2C11 Expression
The time course of 2C11 mRNA induction after TIA in 4 regions within the MCA territory is summarized in Figure 3. Induction of 2C11 mRNA was apparent by day 2 in TIA versus sham groups in medial cortex and throughout striatum. By day 3, mRNA levels were 2 to 3 times higher in ipsilateral cortex and striatum in TIA versus sham groups. Contralateral expression was unchanged by TIA at all time points (data not shown). Increased levels of 2C11 mRNA in TIA versus sham animals persisted after the stroke procedure. Figure 4 demonstrates representative RPA blots illustrating poststroke 2C11 and β-actin RNA expression in ischemic and contralateral hemispheres of sham- and TIA-preconditioned brains. 2C11 mRNA was quantified at 24 hours after 2-hour MCA occlusion with the use of indirect RNase protection assay and normalized to β-actin. 2C11 mRNA after stroke was 2-fold higher in ischemic hemisphere of TIA- vs sham-preconditioned animals. No difference in 2C11 mRNA was observed between the 2 groups in contralateral hemisphere.

Effect of TIA on Regional CBF
End-ischemic regional CBF was not different between TIA- and sham-pretreated animals. Mean CBF rates at the end of 2-hour ischemia were 53±13 and 41±9 mL/100 g per minute in the cerebral cortex and striatum of TIA-pretreated rats compared with 62±13 and 33±7 mL/100 g per minute in sham animals. No differences in CBF rates were observed in the contralateral hemisphere between the 2 groups. Furthermore, cumulative distribution analysis of CBF rates within ischemic hemisphere revealed no differences in the amount of tissue (mm³) perfused with any given flow rate (mL/100 g per minute) between TIA- and sham-pretreated groups (Figure 7).

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Discussion

We demonstrated that prior exposure to TIA decreases infarct size in experimental stroke. The protection coincided with upregulation of brain P450 2C11 AA epoxygenase, which does not alter severity of the ischemic insult. These findings suggest a protective role for EETs, the products of AA epoxygenase, against cerebral ischemia.

We found that three 10-minute periods of MCA occlusion provide protection against lethal ischemia consisting of 2 hours of MCA occlusion within 3 days, which is consistent with previous reports of preconditioning in focal cerebral ischemia. In our study this preconditioning paradigm does not cause tissue injury as identified by TTC. However, in previous studies, more subtle signs of tissue injury, such as selective neuronal necrosis, were identified at various time points after TIA.

The mechanism of TIA-acquired ischemic tolerance in brain is unknown. Acute preconditioning results from rapid posttranslational modifications of existing proteins, while delayed tolerance involves gene induction and de novo protein synthesis. One highly inducible group of proteins involved in cellular adaptation to stress is that of the cytochrome P450 enzymes. During cerebral ischemia, AA is released from membranes, which can then be metabolized via 3 enzymatic pathways: cyclooxygenase, lipoxygenase, or cytochrome P450 pathways. We previously showed that rat brain astrocytes metabolize AA via the P450 pathway to...
EETs, which play an important role in coupling neuronal activity to regional blood flow. We have also demonstrated that astrocytes express cytochrome P450 2C11, a P450 isomorph with AA epoxygenase activity, which is inducible by glutamate and is an important contributor to the epoxygenase activity of brain. Recent reports suggested that EETs are protective against myocardial ischemia. Thus, we examined whether TIA induces expression of P450 2C11 in brain and whether the temporal profile of 2C11 induction in brain coincides with neuroprotection observed in this model.

We measured 2C11 mRNA and protein on days 1, 2, and 3 after TIA or sham operation and at 24 hours after stroke. We found that the level of 2C11 mRNA in TIA-pretreated animals on day 1 was less than that in sham-operated animals, especially in the striatum. P450 2C11 mRNA expression is known to be suppressed by inflammatory mediators. Thus, the relative attenuation of 2C11 mRNA expression on day 1 may reflect a more severe insult and inflammatory response on day 1 after TIA. By day 2 after TIA, 2C11 mRNA recovers to equal or exceed its level of expression in sham-operated animals. By day 3, 2C11 mRNA levels were 2 to 3 times higher in TIA- versus sham-preconditioned animals. In agreement with the changes observed at the mRNA level, 2C11 protein was 2-fold higher in preconditioned compared with sham animals in ischemic, but not contralateral, hemisphere. The observed upregulation of 2C11 beginning on day 2 is in agreement with the finding of Chen et al that a similar preconditioning paradigm confers protection against MCA occlusion on days 2 and 3 but not on day 1. We also measured 2C11 expression 24 hours after stroke onset in TIA-pretreated and sham animals. Measurements performed on total RNA and tissue homogenates demonstrated that the 2-fold increase in 2C11 mRNA and protein expression in ipsilateral hemisphere was maintained during the immediate postischemic period critical to infarct maturation. The 2-fold upregulation of 2C11 at the mRNA and protein levels at this time point is important because this is when infarct was measured and protection was observed. These findings demonstrate that 2C11 upregulation precedes and correlates spatially and temporally with tissue protection from stroke but does not prove that 2C11 upregulation and neuroprotection are mechanistically linked.

We then examined whether TIA preconditioning decreases infarct size by attenuating severity of the ischemic insult. Our previous work has identified EETs as endothelium-dependent hyperpolarizing factors and mediators of functional hyperemia in brain. Furthermore, EETs are vasodilators in the cerebral circulation, and promote capillary angiogenesis. Therefore, we tested the hypothesis that 2C11 upregulation by TIA is associated with higher tissue perfusion during ischemia, attenuating ischemic severity, and accounting for the observed reduction in infarct size. Measurement of LDP on MCA occlusion on day 3 demonstrated that the relative change from baseline in LDP was similar between the 2 groups. However, because the relationship between LDP and true blood flow is unknown and because LDP measures only surface tissue perfusion, we also quantified absolute blood flow rates within MCA territory using quantitative IAP autoradiography. Measurement of regional CBF by this technique at end ischemia indicated that ischemic severity was equivalent between the sham and TIA groups despite the striking difference in infarct size, suggesting that ischemic tolerance in our model is not mediated via a blood flow–enhancing mechanism acting during vascular occlusion. This is in agreement with most studies demonstrating no difference in CBF within protected regions before or during stroke between preconditioned and sham-operated animals. In contrast, Matsushima and Hakim observed higher CBF during stroke in preconditioned versus sham animals; however, the increase in CBF in their study was not associated with a reduction in infarct size. In summary, evidence from our study with the use of LDP and autoradiography corroborates studies by others to support the conclusion that TIA preconditioning does not alter the severity of ischemia. Our study did not address the question of whether TIA preconditioning improves regional CBF during the reperfusion period.

In addition to vasodilation, EETs have other properties that can potentially mediate their neuroprotective effect. These include the antioxidant, anti-inflammatory, and antipyretic properties of EETs. Furthermore, EETs regulate intracellular calcium concentration, inhibit platelet aggregation, and prevent leukocyte adhesion to vascular wall. A related P450 metabolite, (R)-hydroxyeicosatetraenoic acid [16(R)-HETE], suppresses human leukocyte activation and reduces intracranial pressure in a rabbit model of thromboembolic stroke. These mechanisms may be important factors in how upregulation of 2C11 reduces stroke damage.

Several other mechanisms have been proposed to mediate the protection associated with late-phase preconditioning in brain. Tolerance to ischemia is associated with higher expression of neuroprotective genes such as heat shock proteins, superoxide dismutase, and bel-2. Ischemic tolerance has also been induced by the cytokines interleukin-1 and tumor necrosis factor-α (TNF-α) and has been linked to greater ability to buffer cytosolic calcium loads, enhanced ceramide synthesis, and higher capacity to neutralize reactive oxygen species. Several signaling pathways have been shown to be activated by ischemic preconditioning, including the N-methyl-D-aspartate/nitric oxide/p38/ERK/extracellular regulated kinase, the adenosine A1 receptor/protein kinase C, and tumor necrosis factor-α receptor signaling pathways. It is possible that EETs may exert protective effects via some of these mechanisms. For example, EETs may induce bel-2 or other cAMP-responsive genes with neuroprotective properties via their action on cAMP. EETs may also exert neuroprotective effects via their mitogenic actions, such as their role in mediating epidermal growth factor signaling. Furthermore, EETs may mediate the preconditioning effects by regulating intracellular calcium concentrations. EETs also antagonize TNF-α effects, and both TNF-α and interleukin-1β downregulate 2C11 expression, likely via the sphingomyelin/ceramide pathway. Finally, EETs activate ATP-sensitive K⁺ channels in rat cardiac ventricular myocytes.

The clinical implication of our findings is limited by the fact that these studies were conducted in young healthy...
animals and that they measured only short-term outcome. Because stroke and TIA mostly affect the aging population, it is important to determine whether experimental TIA remains effective in eliciting protection in aged animals with underlying vascular disease and whether the protection is long lasting. In heart, some studies demonstrated loss of preconditioning effect with aging, while other studies demonstrated that preconditioning is well preserved in senescent myocardium. One explanation for the discrepancy is that studies that showed loss of preconditioning effect with aging have mostly been conducted on isolated buffer-perfused rat heart. Furthermore, these studies demonstrated loss of early-phase preconditioning, which is not surprising given age-related changes in the vasculature. It is yet to be determined whether late-phase preconditioning in heart or brain is lost with advanced age. In brain, global ischemic preconditioning was more effective in aged versus young gerbils. In elderly humans, antecedent (preinfarction) angina, which presumably is a preconditioning stimulus, has been reported to exert a beneficial effect on outcomes from myocardial infarction in some but not other studies. Finally, diabetes mellitus in humans and hyperglycemia in animals prevented ischemic preconditioning in heart. Interestingly, diabetes also suppresses 2C11 expression, but it is not clear yet whether 2C11 suppression by chronic hyperglycemia alters the preconditioning effect.

In summary, we described a model of experimental TIA and demonstrated its effect to reduce subsequent stroke damage. The protection against stroke was temporally and spatially linked to upregulation of P450 2C11 epoxygenase without affecting ischemic stroke severity. These results suggest that EETs may serve as endogenous neuroprotectants in brain, which are induced by TIA and can be targeted as a novel strategy for ischemic brain injury prevention and treatment. EET levels in brain can be enhanced by increasing their synthesis or by decreasing their breakdown. EET synthesis can be enhanced by inducing expression of P450 2C11 epoxygenase pharmacologically with the use of β-naphthalone. P450 2C11 has also been introduced into cultured cells, such as astrocytes, by viral-mediated gene transfer. Alternatively, endogenous EET levels can be enhanced by inhibiting their breakdown. EETs are inactivated by hydration into dihydroeicosatrienoic acids by the epoxide hydrolase.

Acknowledgments

This study was funded by grants NS33668 and NS20020 and Veterans Affairs merit review grant 3440-06P.

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Stroke. 2002;33:1677-1684
doi: 10.1161/01.STR.0000016332.37292.59

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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