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 [14C]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. However, 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 Ca 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 K channel, and increases Ca influx via L-type Ca 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 dianisobenzidine solution for 1 hour.
In Situ Hybridization

A P-450 4A2 cDNA cloned 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 monoclonal antibody (Amersham). Other sections were stained with Cy3-conjugated anti-smooth muscle α-actin monoclonal 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, 4A8, and GAPDH having the following sequences: 4A1 forward, 5'-CTCTTACCTTGCAATGAGAA-3'; 4A1 reverse, 5'-GACTTGGATACCTTGGGTAAG-3'; 4A2 forward, 5'-AGATCCAAAAGCCTTATCAACT-3'; 4A2 reverse, 5'-CAGCCTTGGGTGTTAGACCT-3'; 4A3 forward, 5'-CAAAGGCTTGGGAAATTATC-3'; 4A3 reverse, 5'-CGACCTTTGGGTTAGACCT-3'; 4A8 forward, 5'-ATCCAGAGGTGTTTGACCCTTAT-3'; 4A8 reverse, 5'-AATGAGATGTTGACAGTTGAGT-3'; GAPDH forward, 5'-CCCTTGCAATTGCACTTACA-3'; and GAPDH reverse, 5'-ATGCATTGCTGACAATCTTGAG-3'. 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 INVcr 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 Metabolism of AA

Microsomes prepared from bulk isolated cerebral microvessels were incubated with [14C]AA in the absence or presence of the inhibitor of 20-HETE production N-methylsulfonyl-12,12-dibromomodec-11-enamide (DDMS) (50 μmol/L). Reaction products were separated using HPLC as described previously. Actions of DDMS, 15-HETE, and 20–20-Hydroxyeicosa-6(Z),15(Z)-Dienoic Acid (HEDE) on the KCl-Induced Contraction of Cerebral Arterial Rings

Rat middle cerebral arterial ring segments were mounted for tension recording in 2-mL muscle chambers filled with physiological salt solution (PSS) bubbled with 95% O2 and 5% CO2 at 37°C and pH 7.4. After re-equilibration at 80 mg applied tension, a control response to 80 mmol/L KCl was determined. After washout, DDMS (10 μmol/L), 15-HETE (1 μmol/L), and 20-HEDE (1 μmol/L) or vehicle was added to the baths (n=7 to 8 for each group), and 30 minutes later the response to 80 mmol/L KCl was re-determined.

Effect of Transmural Pressure on 20-HETE Concentration

Middle cerebral arteries (150 to 200 μm ID) were placed in a pressure myograph filled with PSS and cannulated as described previously. The endothelium was disrupted by passing air through the lumen, and its absence was confirmed by a lack of relaxation to 1 μmol/L acetylcholine. The bathing solution was composed of (in mmol/L) NaCl 130, CaCl2 2.5, NaHCO3 15, MgSO4 1.2, NaHPO4 1.2, KCl 4.7, glucose 5.5, and HEPES 10 and was equilibrated with 95% O2 and 5% CO2 at 37°C and pH 7.4. After a 60-minute equilibration period, a pressure-diameter curve was determined between 20 and 160 mm Hg during control, in Ca2+-free media, after treatment with DDMS (10 μmol/L), or after addition of the 20-HETE antagonist 20-HEDE (1 μmol/L) or 15-HETE (1 μmol/L). We also confirmed the actions of the later compounds on the vasoconstrictor response to 20-HETE in rat isolated middle cerebral arteries. In a separate study, the arteries were pressurized at 20 or 140 mm Hg for 30 minutes, removed, and frozen in liquid N2, and 20-HETE levels were measured by gas chromatography/mass spectrometry (GC/MS).

GC/MS Measurement of 20-HETE

Rat cerebral arterial segments were equilibrated at a transmural pressure of either 20 or 140 mm Hg for 30 minutes, frozen in liquid N2, and then homogenized in 10 μL of PBS (pH 7.4). Deuteriated 20-HETE ([2H2]-20-HETE) that served as an internal standard was added to the homogenate sample, acidified (pH 3.5), extracted with ethyl acetate, and dried under stream of N2 gas. The dried sample was subjected to pentafluorobenzyl-ester derivatization (COOH group) and Bis-(trimethylsilyl) trifluoroacetamide–ether derivatization (OH group) and analyzed by GC/MS. The ratio of peak area of m/z 391 to the peak area of m/z 393 (internal standard) was used to calculate the amount of 20-HETE in the sample.

CBF Autoregulation Studies

Experiments were performed on male Sprague-Dawley rats housed in the animal care facility of the Medical College of Wisconsin, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Rats (250 to 350 g) were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg body weight, IP). The role of 20-HETE in autoregulation of CBF was examined using laser Doppler flowmetry through a thinned cranial window 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 μmol/L, 1 μL/min), subdural infusion of 15-HETE (1 μmol/L, 2 μL/min), or ICV infusion of 20-HEDE (1 μmol/L, 2 μL/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 was used to probe sections of rat brain for the presence of immunoreactive protein. Figure 1A shows the localization of P-450 4A isoforms in the cerebral microvasculature.

To confirm these results, cRNA probes corresponding to P-450 4A2 were used to localize P-450 4A2 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.
a-actin. The antisense cRNA probes hybridized to the wall of cerebral arteries (Figure 1D) and colocalized with the pattern of the a-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±6% 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 vasconstrictor 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

Formation of 20-HETE in Rat Cerebral Vessel Microsomes

Incubation of microsomes prepared from rat cerebral arteries with [14C]AA resulted in the formation of a peak (Figure 3C)
160 mm Hg, respectively, and shifted the pressure-tension curve to the right (Figure 4B). The contractile response of cerebral arterial rings to 80 mmol/L KCl was not altered after treatment with (in μmol/L) DDMS 10, 20-HEDE 1, or 15-HETE 1. KCl increased tension by 66±23% and 87±21%, 70±15% and 86±16%, and 131±46% and 157±45% before and after administration of DDMS, 20-HEDE, and 15-HETE, respectively (P<0.05 for all groups).

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 (10−6 to 10−4 mol/L) on the internal diameter of pressurized (80 mm Hg) cerebral arteries was determined before and after addition of 20-HEDE (1 μmol/L) or 15-HETE (1 μmol/L) to the bath. The average basal diameter of the cerebral arteries pressurized at 80 mm Hg was 64.2±3.0 μm (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 10−4 mol/L 20-HETE. Prior application of 1 μmol/L 20-HEDE or 1 μmol/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 chloralose/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 μmol/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 chloralose/urethane (225 mg/kg body weight) to rule out any effect of the anesthetic on the response to DDMS. In these experiments, DDMS (25 μmol/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 vasconstriction. 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 PKC17,27 and modulates the activities of K Ca and L-type Ca2+ channels,12,17,18,20,28 thereby depolarizing cerebral VSM cells and promoting Ca2+ influx, effects that are similar in character to that of pressure-induced myogenic vasconstriction. 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.

Figure 4. A, Effects of DDMS (●, 10 μmol/L), the 20-HETE antagonists 20-HEDE (▲, 1 μmol/L) and 15-HETE (Δ, 1 μmol/L), and Ca2+-free PSS (○) on the pressure-induced constriction of cerebral arteries. Incubation of middle cerebral arteries with DDMS or addition of 20-HEDE or 15-HETE to the bath eliminated the pressure-induced constriction of these arteries, whereas the arterial segments displayed passive dilation during increases in transmural pressure in Ca2+-free medium. B, Pressure-active tension relationship in cerebral arteries. Preincubation with DDMS (10 μmol/L, ●) or addition of 1 μmol/L 20-HEDE (▲) or 1 μmol/L 15-HETE (Δ) significantly reduced the active tension generated by arteries subjected to elevation in intravascular pressure. C, 20-HETE concentrations in cerebral arterial segments maintained at a transmural pressure of 20 or 140 mm Hg for 30 minutes. *P<0.01, n=5 for each pressure. D, Line graphs depicting the effects of 20-HEDE (●) and 15-HETE (▲) on the cerebral vasoconstrictor action of 20-HETE. Addition of 20-HEDE (1 μmol/L) or 15-HETE (1 μmol/L) to the bath blocked reduction in diameter caused by 20-HETE. Each data point is mean±SEM. *P<0.05, n=4 to 5 for each group.
study suggests that this endogenous metabolite contributes to the formation 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 ω-hydroxylase and endogenous 20-HETE in the development of pressure-induced myogenic vasoconstriction.

DDMS and another P-450 4A ω-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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