Arachidonic acid metabolites, hydrogen peroxide, and EDHF in cerebral arteries

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You, Junping, Elke M. Golding, and Robert M. Bryan, Jr. Arachidonic acid metabolites, hydrogen peroxide, and EDHF in cerebral arteries. Am J Physiol Heart Circ Physiol 289: H1077–H1083, 2005. First published April 29, 2005; doi:10.1152/ajpheart.01046.2004.—We tested the hypotheses that EDHF in rat middle cerebral arteries (MCAs) involves 1) metabolism of arachidonic acid through the epoxygenase pathway, 2) metabolism of arachidonic acid through the lipoxygenase pathway, or 3) reactive oxygen species. EDHF-mediated dilations were elicited in isolated and pressurized rat MCAs by activation of endothelial P2Y2 receptors with either UTP or ATP. All studies were conducted after the inhibition of nitric oxide synthase with Nω-nitro-L-arginine methyl ester (10 μM) and indomethacin (10 μM), respectively. The inhibition of epoxygenase with miconazole (30 μM) did not alter EDHF dilations to UTP, whereas the structurally different epoxygenase inhibitor N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanoic acid (20 or 40 μM) only modestly inhibited EDHF at the highest concentration of UTP. An antagonist of epoxyeicosatrienoic acids, 14,15-epoxyeicosa-5(12)-enoic acid, had no effect on EDHF dilations to UTP. Chronic inhibition of epoxygenase in the rat with 1-aminobenzotriazol (50 mg/kg twice daily for 5 days) did not alter EDHF dilations. The inhibition of the lipoxygenase pathway with either 10 μM baicalein or 10 μM nordihydroguaiaretic acid produced no major inhibitory effects on EDHF dilations. The combination of superoxide dismutase (200 U/ml) and catalase (140 U/ml) had no effect on EDHF dilations. Neither tiron (10 mM), a cell-permeable scavenger of reactive oxygen species, nor deferoxamine (1 or 10 mM), an iron chelator that blocks the formation of hydroxyl radicals, altered EDHF dilations in rat MCAs. We conclude that EDHF dilations in the rat MCA do not involve the epoxygenase pathway, lipoxygenase pathway, or reactive oxygen species including H2O2.

adrenosine triphosphate; endothelium; endothelium-derived hyperpolarizing factor; epoxyeicosatrienoic acids; epoxygenase; hydroxyl radical; lipoxygenase; reactive oxygen species; superoxide anion; uridine triphosphate

STIMULATION OF ENDOTHELIAL RECEPTORS by a number of agonists including ACh, bradykinin, ATP, or UTP can dilate arteries and arterioles. Depending on the vessel, these dilations involve nitric oxide (NO), prostacyclin (PGI2), and/or EDHF. Of these three dilator processes, EDHF is the least understood and thus the most controversial. While the definition of EDHF varies, we define it as a dilator process with the following characteristics: 1) endothelium dependent, 2) not NO or PGI2, 3) dilates by hyperpolarizing vascular smooth muscle, and 4) involves Ca2+-activated K+ (KCa) channels (9, 25).

Presently, very little is known about the mechanism of EDHF in cerebral arteries and arterioles. It is clear that mechanistic differences between cerebral and peripheral vessels do exist (17). For example, in many peripheral vessels, EDHF requires activation of both small and intermediate-conductance KCa channels (65). In contrast, EDHF in cerebral arteries and arterioles involves only intermediate-conductance KCa channels (34, 61, 64). Additionally, NO inhibits EDHF dilations in peripheral vessels (5, 47) but has no effect on EDHF dilation in cerebral arteries (53).

Given the limited understanding of the mechanism of EDHF dilations in cerebral vessels, we tested the following three hypotheses: 1) metabolites of arachidonic acid through the epoxygenase pathway are involved in EDHF-mediated dilations in the rat middle cerebral artery (MCA), 2) metabolites of arachidonic acid through the lipoxygenase pathway are involved in EDHF-mediated dilations in the rat MCA, and 3) H2O2 or other reactive oxygen species (ROS) are involved in EDHF-mediated dilations in the rat MCA.

These three hypotheses are based on mechanisms reported to be involved with EDHF in peripheral arteries (12, 13, 21, 36). The predominant product of phospholipase A2-catalyzed reactions is arachidonic acid, the substrate for epoxygenase or lipoxygenase, and we have previously demonstrated that activation of phospholipase A2 is required for EDHF dilations in the rat MCA (62). Thus the hypotheses involving arachidonic acid metabolism through the epoxygenase or lipoxygenase pathways were particularly appealing.

MATERIALS AND METHODS

The Animal Protocol Review Committee at Baylor College of Medicine approved the experimental protocol. Male Long-Evans rats (250–350 g body wt) were anesthetized with 3% isoflurane and killed by decapitation. The brain was rapidly removed and placed in chilled physiological salt solution (PSS). MCAs were carefully dissected from the brain and placed in a vessel chamber. Micropipettes were inserted into each end of the MCA and tied in place (10). Each MCA was bathed with 37°C PSS equilibrated with a gas mixture of 20% O2-5% CO2-balance N2. The pH of the PSS was ~7.4, PCO2 was ~35 mmHg, and P02 was ~130 mmHg. MCAs were pressurized to 85 mmHg by a column of PSS that was raised to the appropriate height above the vessel. Luminal flow was adjusted to 150 μl/min by setting the inflow and outflow reservoirs at different heights. Pressure transducers on either side of the MCA allowed measurement of the perfusion pressure across the vessel. Each MCA was magnified ×550, recorded on videotape, and dis-
played on a video monitor. Changes in the diameter were continually measured using Optimas image-analysis software (Bothell, WA) either online or after the videotapes were replayed.

After being warmed and pressurized to 85 mmHg, MCAs developed spontaneous tone by constricting to ~75% of their initial diameters. Note that MCAs had two distinct and independent compartments consisting of the luminal perfusate and the abluminal bath. Drugs and reagents added to the luminal compartment had preferential access to the endothelium; conversely, drugs and reagents added to the abluminal bath had preferential access to vascular smooth muscle (10, 63).

The EDHF response was elicited by stimulating endothelial P2Y2 receptors with luminally applied UTP or ATP after inhibition of NO synthase and cyclooxygenase with 10 μM N-nitro-l-arginine methyl ester (l-NAME) and 10 μM indomethacin, respectively (61, 64). Note that l-NAME and indomethacin were present in the luminal perfusate and abluminal bath in all vessels. In some studies (Figs. 1, 3, and 4), MCAs were subjected to only one concentration-response curve where UTP or ATP was administered over a concentration range. In two studies (Fig. 2 and RESULTS), repeated dilations were elicited by the luminal application of 10−5 M UTP.

In the rat, the formation of epoxyeicosatrienoic acids (EETs) was chronically inhibited using 1-aminobenzotriazole (ABT; 50 mg/kg bid ip) for 5 days (18). ABT has been demonstrated to inhibit EET production in vivo in the liver, lung, and kidney (3, 18, 32). Furthermore, intraperitoneal injections of ABT in mice significantly inhibited cytochrome P-450 activity in the brain (14). Although ABT inhibits both ω-hydroxylase and epoxygenase, it was chosen for these studies because it irreversibly inactivates epoxygenase in vivo and the chronic administration of ABT is well tolerated by rats (38, 51). After 5 days of the ABT administration, rat MCAs were harvested and mounted in the vessel baths as described above. 1-[2,4-Dichloro-β-[(2,4-dichloro-robzenyl)-oxy]phenethyl}imidazzole (miconazole; 30 μM) was present in the luminal perfusate and abluminal bath in the chamber for the MCAs harvested from ABT-treated rats.

Drugs and reagents. UTP, ATP, l-NAME, indomethacin, miconazole, ABT, 17-octadecynoic acid (17-ODYA), Phenylephrine, superoxide dismutase (SOD), catalase, 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), and deferoxamine were purchased from Sigma. 5,6,7-Trihydroxystilva (baicalein) and nordihydroguaiaretic acid (NDGA) were purchased from BioMol. N-methylsulfonyl-6-(2-proparglyoxyphenyl)hexanoic acid (MS-PPOH) and 14,15-epoxyeicos-5(Z)-enoic acid (14,15-EEZE) were gifts from Dr. John R. Falck (University of Texas Southwestern Medical Center, Dallas, TX).

Indomethacin was dissolved in a 15 mM Na2CO3 solution. Miconazole and baicalein were dissolved in DMSO. MS-PPOH and NDGA were dissolved in ethanol. 14,15-EEZE was dissolved in 95% ethanol (EtOH). ABT was dissolved in PSS. All other reagents were dissolved in distilled water. The composition of the PSS was as previously described (10).

Data analysis. All data are reported as means ± SE. Dilations of the MCAs are expressed as the percentage of the maximum diameter (%Dmax) using the following equation:

\[ \%D_{\text{max}} = \left( \frac{D_{\text{agonist}} - D_{\text{basal}}}{D_{\text{max}} - D_{\text{basal}}} \right) \times 100 \]

where Dagonist is the diameter after luminal administration of either UTP or ATP, Dbasal is the baseline diameter before the addition of the agonist, and Dmax is the maximum diameter at 85 mmHg, which was obtained in the presence of Ca2+-free PSS-containing EGTA (2 mM).

For statistical analysis, two-way repeated-measures ANOVA was used followed by the Tukey test for individual comparisons when appropriate. P < 0.05 defined the acceptable level of significance.

RESULTS

Figure 1 shows the effects of two structurally different inhibitors of epoxygenase, miconazole and MS-PPOH, on

EDHF dilations. EDHF responses were elicited by the luminal application of UTP after the inhibition of NO synthase and cyclooxygenase with l-NAME (10 μM) and indomethacin (10 μM), respectively (61, 64). l-NAME and indomethacin were added to both the luminal perfusate and abluminal bath. Figure 1A shows EDHF dilations to UTP in rat MCAs in the presence of DMSO (vehicle for miconazole, n = 8) or 30 μM miconazole (n = 6). Figure 1B shows EDHF dilations in the presence of EtOH (vehicle for MS-PPOH, n = 8), 20 μM MS-PPOH (n = 4), or 40 μM MS-PPOH (n = 6). The vehicle or epoxygenase inhibitors were added to both the luminal perfusate and abluminal bath. Miconazole had no effect on EDHF dilation compared with the vehicle control. MS-PPOH, a structurally distinct and more specific epoxygenase inhibitor, produced a small but significant inhibition of EDHF dilation with 10−4 M UTP (P = 0.048 and 0.002 for 20 and 40 μM MS-PPOH, respectively) but had no effect with 10−5 M UTP.

In preliminary studies, the efficacy of miconazole in blocking epoxygenase was determined in rat interlobar arteries after preconstriction with abuminally applied UTP (with 10 μM l-NAME and 10 μM indomethacin present). Control arteries treated with the DMSO vehicle dilated 95 ± 3% (n = 3) to ACh; arteries treated with 30 μM miconazole only dilated 8 ± 6% (n = 3). This study in interlobar arteries confirmed the ability of miconazole to block epoxygenase in our hands.

For EETs to be involved with EDHF dilations, they do not necessarily have to be synthesized on demand because EETs can be stored in membrane phospholipids (50, 57–60). Activation of phospholipase A2 could, therefore, directly liberate EETs from the phospholipid pool to act as EDHFs. Thus epoxygenase would not be required until the EET pool had been depleted (43).
To investigate the possibility that stored EETs are involved, we used multiple experimental approaches. First, we used 14,15-EEZE, an antagonist of EETs (22). If EETs are involved, then 14,15-EEZE should block their effects regardless of whether they were made on demand or released from a stored pool. Figure 1B shows that 20 μM 14,15-EEZE had no effect on the EDHF response (n = 6). The efficacy of 14,15-EEZE was demonstrated in pressurized small mesenteric arteries, which were preconstricted with phenylephrine as previously described (39). The addition of 3 × 10⁻⁷ M 14,15-EET diluted mesenteric arteries by 20 ± 6%. The addition of 10 μM 14,15-EEZE attenuated the dilation by 85% to 3 ± 1% (n = 3). We choose to demonstrate the efficacy of 14,15-EEZE in mesenteric arteries because they dilate to 14,15-EET and other EETs (39). The direct effects of EETs, especially 14,15-EET, on cerebral arteries is not well established (19, 23).

Second, we conducted repeated EDHF dilations in MCAs in the presence and absence of miconazole (Fig. 2). The idea was to inhibit synthesis and deplete any stored EETs with repeated EDHF dilations. Figure 2 shows traces of repeated EDHF dilations in two MCAs, one treated with vehicle (Fig. 2A) and one treated with 30 μM miconazole (Fig. 2B). All dilations were elicited by luminal applications of 10⁻⁵ M UTP in the presence of L-NAME and indomethacin at intervals of ~30 min. An enlargement of a single dilation is shown in Fig. 2, inset. After the initial EDHF dilation, either EtOH or miconazole was added luminally and abluminally for six subsequent dilations (Fig. 2). The EDHF dilation to UTP produced a near-maximal response, which was not affected with subsequent dilations in the presence of either the vehicle (EtOH, n = 3) or 30 μM miconazole (n = 5, Fig. 2).

We also used a fourth method in an attempt to deplete EETs from stored pools. ABT (50 mg/kg ip twice daily), an inhibitor of epoxygenase and ω-hydroxylase, was injected intraperitoneally for 5 days (n = 4 rats). Control rats (n = 3) were injected with vehicle (PSS) only. After 5 days, MCAs were harvested and mounted in a vessel bath containing 30 μM miconazole, 10 μM L-NAME, and 10 μM indomethacin. Luminally applied UTP was used to elicit EDHF dilations. ABT had no effect on EDHF-mediated dilation in MCAs (data not shown).

Figure 3 shows the effects of inhibition of the lipoxygenase pathway on EDHF-mediated dilations to UTP. EDHF dilations were elicited by the luminal application of UTP in the presence of L-NAME and indomethacin. The inhibitors of the lipoxygenase pathway baikalein or NDGA were added to the luminal and abluminal bath at a concentration of 10 μM for 30 min before the administration of UTP. Baicalein had no effect on EDHF-mediated dilations (Fig. 3A). In the presence of NDGA (Fig. 3B), EDHF dilations were potentiated at UTP concentrations from 10⁻⁸ to 10⁻⁶ M. At 10⁻⁴ M UTP, there was a slight but significant (P < 0.001) inhibition of the EDHF response. Repeated EDHF dilations (with L-NAME and indomethacin present) were conducted in the presence of 10 μM baicalein. The first EDHF dilation was in the absence of either vehicle or baicalein. Subsequent dilations were in the presence of the vehicle DMSO (n = 4) or 10 μM baicalein (n = 5). Baicalein had no significant effect on EDHF dilations in six subsequent repeated dilations (data not shown).

Figure 4A shows that the combination of SOD (200 U/ml) and catalase (140 U/ml) (n = 4) had no significant effect on EDHF-mediated dilation (with L-NAME and indomethacin present) was exposure to UTP.
inhibited by catalase. Additionally, tiron (10 mM), a chelator of iron, had no effect on EDHF dilations elicited by ATP. L-NAME (10 μM) and indomethacin (10 μM) were present at all times. SOD produces the dismutation of superoxide to hydrogen peroxide and oxygen. Catalase is responsible for decomposing hydrogen peroxide to oxygen and water. Tiron scavenges reactive oxygen species. B: effects of deferoxamine, an iron chelator, on EDHF-mediated dilations to ATP. L-NAME (10 μM) and indomethacin (10 μM) were present in all experiments.

DISCUSSION

The purpose of this study was to gain insight into the mechanism of EDHF dilations in cerebral arteries. We report three new findings involving EDHF in cerebral arteries: 1) EETs or other metabolites of arachidonic acid through the epoxygenase pathway are not involved with EDHF dilations in the rat MCA, 2) metabolites of arachidonic acid through the lipoxigenase pathway are not involved with EDHF dilations in the rat MCA, and 3) H₂O₂ or other ROS are not involved with EDHF dilations in the rat MCA.

**Epoxygenase pathway.** Evidence for a role for EETs as EDHFs comes mostly from coronary and renal arteries (11, 12, 22, 28, 30, 41), although EETs have been shown to be involved with other peripheral vessels (7, 30). Unlike coronary or renal arteries, we concluded that EDHF in the rat MCA does not involve EETs or other metabolites of the epoxygenase pathway.

Our conclusion is based on several lines of evidence. Miconazole, an epoxygenase inhibitor, had no effect on EDHF dilations elicited by the P2Y₂ agonist UTP (Fig. 1A). A ATP concentration of 10⁻⁴ M and MS-PPOH slightly but significantly attenuated EDHF dilation. If these data were indicative of epoxygenase involvement, it would only suggest a minor role in EDHF dilations. The concentrations of MS-PPOH (20 and 40 μM) used in our study were greater than those required for the inhibition of epoxygenase in perfused rat kidneys (12 μM) or rat preglomerular microvessels (12 μM) (16, 49). Because structurally different epoxygenase inhibitors did not substantially attenuate EDHF dilations in rat MCAs, we conclude that EETs, which are synthesized on demand, are not involved with EDHF-mediated dilations in rat MCAs.

The studies using the epoxygenase inhibitors miconazole and MS-PPOH only address those EETs that would be synthesized upon stimulation of the P2Y₂ receptors with UTP. Because EETs can be stored in membrane phospholipids (50, 57–60), activation of phospholipase A₂ could directly liberate them to act as EDHFs without the involvement of epoxygenase. Epoxygenase inhibitors would only have an effect on the EDHF response after the EET pool had been depleted (43). To determine whether stored EETs could be involved with EDHF dilation in rat MCAs, we used three strategies. First, repeated dilations after epoxygenase inhibition should eventually deplete the stored EETs, resulting in diminished EDHF dilation (43). EDHF dilations were not diminished after repeated applications of luminally applied UTP (with L-NAME and indomethacin present) in the presence of the epoxygenase inhibitor miconazole (Fig. 2). Second, chronically inhibiting epoxygenase in the whole animal with ABT for 5 days had no effect on EDHF dilations in isolated MCAs. Finally, the EET antagonist 14,15-EEZE should inhibit the effects of EETs regardless of whether it was synthesized on demand or released from stored pools. The EET antagonist 14,15-EEZE (20 μM) had no effect on EDHF dilations in the rat MCA (Fig. 1B). In bovine coronary arteries, 10 μM 14,15-EEZE substantially attenuated dilations to EETs and arachidonic acid and EDHF dilations elicited by either methacholine or bradykinin (22). Furthermore, we demonstrated that 10 μM 14,15-EEZE blocked dilations to 14,15-EEET in small mesenteric arteries. These three strategies provide corroborating evidence demonstrating that stored EETs are not involved with EDHF dilations in the rat MCA.

One limitation to our study was the inability to measure EETs. The very small size of the MCA combined with the small concentrations of EETs necessary to have a dilator response made it impractical to measure EETs in the rat MCA. Because of this limitation, we chose to test the hypothesis using multiple approaches as described above. In summary, our data support the conclusion that EETs or other metabolites of the epoxygenase pathway have no major role in the mechanism for EDHF dilations in rat MCAs. This conclusion pertains to both EETs made on demand and those stored in membrane phospholipids. Of note, our conclusions regarding EETs and EDHF are not at odds with other studies demonstrating a

![Graph](http://ajpheart.physiology.org/Downloadedfrom/article.fig8a.png)
non-EDHF dilator role for EETs produced in astrocytes (1, 2, 6, 48).

Lipoxygenase pathway. Although EETs are the major arachidonic acid metabolite linked to EDHF in peripheral vessels, the lipoxygenase pathway can produce dilator metabolites (20), and these dilator metabolites have been linked to EDHF in the rabbit aorta and rat coronary microcirculation (13, 37). Thus we tested the hypothesis that the lipoxygenase pathway is involved with EDHF dilations in the rat MCA.

The major arachidonic acid metabolite produced by lipoxygenase in cerebral microvessels from rats and mice is 12-hydroxyeicosatetraenoic acid (12-HETE), produced by the 12-lipoxygenase pathway (4, 26, 44–46). Either 10 μM baicalin or 10 μM NDGA blocked both dilations to arachidonic acid and the production of 12-HETE in the rat basilar artery (20). The same concentration of baicalin (10 μM) had no effect on EDHF dilations in the rat MCA, regardless of whether single or repeated dilations were elicited. Interestingly, NDGA potentiated EDHF dilations to UTP at concentrations of 10−4 M UTP and slightly but significantly inhibited the dilations with 10−4 M UTP. We concluded that the lipoxygenase pathway has no major role in EDHF dilations in the rat MCA.

Possible role for arachidonic acid as a signaling molecule for EDHF dilations in rat MCAs. Our previous study (62) showed that phospholipase A2 has a major role in EDHF dilations in the rat MCA. Because arachidonic acid is the predominant product of phospholipase A2-catalyzed reactions, it was surprising that none of the metabolic pathways for arachidonic acid appeared to be involved with the EDHF response. If arachidonic acid metabolites are not involved, then perhaps it is arachidonic acid itself that is the signaling molecule involved with EDHF dilations. Arachidonic acid is known to be a signaling molecule in a number of cellular processes (8). Therefore, we speculate that arachidonic acid might serve as a signaling molecule in EDHF-mediated dilations in the rat MCA.

ROS. H2O2 has been reported to act as an EDHF in mice mesenteric arteries, small piglet pial arteries, human coronary arterioles, and human mesenteric arteries (33, 35, 36, 42). In human submucosal intestinal microvessels, H2O2 is not EDHF but is involved with EDHF dilations by possibly stimulating EDHF (27). Dilations of rat cerebral arterioles to bradykinin or arachidonate were abolished by catalase (54, 55). The primary determinant for H2O2 involvement is the ability of catalase to inhibit the dilation. Catalase degrades H2O2 to O2 and H2O. In cells, H2O2 can be generated by the dismutase of superoxide by SOD. In the present study, catalase combined with SOD did not alter EDHF-mediated dilation (Fig. 4A), indicating that neither superoxide nor H2O2 was involved with EDHF-mediated dilation. Tiron, a cell-permeable scavenger of ROS, did not affect EDHF dilation (Fig. 4A), and deferoxamine, an iron chelator, had no effect on EDHF dilation elicited by ATP (Fig. 4B). H2O2 reacts with iron to form hydroxyl radicals, which can dilate arteries and arterioles (52). Deferoxamine inhibits the production of hydroxyl radicals by chelating iron. We concluded that EDHF dilations in rat MCAs do not involve H2O2, superoxide radicals, or other ROS.

Concluding remarks. The major candidates for EDHF in arteries and arterioles are 1) EETs, 2) H2O2, and 3) K+. Metabolites of arachidonic acid through the lipoxygenase pathway have been implicated in the mechanism of EDHF in the rabbit aorta and rat coronary circulation (13, 37), through they have not been as widely studied as EETs. In addition to the above, myoendothelial gap junctions have been proposed as an integral part of the EDHF mechanism (15). Gap junctions would serve as either the conduit for EDHF to pass from endothelial cells to vascular smooth muscle or as a means of conducting hyperpolarizing current from the endothelium to vascular smooth muscle. In the present study, we have shown that neither EETs nor H2O2 are involved in EDHF dilations in the rat MCA. We further demonstrated that metabolites of arachidonic acid through the lipoxygenase pathway are not involved with EDHF dilations. In previous studies (31, 64), we demonstrated that K+ was not EDHF in rat MCAs. This latter conclusion was based on the fact that Ba2+, an inhibitor of inwardly rectifying K+ channels, could block dilations to increases in extracellular K+ but could not block EDHF-mediated dilations (31, 64). On the other hand, EDHF dilations were completely blocked by peptides that inhibit gap junctions involving connexin37, -43, and -40 (24, 56). Thus the best evidence to date for the mechanism of EDHF dilations in cerebral vessels involves gap junctions, presumably myoendothelial gap junctions.

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REFERENCES

13. Campbell WB, Spitzyhartz N, Gautheimer KM, and Pfister SL. 11,12,15-


