Production of 20-HETE and Its Role in Autoregulation of Cerebral Blood Flow


Abstract—In the brain, pressure-induced myogenic constriction of cerebral arteriolar muscle contributes to autoregulation of cerebral blood flow (CBF). This study examined the role of 20-HETE in autoregulation of CBF in anesthetized rats. The expression of P-450 4A protein and mRNA was localized in isolated cerebral arteriolar muscle of rat by immunocytochemistry and in situ hybridization. The results of reverse transcriptase–polymerase chain reaction studies revealed that rat cerebral microvessels express cytochrome P-450 4A1, 4A2, 4A3, and 4A8 isoforms, some of which catalyze the formation of 20-HETE from arachidonic acid. Cerebral arterial microsomes incubated with [14 C]arachidonic acid produced 20-HETE. An elevation in transmural pressure from 20 to 140 mm Hg increased 20-HETE concentration by 6-fold in cerebral arteries as measured by gas chromatography/mass spectrometry. In vivo, inhibition of vascular 20-HETE formation with N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), or its vasoconstrictor actions using 15-HETE or 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20-HEDE), attenuated autoregulation of CBF to elevations of arterial pressure. In vitro application of DDMS, 15-HETE, or 20-HEDE eliminated pressure-induced constriction of rat middle cerebral arteries, and 20-HEDE and 15-HETE blocked the vasoconstriction action of 20-HETE. Taken together, these data suggest an important role for 20-HETE in the autoregulation of CBF. (Circ Res. 2000;87:60-65.)

Key Words: cerebral blood flow ■ homeostasis ■ HETE ■ cytochrome P-450 ■ arachidonic acid

Blood flow in the brain is normally maintained within narrow limits despite increases in vascular perfusion pressures caused by active reductions in arteriolar diameter. This pressure-induced vasoconstriction, known as the myogenic response, has been intensely investigated since its first description by Bayliss nearly a century ago. The nature of the cellular mechanisms involved have yet to be defined. Vasoconstriction in response to increased intravascular pressure is mediated by changes in the activation state of K+ and/or Ca2+ channels resulting in depolarization of vascular smooth muscle (VSM) and an influx of calcium. Activation of phospholipases and protein kinase C (PKC) have also been correlated with the development of myogenic tone, implicating lipid mediators such as diacylglycerol and arachidonic acid (AA) in this response. Prior studies have also suggested an important role for cytochrome P-450 metabolites of AA in the pressure-induced arterial constriction of cerebral and renal arteries in vitro. The P-450 metabolite of AA, 20-HETE, is a potent vasoconstrictor, activates PKC and depolarizes VSM by inhibiting the large-conductance KCa channel, and increases Ca2+ influx via L-type Ca2+ channels. Given that the effects of 20-HETE on ion channels, membrane potential, and PKC mimic those involved in the pressure-induced myogenic response, we hypothesize that elevations in transmural pressure increase the concentration of 20-HETE in VSM cells, which enhances myogenic constriction of cerebral arterioles. This pressure-induced constriction of cerebral arteries then plays a critical role in autoregulation of cerebral blood flow (CBF) during elevations in arterial pressure.

Materials and Methods

Immunohistochemistry

Cryosectioned (10 to 20 μm) rat brain sample slides were incubated with a polyclonal antibody to P-450 4A enzyme. The slides were washed with PBS and incubated with a secondary antibody conjugated to horseradish peroxidase, and P-450 4A immunoreactivity was detected by covering the slides with a 3% solution of H2O2 followed by diaminobenzidine solution for 1 hour.

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In Situ Hybridization
A P-450 4A2 cDNA cloned\(^9\) from rat kidney was linearized for in vitro transcription of sense or antisense cRNA. Brain sections were probed with the labeled cRNA probe, blocked, and incubated with alkaline phosphatase–conjugated anti-fluorescein monomolecular antibody (Amersham). Other sections were stained with Cy3-conjugated anti–smooth muscle \(\alpha\)-actin monomolecular antibody simultaneously with treatment with the anti-fluorescein antibody and counterstained with 1% fast green FCF (Fischer).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)
Poly A\(^{+}\) mRNA was extracted from rat cerebral microvessels reverse transcribed using poly T primers and amplified by RT-PCR using forward and reverse primers specific for cytochrome P-450 4A1, 4A2, 4A3, and GAPDH\(^9\) having the following sequences: 4A1 forward, 5\(^\prime\)-CTCTTTACCTTGCCAGAATGGA\(_3\); 4A1 reverse, 5\(^\prime\)-GACTTGGATACCTTGGGTAAAG-3\(^\prime\); 4A2 forward, 5\(^\prime\)-AGATCCAAAAGCCTTATCACA\(_3\); 4A2 reverse, 5\(^\prime\)-CAGCCTTGGTGTTAGGACCT-3\(^\prime\); 4A3 forward, 5\(^\prime\)-CAAGGCCTCTGGAAATTATC-3\(^\prime\); 4A3 reverse, 5\(^\prime\)-CAGCCTTGGTGTTAGGACCT-3\(^\prime\); 4A8 forward, 5\(^\prime\)-ATCACGAGGTGTTGGACCTTAT-3\(^\prime\); 4A8 reverse, 5\(^\prime\)-AATGAGATGTGAGCAGATGGAGT-3\(^\prime\); GAPDH forward, 5\(^\prime\)-CCCCTTCATTGACCTCAACTA; and GAPDH reverse, 5\(^\prime\)-ATGACGTGGTGAATTCTGAG-3\(^\prime\). The specificity of these primer pairs was tested by amplifying each against 10 ng of the full-length P-450 4A1, 4A2, 4A3, and 4A8 cDNAs we have previously cloned. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

Cloning and Sequencing of PCR Products
The P-450 4A1, 4A2, 4A3, and 4A8 PCR products were isolated, ligated into a PCR-2.1 vector (Invitrogen), and used to transfect INVraf competent cells. Plasmid DNA was isolated and sequenced using the Thermo Sequence dye termination cycle sequencing kit (Amersham) and a Research Biochemical International (RBI; model 377) sequencer (Applied Biosystems).

Assays of P-450 450 Metabolism of AA
Microsomes prepared from bulk isolated cerebral microvessels\(^9\)–22 were incubated with \([\text{H}]\)AA in the absence or presence of the inhibitor of 20-HETE production N-methylsulfonil-12,12-dibromododec-11-enamide (DDMS) (50 \(\mu\)mol/L). Reaction products were subjected to pentafluorobenzyl-ester derivatization (window25,26) in combination with subdural or intracerebroventricular (ICV) infusion of agents that inhibit the formation or the action of 20-HETE. In 10 rats, autoregulation of CBF was measured during the control period and after ICV infusion of DDMS (50 \(\mu\)mol/L, 1 \(\mu\)L/min), subdural infusion of 15-HETE (1 \(\mu\)mol/L, 2 \(\mu\)L/min), or ICV infusion of 20-HETE (1 \(\mu\)mol/L, 2 \(\mu\)L/min).

Statistical Analysis
Data are presented as mean±SEM. The difference in mean values was determined by 1-way ANOVA with repeated measures, followed by a Tukey least-significant difference post hoc test. Paired and unpaired \(t\) tests were used where required. \(P<0.05\) was considered statistically significant.

Drugs and Chemicals
All chemicals were of analytical grade, except where indicated, and were obtained from Sigma. 15-HETE was purchased from BIOMOL. DDMS and 20-HETE were synthesized by J.R.F.

Results
Localization of P-450 4A Isoforms in the Cerebral Vasculature
A polyclonal antibody that cross-reacts with rat P-450 4A1, 4A2, and 4A3 isoforms was used to probe sections of rat cerebral microvessels. Preincubation of the antibody with the corresponding antigenic peptide eliminated the staining. The staining was localized in the smooth muscle layers of the cerebral arteries, and demonstrated that there is very little nonspecific binding. Preimmune serum presented the staining pattern obtained with preimmune serum.

Localization of P-450 4A Isoforms in the Cerebral Vasculature
A polyclonal antibody that cross-reacts with rat P-450 4A1, 4A2, and 4A3 isoforms was used to probe sections of rat brain for the presence of immunoreactive protein. Figure 1A presents the staining pattern obtained with preimmune serum and demonstrates that there is very little nonspecific binding. Figure 1B indicates that there is intense staining for P-450 4A protein in the cerebral microvasculature.

To confirm these results, cRNA probes corresponding to P-450 4A2 were used to localize P-450 4A mRNA in sections of rat brain using in situ hybridization. Sections of the brain were probed with P-450 sense and antisense cRNA probes and stained with antibody against vascular smooth muscle.
actin. The antisense cRNA probes hybridized to the wall of cerebral arteries (Figure 1D) and colocalized with the pattern of the actin staining (Figures 1C and 1D). No detectable signal was observed in rat brain sections hybridized with sense cRNA probes.

The high degree of homology between the P-450 4A isoforms suggests that the antibody and cRNA probes used in the in situ hybridization and immunohistochemical localization studies likely cross-react with all members of the P-450 4A family. Therefore, RT-PCR was used to specifically identify the P-450 4A isoforms expressed in rat cerebral microvessels. The results presented in Figure 2 demonstrate that comigrates with 20-HETE standard.20 Previous GC/MS analysis confirmed that this peak is 20-HETE.20 Addition of 50 μmol/L DDMS to the incubation blocked the formation of 20-HETE by cerebral arterial microsomes (Figure 3C). These results indicate that rat cerebral microvessels synthesize 20-HETE, the formation of which is inhibited by DDMS.

**Effect of Transmural Pressure on Vascular 20-HETE Concentration**

To determine whether elevation in transmural pressure increases 20-HETE concentration, we measured 20-HETE levels by GC/MS analysis in pressurized cerebral arteries. Negative ion chemical ionization GC/MS analysis revealed the presence of a major ion with a mass-to-charge ratio of 393 for the internal standard [2H2]20-HETE and 391 for the biological sample extracted from the pressurized cerebral vessels, confirming the presence of 20-HETE. As depicted in Figure 4C, an increase in intravascular pressure from 20 to 140 mm Hg produced a 6-fold increase in 20-HETE concentration in cerebral arteries (n=5 vessels, *P<0.01).

**Effects of DDMS and Antagonists of 20-HETE on the Pressure-Induced Constriction of Isolated Cerebral Arteries**

The effect of step increases in transmural pressure from 20 to 160 mm Hg on the diameter and active tension of cerebral arterial segments was determined in the presence and absence of the cytochrome P-450 inhibitor DDMS or the 20-HETE antagonists, 15-HETE and 20-HEDE.24 Under control conditions, increases in transmural pressure reduced arteriolar diameter by 48±5% and 53±6% (n=12) at 140 and 160 mm Hg, respectively (Figure 4A). Pretreatment of the vessels with DDMS (10 μmol/L) for 15 minutes blocked the pressure-induced constriction and increased diameter to a maximum of 23±5% and 24±6% (n=5) above control at 140 and 160 mm Hg, respectively (Figure 4A). To rule out the possibility that the inhibitory actions of DDMS were due to a nonspecific action of this inhibitor, additional experiments were performed using structurally and mechanistically different inhibitors of the vasoconstrictor actions of 20-HETE. In these experiments, addition of the 20-HETE antagonist 20-HEDE (1 μmol/L, n=4) or 15-HETE (1 μmol/L, n=5) to the bath also attenuated the pressure-induced constriction of cerebral arteries (Figure 4A). Application of (in μmol/L) DDMS 10, 20-HEDE 1, or 15-HETE 1 to the bath reduced the increase in active wall tension by 70%, 49%, and 61% at
HEDE, and DDMS, respectively (645% before and after administration of DDMS, 20-HETE 21%, 70–87% and 15-HETE 46% and 86% before and after administration of DDMS, 20-HEDE, and 15-HETE, respectively). KCl increased tension by 66 m(80 mm Hg) cerebral arteries was determined before and after addition of 20-HEDE (1 m mol/L) or 15-HETE (1 m mol/L) to the bath. The average basal diameter of the cerebral arteries pressurized at 80 mm Hg was 64.2±3.0 mm (n=7). Under control conditions, 20-HETE caused concentration-related reductions in diameter that reached a maximum of 25.3±2% of control in response to 10–6 mol/L 20-HETE. Prior application of 1 m mol/L 20-HEDE or 1 m mol/L 15-HETE to the bath completely blocked the vasoconstrictor response to 20-HETE (Figure 4D, n=7).

Confirmation that 20-HEDE and 15-HETE Block the Vasoconstrictor Effect of 20-HETE in Cerebral Arteries

The effects of increasing concentrations of 20-HETE (10–4 to 10–6 mol/L) on the internal diameter of pressurized (80 mm Hg) cerebral arteries was determined before and after addition of 20-HEDE (1 m mol/L) or 15-HETE (1 m mol/L) to the bath. The average basal diameter of the cerebral arteries pressurized at 80 mm Hg was 64.2±3.0 mm (n=7). Under control conditions, 20-HETE caused concentration-related reductions in diameter that reached a maximum of 25.3±2% of control in response to 10–6 mol/L 20-HETE. Prior application of 1 m mol/L 20-HEDE or 1 m mol/L 15-HETE to the bath completely blocked the vasoconstrictor response to 20-HETE (Figure 4D, n=7).

Inhibition of 20-HETE Formation or Its Action Impairs Autoregulation of CBF

Autoregulation of CBF was studied using a bilateral closed-cranial window technique25,26 in either pentobarbital- or chlorolose/urethane-anesthetized rats in vivo. CBF in response to elevations of systemic arterial blood pressure was measured using laser Doppler flowmetry. Artificial cerebrospinal fluid (aCSF) containing 50 m mol/L DDMS was superfused over 1 cerebral hemisphere, whereas vehicle (aCSF alone) was superfused over the contralateral hemisphere. Figure 3A depicts representative tracing of mean arterial pressure (MAP) and laser Doppler perfusion units (LDPU) for the right and left hemispheres obtained from a single experiment. Figure 3B summarizes the results from 7 experiments in which intracranial infusion of DDMS impaired autoregulation of CBF in pentobarbital-anesthetized rats. The autoregulatory index (AI) (AI=percentage change in CBF divided by percentage change in MAP) for these data indicated that blood flow within the control hemisphere was tightly autoregulated (AI=0.10±0.06; perfect autoregulation is exhibited at an AI of 0, and no autoregulation is exhibited at an AI of 1.0) over the range of pressures from 70 to 150 mm Hg, whereas the hemisphere superfused with DDMS displayed a greatly attenuated autoregulatory response (AI=0.92±0.09). Autoregulation of CBF recovered after “washout” of DDMS for 30 to 60 minutes. DDMS did not alter baseline CBF. Similar experiments (n=4) were repeated in rats anesthetized with chlorolose/urethane (225 mg/kg body weight) to rule out any effect of the anesthetic on the response to DDMS. In these experiments, DDMS (25 m mol/L) also blocked autoregulation of CBF. Thus, changing the anesthetic did not influence the results.

The results of the experiments looking at the effects of 20-HETE antagonists (subdural 15-HETE or DDMS, and ICV 20-HEDE) on CBF autoregulatory responses are presented in Figure 5. In all animals studied, 15-HETE, 20-HEDE, and DDMS increased the AI. The change in the AI was significantly greater for 15-HETE, 20-HEDE, or DDMS than that seen in the time control studies (P<0.05).

Discussion

The results of the present study demonstrate that 20-HETE is produced by rat cerebral microvessels, and immunoreactive protein and mRNA for P-450 4A1, 4A2, 4A3, and 4A8 isoforms are expressed in these vessels. Subsequent GC/MS analysis confirmed the presence of 20-HETE in cerebral arteries and that an elevation in the transmural pressure from
20 to 140 mm Hg increased 20-HETE concentration in these vessels by 6-fold. The rise in 20-HETE concentration with elevation in transmural pressure suggests a role for this endogenous vasoconstrictor in the generation of pressure-induced cerebral vasoconstriction. Our findings that inhibitors of the formation of 20-HETE or blockers of its vasoconstrictor action eliminate the pressure-induced constriction of isolated cerebral arteries indicate that endogenous 20-HETE is an important component of pressure-induced cerebral arterial constriction. The idea that 20-HETE plays an important role in autoregulation of CBF is further supported by the observation that 15-HETE and 20-HEDE, which antagonize the cerebral vasoconstrictor effect of 20-HETE, also attenuated autoregulation of CBF in rats in vivo. Similarly, inhibition of autoregulation of CBF was observed on inhibition of P-450 ω-hydroxylase with DDMS. Taken together, these findings suggest that the cytochrome P-450 4A enzymes and 20-HETE play an important role in the autoregulation of CBF. 20-HETE activates PKC and modulates the activities of KCa and L-type Ca2+ channels, thereby depolarizing cerebral VSM cells and promoting Ca2+ influx, effects that are similar in character to that of pressure-induced myogenic vasoconstriction. Activation of PKC leads to sustained VSM contraction, depolarization, and increased calcium sensitivity of myofilaments.4,29–31 The pressure-induced increase in vascular 20-HETE concentration of the present

Figure 5. Change in AI between successive determinations when the perfusate is changed from aCSF to aCSF (time control) or to (in μmol/L) 15-HETE 1, 20-HEDE 1, or DDMS 50. Changes recorded for 15-HETE, 20-HEDE, and DDMS were significantly (P<0.05) greater than those recorded for aCSF.
study suggests that this endogenous metabolite contributes to the regulation of pressure-induced myogenic vasoconstriction. The recent findings of Dr Michael L. Schwartzman’s laboratory that overexpression of the cytochrome P-450 4A1 protein and activity enhances pressure-induced constriction of arteries in vitro (personal communication, March 2000) also supports our present findings and further strengthens the role of P-450 4A α-hydroxylase and endogenous 20-HETE in the development of pressure-induced myogenic vasoconstriction.

DDMS and another P-450 4A α-hydroxylase inhibitor, 17-octadecenoic acid, inhibit pressure-dependent vasoconstriction through blockade of the formation of endogenous 20-HETE (References 14–16, this study). Consistent with a previous study using 17-octadecenoic acid, inhibition of enzymatic formation of 20-HETE by DDMS did not alter baseline blood flow in the present study. The lack of effect of DDMS on baseline CBF is unknown. One possible explanation is that 20-HETE may be stored in tissue or activate a signaling cascade with sustained effects on vascular tone. Thus, it may take considerable time to alter baseline tone.

In summary, the present results suggest that cerebral arteries normally produce 20-HETE and that elevation in transmural pressure increases 20-HETE concentration in these vessels. Moreover, inhibitors of the formation of 20-HETE or antagonists of its action attenuate the development of pressure-induced constriction of cerebral arteries in vitro and impair autoregulation of CBF in vivo. These studies further suggest that alterations in the cytochrome P-450 4A activity will alter autoregulation of CBF, which could have a negative impact on neuronal function or result in cerebrovascular pathologies.

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