

Epoxyeicosatrienoic acid-dependent cerebral vasodilation evoked by metabotropic glutamate receptor activation in vivo

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¹Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, Maryland; ²Cardiovascular Research Center and Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin; ³Department of Entomology and Cancer Center, University of California, Davis, California; ⁴Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas; and ⁵Department of Pharmacology, University of Mississippi Medical Center, Jackson, Mississippi

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Liu X, Li C, Gebremedhin D, Hwang SH, Hammock BD, Falck JR, Roman RJ, Harder DR, Koehler RC. Epoxyeicosatrienoic acid-dependent cerebral vasodilation evoked by metabotropic glutamate receptor activation in vivo. *Am J Physiol Heart Circ Physiol* 301: H373–H381, 2011. First published May 20, 2011; doi:10.1152/ajpheart.00745.2010.—Group I metabotropic glutamate receptors (mGluR) on astrocytes have been shown to participate in cerebral vasodilation to neuronal activation in brain slices. Pharmacological stimulation of mGluR in brain slices can produce arteriolar constriction or dilation depending on the initial degree of vascular tone. Here, we examined whether pharmacological stimulation of mGluR in vivo increases cerebral blood flow. A 1-mM solution of the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG) superfused at 5 μ l/min over the cortical surface of anesthetized rats produced a $30 \pm 2\%$ (\pm SE) increase in blood flow measured by laser-Doppler flowmetry after 15–20 min. The response was completely blocked by superfusion of group I mGluR antagonists and attenuated by superfusion of an epoxyeicosatrienoic acid (EET) antagonist ($5 \pm 4\%$), an EET synthesis inhibitor ($11 \pm 3\%$), and a cyclooxygenase-2 inhibitor ($15 \pm 3\%$). The peak blood flow response was not significantly affected by administration of inhibitors of cyclooxygenase-1, neuronal nitric oxide synthase, heme oxygenase, adenosine A_{2B} receptors, or an inhibitor of the synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE). The blood flow response gradually waned following 30–60 min of DHPG superfusion. This loss of the flow response was attenuated by a 20-HETE synthesis inhibitor and was prevented by superfusion of an inhibitor of epoxide hydrolase, which hydrolyzes EETs. These results indicate that pharmacological stimulation of mGluR in vivo increases cerebral blood flow and that the response depends on the release of EETs and a metabolite of cyclooxygenase-2. Epoxide hydrolase activity and 20-HETE synthesis limit the duration of the response to prolonged mGluR activation.

astrocyte; cerebral blood flow; epoxide hydrolase; epoxygenase; neurovascular coupling

ASTROCYTES PLAY A KEY ROLE in coupling the increase in cerebral blood flow (CBF) with neuronal activity (19). Studies (47) in brain slices indicate that an increase in astrocyte Ca²⁺ during neuronal stimulation largely depends on stimulation of group I metabotropic glutamate receptors (mGluR) and that nearby arterioles with preexisting tone frequently dilate. The coupling of increased astrocyte Ca²⁺ with vasodilation has been attrib-

uted to astrocytic release of a cyclooxygenase (COX) product (5, 16, 41, 47, 48), epoxyeicosatrienoic acids (EETs) (1, 2, 5), and K⁺ flux through Ca²⁺-sensitive K⁺ (K_{Ca}) channels (11). Results in vivo with physiological neuronal activation have confirmed rapid increases in astrocyte Ca²⁺ (45) and an increase in CBF that is partially inhibited by mGluR antagonists (38, 47), COX-2 inhibitors, knockout of the COX-2 gene (29, 39), and administration of inhibitors and antagonists of EETs (32, 33, 38). Furthermore, astrocyte-evoked vasoconstriction has also been reported in arterioles with low baseline tone in brain slices. The constriction depends on the synthesis of the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE; Refs. 5, 26). Astrocytes stimulated with glutamate release arachidonic acid (1), which serves as a substrate for the synthesis of EETs by epoxygenases in astrocytes (1, 2, 28, 33), prostaglandin E₂ by COX-1 in astrocytes (48), and the synthesis of 20-HETE by CYP4A enzymes in cerebral vascular smooth muscle (9). EETs promote opening of astrocyte K_{Ca} channels (14, 46), which are thought to release sufficient K⁺ to open inward-rectifier K⁺ channels in vascular smooth muscle and thereby elicit hyperpolarization (11, 15). EETs may also diffuse from astrocytes to the adjacent smooth muscle cells and promote opening of vascular K_{Ca} channels (13). COX-2 is thought to be constitutively expressed in neurons and not astrocytes (41, 43). Nearly all of the work on the vasodilatory response to mGluR activation has been done in vitro. One study (15) has demonstrated a role of activation of vascular K_{Ca} channels in mediating the CBF response to a mGluR agonist in vivo.

The present study examined the hypothesis that the increase in CBF following activation of mGluR is mediated by the formation and release of COX products and EETs, limited by the breakdown of EETs by soluble epoxide hydrolase (sEH), and modulated by the formation of the vasoconstrictor 20-HETE. This hypothesis was tested by measuring CBF to superfusion of the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG; Ref. 37) over the surface of cerebral cortex in rats and then determining the effect of coadministration of the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), the EET synthesis inhibitor *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH), the COX-1 inhibitor 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole (SC-560), the COX-2 inhibitor *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398), the sEH inhibitor *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB), and the 20-HETE synthesis inhibitor *N*-hydroxy-*N'*-(4-*n*-butyl-2-methylphenyl)forma-

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midine (HET0016). Because adenosine A_{2B} receptors (38) and neuronal nitric oxide (NO) synthase (nNOS; Ref. 22) are known to play a role in the vasodilation elicited by neuronal activation and because heme oxygenase in astrocytes plays a role in the vasodilation elicited by glutamate in piglet pial arteries (20, 21), we also evaluated the effects of the A_{2B} receptor antagonist alloxazine, the nNOS inhibitor 7-nitroindazole (7-NI), and the heme oxygenase inhibitor chromium mesoporphyrin IX (CrMPiX) on the CBF response to DHPG.

MATERIALS AND METHODS

All animal procedures were conducted in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Johns Hopkins University Animal Care and Use Committee.

Surgical preparation. Data are reported from *in vivo* experiments performed on 76 adult male Wistar rats (250–350 g; Harlan, Indianapolis, IN) that were maintained in a climate-controlled room on a 12-h light-dark cycle with food and water available *ad libitum*. The rats were anesthetized with 1.5% isoflurane during the surgical procedure. A femoral artery and femoral vein were catheterized, and mean arterial blood pressure was monitored. A tracheostomy was performed for mechanical ventilation with 30–40% O₂ and ~1.5% isoflurane. Rectal temperature was maintained at 37°C with a heating blanket. Arterial pH, PCO₂, and PO₂ were measured with a blood gas analyzer (Chiron Diagnostics, Halstead, Essex, UK), and hemoglobin concentration was measured with a hemoximeter (OSM3; Radiometer, Copenhagen, Denmark).

The rat was placed in the prone position, and the head was fixed with a stereotaxic holder. A 3 × 3-mm region on the left side of the skull was thinned to translucency by careful drilling for placement of a laser-Doppler flow (LDF) probe, which was located 2–3 mm posterior and 7 mm lateral to bregma. Some inhibitors were administered by subarachnoid superfusion over the cortical surface at a constant rate of 5 μl/min (38). A small drill hole was made superior to the LDF probe site to expose the dura. A PE-10 catheter, with the tip tapered to ~120 μm, was carefully inserted subdurally. Another hole was made inferior to the flow probe site, and the dura was incised for passive drainage of the superfused fluid. At a superfusion rate of 5 μl/min, drug outflow concentration can attain a quasi-steady state within 10–15 min (38).

Experimental protocol. The LDF response to 1 h of 1 mM DHPG superfusion in rats was recorded with or without treatment of the animals with various inhibitors. The cortical surface was superfused with artificial cerebrospinal fluid (CSF) starting 1 h after completion of the surgery at a constant rate of 5 μl/min. The artificial CSF constituents were as follows (in mM): 156 Na⁺, 3 K⁺, 1.25 Ca²⁺, 0.66 Mg²⁺, 133 Cl⁻, 25 HCO₃⁻, 6.7 urea, and 3.7 dextrose. The CSF was warmed to 37°C. After 15 min of CSF superfusion, various inhibitors or vehicle was added to the superfusate for up to 1 h. Then, 1 mM DHPG was added to the superfusate together with a particular inhibitor or vehicle for 1 h. In previous work, no additional inhibition of vascular responses to neural activation was observed with superfusion of inhibitors for more than 1 h (23, 32, 38).

Eleven groups (6 rats per group) were treated with various inhibitors. To test for specificity of DHPG, the group I mGluR subtype 1 antagonist (S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY-367385; 300 μM) and the subtype 5 antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP; 100 μM) were superfused together before DHPG was added to the superfusate. Combined administration of LY-367385 and MPEP has been shown to reduce the astrocyte Ca²⁺ and the vasodilator response to neuronal activation in brain slices and to reduce the *in vivo* LDF response following whisker stimulation (47). Other groups were treated by superfusion of the cortical surface with 30 μM 14,15-EEZE, 20 μM MS-PPOH, or 100

μM NS-398. These concentrations have been shown to maximally inhibit the LDF response to whisker stimulation *in vivo* (29, 32, 38). For SC-560, a concentration of 25 μM, which produces maximum inhibition of the LDF response to bradykinin and hypercapnia (30), and a concentration of 500 μM, which inhibits the arteriolar dilation induced by astrocyte activation (41), were tested in two additional groups. In other experiments, alloxazine was administered both systemically (1 mg/kg *iv*) and by CSF superfusion (1 μM) based on previous work (38) showing inhibition of the LDF response to whisker stimulation. Because 7-NI is relatively insoluble in aqueous solution, it was given intraperitoneally at a dose of 40 mg/kg 1 h before DHPG superfusion. This dose produces stable reductions in the LDF response to whisker stimulation for at least 2 h (23). CrMPiX was superfused at a concentration of 15 μM, which has been shown to inhibit pial arteriolar dilation and CO production in response to glutamate in the piglet (36) and pial arteriolar dilation in response to AMPA in the rat (31). HET0016 was superfused at a concentration of 1 μM, which has been found to increase the LDF responses to whisker stimulation after nNOS inhibition (23) and to block cerebral vasoconstriction after transfusion of cell-free polymeric hemoglobin (35). The sEH inhibitor *t*-AUCB was chosen based on its relatively high potency, solubility, and bioavailability (18, 42) and was superfused at a concentration of 0.5 μM. This concentration in the perfusate of postschemic, isolated hearts produced the maximum recovery of developed pressure (7).

A control group consisting of 10 rats was superfused with one of three vehicles in artificial CSF for 1 h followed by 1 h of DHPG superfusion. The vehicles were 0.1% ethanol (vehicle for 14,15-EEZE, MS-PPOH, HET0016, and *t*-AUCB), 0.1% DMSO (vehicle for NS-398), and 1 mM NaOH (vehicle for LY-367385, alloxazine, and CrMPiX). Because none of the vehicles had a significant effect on the LDF response to DHPG compared with a preliminary group superfused with DHPG in artificial CSF alone, the responses of all the vehicle treatment groups were pooled.

Isolated cerebral artery studies. To assess the potential direct effects of DHPG on cerebral arterial tone, isolated small branches of rat middle cerebral arterial segments (45–120 μm inner diameter; 0.5–1 mm in length) were placed in a perfusion chamber, cannulated with glass micropipettes, and secured in place with 8-0 polyethylene suture. Side branches were tied off with 10-0 polyethylene suture using a stereomicroscope. The cannulated arterial segments were bathed in physiological salt solution equilibrated with a 95% O₂-5% CO₂ gas mixture at 37°C. The physiological salt solution bathing the arterial segments was composed of the following (in mM): 130 NaCl, 2.5 CaCl₂, 15 NaHCO₃, 1.2 MgSO₄, 1.2 NaH₂PO₄, 4.7 KCl, 5.5 glucose, 10 HEPES, and 0.026 EDTA, pH 7.4. The inflow cannula was connected in series with a volume reservoir and a pressure transducer so that intraluminal pressure was controlled and monitored. The small cerebral arterial segments were stretched to their *in vivo* length using a micrometer, the outflow cannula was clamped off, and intramural pressure was adjusted to 40 mmHg. Following a 30-min equilibration period, the arterial segments were pressurized to 80 mmHg. The internal diameter was measured with a videomicrometer system (Living Systems Instrumentation, Burlington, VT) for 10 min before and after abluminal application of 1 mM DHPG. Following repeated washout of DHPG, 80 mM KCl were added to the bath to examine the maximal contractile response of the cerebral arterial segments.

In separate studies, the cerebral vasodilatory actions of the EET isomers 11,12-EET and 14,15-EET were examined following incubation of the cannulated and pressurized (40 mmHg) cerebral arterial segments with 17-octadecynoic acid (10 μM) for 30 min to inhibit endogenous formation of both 20-HETE and EETs. In addition, the cerebral arterial segments were pretreated with the sEH inhibitor *t*-AUCB (0.5 μM) to prevent hydrolysis of exogenously added EETs. Following these conditional treatments, the cannulated cerebral arterial segments were pressurized to 80 mmHg and constricted by

application of 50 μM serotonin to the bath. The percent change in vessel diameter was measured in response to stepwise increasing concentrations of 1, 10, 100, and 300 nM of 11,12-EET and 14,15-EET.

Statistical analysis. The LDF response to DHPG was averaged in 5-min intervals over the 60-min superfusion period. The LDF response was calculated as a percentage of the baseline value taken as the mean value over 20-min period before DHPG superfusion was initiated. For each treatment group, LDF responses were compared with the combined vehicle control group by two-way ANOVA for repeated measures followed by a Newman-Keuls multiple range test. A $P < 0.05$ was considered to be significant. Data are presented as means \pm SE.

RESULTS

Subarachnoid superfusion of 1 mM DHPG produced a gradual increase in LDF that reached $25 \pm 3\%$ 5–10 min after initiation of the infusion and a peak value of $30 \pm 2\%$ after 15–20 min of superfusion (Fig. 1). The relatively slow time course for the onset of the increase in LDF is probably related to the time required to wash out the endogenous CSF and the time required for diffusion of DHPG into the tissue sampled by the LDF probe. In previous work (38), the CSF outflow concentration of a radiolabeled adenosine A_{2A} antagonist required 10–15 min to achieve 80% of the inflow concentration when superfused at the same rate of 5 $\mu\text{l}/\text{min}$. Thus a similar amount of time is likely required for equilibration of the inflow and outflow concentration of DHPG. The LDF response gradually waned to $12 \pm 4\%$ after 30 to 60 min of DHPG superfusion. Astrocytes express type I mGluR1 and mGluR5 (14). Superfusion of the mGluR1 antagonist LY-367385 (300 μM) plus the mGluR5 antagonist MPEP (100 μM) blocked the increase in LDF during DHPG superfusion (Fig. 1), thereby confirming that DHPG was acting via mGluR.

The LDF response to DHPG was markedly suppressed by the EET antagonist 14,15-EEZE (30 μM ; Fig. 2). Furthermore, the response was partially attenuated by the COX-2 inhibitor NS-398 (100 μM) during the first 30 min of DHPG

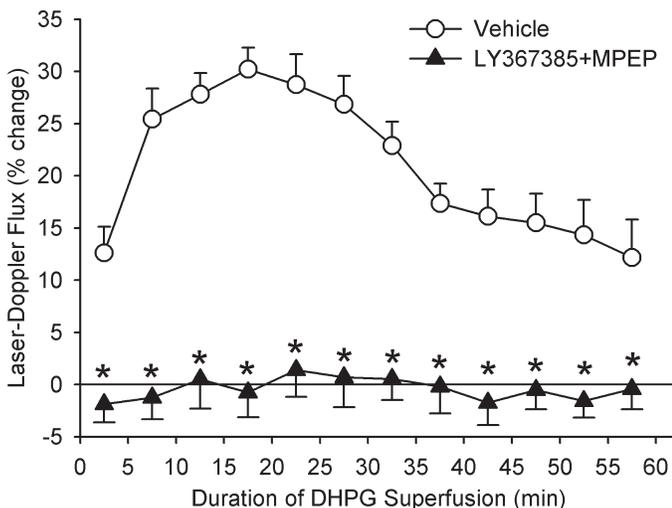


Fig. 1. Percent change in cortical laser-Doppler flux (\pm SE) during 60 min of subarachnoid superfusion of dihydroxyphenylglycine (DHPG) in the presence of vehicle ($n = 10$) or the metabotropic glutamate receptor antagonists LY-367385 and 2-methyl-6-(phenylethynyl)pyridine (MPEP; $n = 6$). * $P < 0.05$, from vehicle treatment.

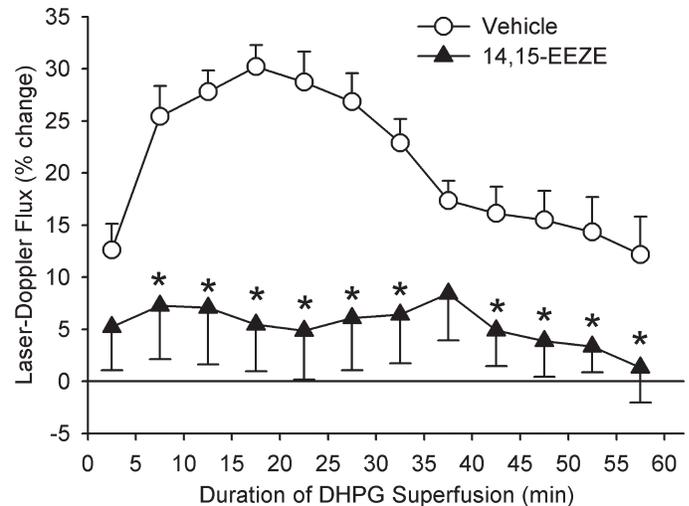


Fig. 2. Percent change in cortical laser-Doppler flux (\pm SE) during 60 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$) or the epoxyeicosatrienoic acid antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE; $n = 6$). * $P < 0.05$, from vehicle treatment.

superfusion (Fig. 3). However, the COX-1 inhibitor SC-560 at a concentration of 25 μM had no significant effect on the response (Fig. 4). Increasing the concentration to 500 μM produced a variable response that was significantly different from the control response only during 5–10 min of superfusion.

The effects of the various treatments on the LDF responses to DHPG at 15–20 min of superfusion are summarized in Fig. 5. In addition to 14,15-EEZE and NS-398, the response to DHPG was significantly reduced by the EET synthesis inhibitor MS-PPOH (20 μM). In contrast, the response to DHPG at 15–20 min was not affected by administration of the A_{2B} receptor antagonist alloxazine, the nNOS inhibitor 7-NI, the heme oxygenase inhibitor CrMPIX, or the 20-HETE synthesis inhibitor HET0016 (Fig. 5).

Although alloxazine, 7-NI, and CrMPIX had no effect on the peak vasodilator response seen 15–20 following administration

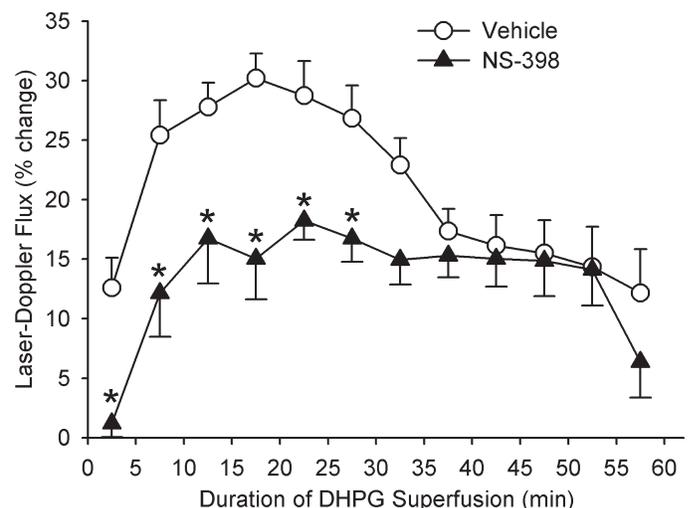


Fig. 3. Percent change in cortical laser-Doppler flux (\pm SE) during 60 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$) or the cyclooxygenase-2 inhibitor NS-398 ($n = 6$). * $P < 0.05$, from vehicle treatment.

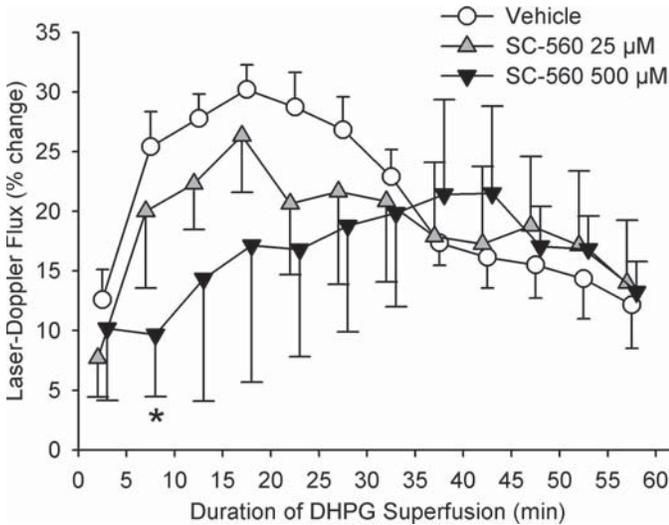


Fig. 4. Percent change in cortical laser-Doppler flux (\pm SE) during 60 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$) or the cyclooxygenase-1 inhibitor SC-560 at a concentration of 25 μ M ($n = 6$) or 500 μ M ($n = 6$). * $P < 0.05$, from vehicle treatment.

of DHPG, these inhibitors had significant effects at earlier time points. Alloxazine and CrMPIX significantly attenuated the increase in LDF 5–15 min after the start of DHPG superfusion, and 7-NI significantly attenuated the LDF response at 5–10 min (Fig. 6). However, alloxazine, 7-NI, and CrMPIX did not significantly attenuate the LDF response beyond 15 min of DHPG superfusion. HET0016 attenuated the LDF response at

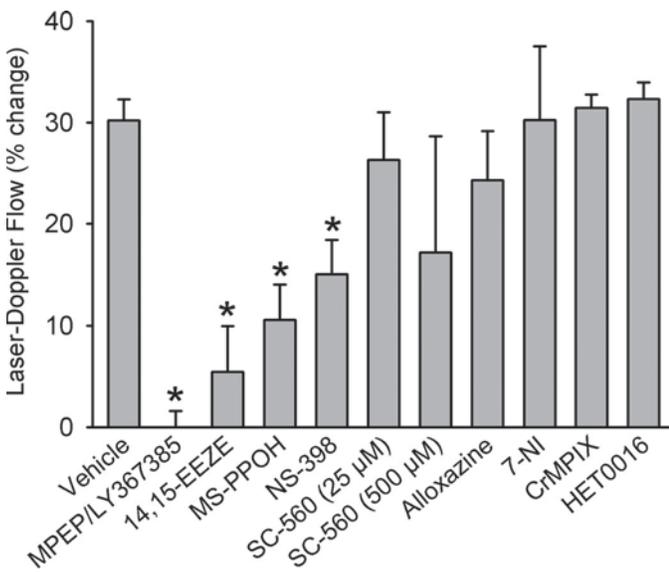


Fig. 5. Comparison of percent change in cortical laser-Doppler flux (\pm SE) at 15–20 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$), LY-367385 + MPEP ($n = 6$), the EETs antagonist 14,15-EEZE ($n = 6$), the EETs synthesis inhibitor *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH; $n = 6$), the cyclooxygenase-2 inhibitor NS-398 ($n = 6$), the cyclooxygenase-1 inhibitor SC-560 at a concentration of 25 μ M ($n = 6$) or 500 μ M ($n = 6$), the A_{2B} receptor antagonist alloxazine ($n = 6$), the neuronal nitric oxide synthesis inhibitor 7-nitroindazole (7-NI; $n = 6$), the heme oxygenase inhibitor chromium mesoporphyrin IX (CrMPIX; $n = 6$), or the 20-hydroxyeicosatetraenoic acid (20-HETE) synthesis inhibitor *N*-hydroxy-*N'*-(4-*n*-butyl-2-methylphenyl)formamidine (HET0016; $n = 6$). * $P < 0.05$, from vehicle treatment.

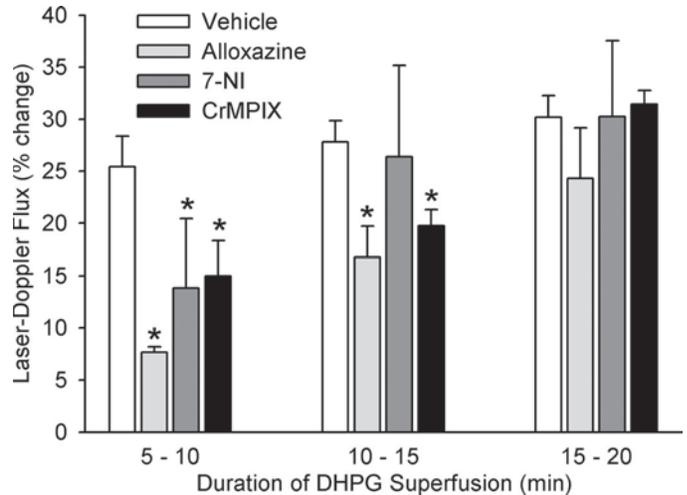


Fig. 6. Percent change in cortical laser-Doppler flux (\pm SE) during 5–10, 10–15, and 15–20 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$), alloxazine ($n = 6$), 7-NI ($n = 6$), or CrMPIX ($n = 6$). Although these inhibitors had no significant effect on the peak response compared with vehicle, they attenuated the early increase at specific times. * $P < 0.05$, from vehicle treatment.

0–5 min of DHPG superfusion (Fig. 7). HET0016 also prevented the fall in the DHPG response seen between 35 and 60 min of DHPG administration.

To determine if the response to DHPG was limited by degradation of EETs by sEH, the sEH inhibitor *t*-AUCB was superfused. The sEH inhibitor had no effect on the LDF response over the first 25 min of DHPG superfusion. However, the LDF response was enhanced by 25–30 min, and the decrease in LDF seen between 30 and 60 min in the vehicle group was prevented by *t*-AUCB superfusion (Fig. 8).

Arterial pH, blood gases, and hemoglobin concentration remained within the normal physiological range in all groups during DHPG superfusion (Table 1). Administration of the various inhibitors and superfusion of DHPG did not significantly change arterial blood pressure (Table 2). Moreover,

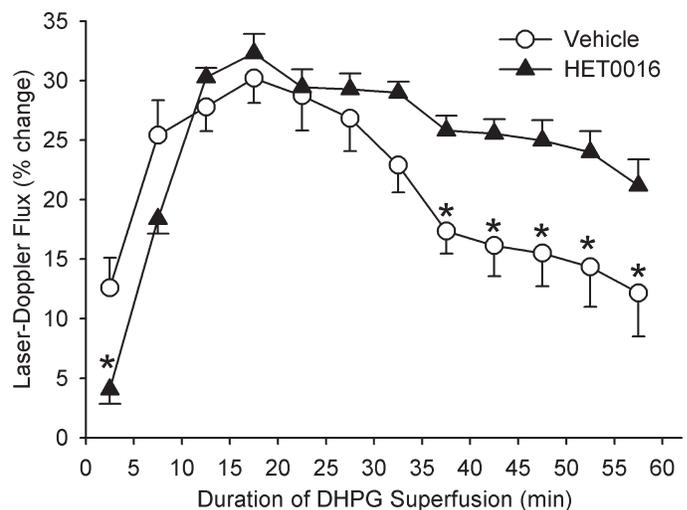


Fig. 7. Percent change in cortical laser-Doppler flux (\pm SE) during 60 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$) or the 20-HETE synthesis inhibitor HET0016 ($n = 6$). * $P < 0.05$, from vehicle treatment.

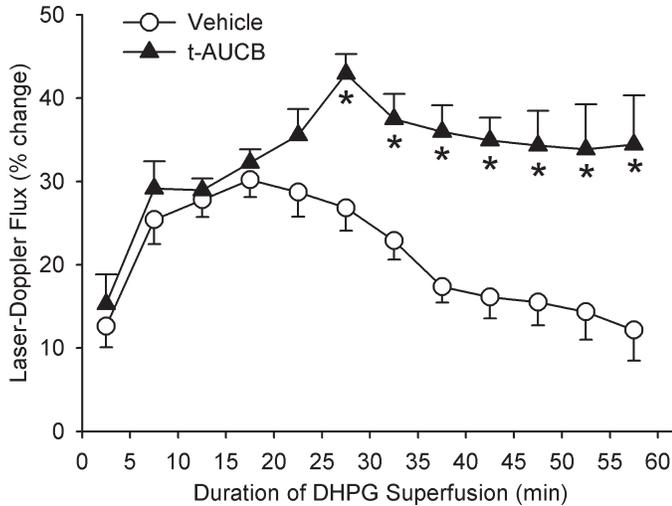


Fig. 8. Percent change in cortical laser-Doppler flux (\pm SE) during 60 min of subarachnoid superfusion of DHPG in the presence of vehicle ($n = 10$) or the soluble epoxide hydrolase inhibitor *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB; $n = 6$). * $P < 0.05$, from vehicle treatment.

baseline LDF was not significantly altered by administration of LY-367385 + MPEP, 14,15-EEZE, MS-PPOH, NS-398, alloxazine, CrMPIX, or *t*-AUCB (Table 2). However, baseline LDF decreased significantly following administration of 25 and 500 μ M SC-560 and by 7-NI. Baseline LDF was slightly increased following administration of HET0016.

To determine if DHPG has a direct effect on cerebral arteries, branches of the middle cerebral artery were isolated and pressurized to 80 mmHg. Arterial diameter was not affected by abluminal exposure to 1 mM DHPG for 10 min (Fig. 9). After washout of DHPG, addition of 80 mM KCl produced a $71 \pm 3\%$ constriction, thereby indicating that the arterial segments were functional.

The direct effect of 11,12-EET and 14,15-EET on arterial diameter was measured in isolated, pressurized branches of the middle cerebral artery after preconstruction with serotonin. Application of either regioisomer induced similar concentration-dependent increases in diameter of the cerebral arterial segments (Fig. 10). The dilation was significant over the 1- to 300-nM range of concentrations that was tested for each regioisomer ($P < 0.01$).

DISCUSSION

This study demonstrates that the group I mGluR agonist DHPG increases CBF in rat cerebral cortex following in vivo superfusion of the cortical surface, but it has no direct effect on the tone of isolated cerebral arteries in vitro. The increase in CBF is largely dependent on the synthesis and release of EETs because the response was greatly attenuated by administration of the selective epoxygenase inhibitor MS-PPOH and the putative EET receptor antagonist 14,15-EEZE. Moreover, a COX-2 metabolite is required for full expression of the CBF response, whereas a COX-1 metabolite is less important for the in vivo response. In contrast, blockade of adenosine A_{2B} receptors, nNOS activity, and heme oxygenase activity had no effect on the peak blood flow response, although the initial response was attenuated. With prolonged mGluR activation, the CBF response gradually fades and this waning appears to be related to breakdown of EETs by sEH and a gradual accumulation of the vasoconstrictor, 20-HETE.

With neuronal activation, mGluR antagonists attenuate but do not completely block the in vivo CBF response (47). Administration of an EET antagonist or an inhibitor of the synthesis of EETs also attenuates the CBF response to neuronal activation, and combining these agents with a mGluR antagonist does not produce a substantial additional decrement in the response (38). These findings suggest that the mGluR-dependent component of the CBF response to neuronal activation is associated with the formation and actions of EETs. The present findings, which showed that the CBF response to selective mGluR activation is largely suppressed by 14,15-EEZE and MS-PPOH, are also consistent with the concept that the mGluR-activated component of vasodilation is highly dependent on EETs. Astrocyte-derived EETs may act in an autocrine role to promote increases in astrocyte Ca^{2+} and opening of astrocytic K_{Ca} channels (5, 11, 14, 46). EETs may also be released from astrocytes to act in a paracrine manner to open K_{Ca} channels and hyperpolarize vascular smooth muscle cells (1). Here, we show that 11,12-EET and 14,15-EET are direct, potent dilators of isolated cerebral arteries in the nanomolar concentration range and thus are capable of acting in a paracrine manner.

The concentration of 14,15-EEZE (30 μ M) used in vivo was chosen to slightly exceed the 10- μ M concentration previously shown to antagonize the vasodilator response to EETs in

Table 1. Arterial pH and blood gas values in rats during superfusion of dihydroxyphenylglycine

Treatment	pH	Paco ₂ , Torr	PaO ₂ , Torr	Hemoglobin, g/dl
Vehicle	7.40 \pm 0.01	39 \pm 0.4	113 \pm 4	11.8 \pm 0.2
MPEP/LY-367385	7.43 \pm 0.01	36 \pm 0.7	113 \pm 3	12.7 \pm 0.3
14,15-EEZE	7.41 \pm 0.01	38 \pm 0.7	120 \pm 3	12.3 \pm 0.4
MS-PPOH	7.39 \pm 0.01	38 \pm 0.9	118 \pm 4	12.0 \pm 0.2
NS-398	7.41 \pm 0.01	38 \pm 0.4	115 \pm 6	11.5 \pm 0.4
SC-560 (25 μ M)	7.40 \pm 0.01	38 \pm 0.7	102 \pm 3	11.8 \pm 0.3
SC-560 (500 μ M)	7.40 \pm 0.01	37 \pm 0.8	112 \pm 5	11.6 \pm 0.4
Alloxazine	7.39 \pm 0.01	38 \pm 0.7	125 \pm 6	11.6 \pm 0.2
7-Nitroindazole	7.39 \pm 0.01	37 \pm 0.8	129 \pm 4	12.4 \pm 0.2
CrMPIX	7.40 \pm 0.01	38 \pm 0.7	119 \pm 2	12.0 \pm 0.2
HET0016	7.41 \pm 0.01	38 \pm 0.5	116 \pm 4	12.5 \pm 0.2
<i>t</i> -AUCB	7.39 \pm 0.01	38 \pm 0.6	116 \pm 5	11.3 \pm 0.3

Values are means \pm SE. MPEP, 2-methyl-6-(phenylethynyl)pyridine; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; MS-PPOH, *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide; CrMPIX, chromium mesoporphyrin IX; *t*-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid.

Table 2. MABP and percent change in LDF with drug treatment before DHPG superfusion in rats

Treatment	MABP, mmHg			LDF with Drug Treatment, %baseline
	Baseline	Drug treatment	15- to 20-min DHPG	
Vehicle	96 ± 3	96 ± 3	98 ± 3	100 ± 1
MPEP/LY-367385	100 ± 2	98 ± 1	97 ± 2	102 ± 2
14,15-EEZE	91 ± 2	90 ± 2	90 ± 2	101 ± 2
MS-PPOH	93 ± 3	90 ± 3	92 ± 4	98 ± 1
NS-398	94 ± 3	93 ± 2	93 ± 3	100 ± 1
SC-560 (25 μM)	97 ± 3	98 ± 2	98 ± 2	91 ± 2*
SC-560 (500 μM)	96 ± 2	96 ± 1	99 ± 2	77 ± 9*
Alloxazine	88 ± 2	90 ± 3	90 ± 2	101 ± 1
7-Nitroindazole	106 ± 2	104 ± 1	104 ± 1	92 ± 2*
CrMPIX	96 ± 3	95 ± 3	96 ± 3	101 ± 2
HET0016	100 ± 2	99 ± 2	98 ± 2	104 ± 1*
t-AUCB	91 ± 2	92 ± 3	91 ± 2	101 ± 1

Values are means ± SE. MABP, mean arterial blood pressure; LDF, laser-Doppler flux; DHPG, dihydroxyphenylglycine. * $P < 0.05$, from baseline before drug treatment.

vitro (12, 38). The concentration of MS-PPOH (20 μM) superfused in vivo is slightly above the 13 μM concentration required to achieve a 50% inhibition (IC_{50}) of EET synthesis in vitro (44) and is the same as that which was previously shown to inhibit the CBF response to neuronal activation in vivo (32). Moreover, this concentration of MS-PPOH does not significantly inhibit 20-HETE synthesis or COX activity (44). Thus these structurally different drugs likely act by inhibiting the synthesis and/or actions of EETs in the present experiment.

COX can metabolize 5,6-EET but not the other EET regioisomers (24). However, 14,15-EET and its metabolites are the most abundant epoxygenase metabolites formed in astrocytes (3, 28). Moreover, MS-PPOH is still effective in inhibiting vasodilation to whisker stimulation after COX inhibition by indomethacin. Thus it is unlikely that a COX metabolite of 5,6-EET mediates the vasodilator response to DHPG.

Inhibition of COX-2 with a superfused concentration of 100 μM of NS-398 is known to attenuate but not abolish the CBF response to neuronal activation (29, 39). This concentration is considered to be selective for COX-2 because it has no effect

on COX-1- or NO-dependent vascular responses to bradykinin, acetylcholine, or hypercapnia, and it has no effect in COX-2-null mice (29). Constitutive expression of COX-2 has been found in a subpopulation of cortical neurons (43) but has not been detected in cortical astrocytes (41). Thus a COX-2 metabolite released from neurons might act directly on vascular smooth muscle to directly produce vasodilation during neuronal activation. However, our results with 100 μM NS-398 show that a COX-2 metabolite likely contributes to the vascular response to a mGluR agonist. Assuming that the mGluR agonist is acting solely on astrocytes, a COX-2 metabolite released from neurons might interact with mGluR-evoked signaling within astrocytes or within vascular smooth muscle.

At a concentration of 25 μM, the COX-1 inhibitor SC-560 has been reported to inhibit cortical vasodilation induced by bradykinin and hypercapnia but not that evoked by sensory stimulation (30). In the present study, a concentration of 25 μM SC-560 was sufficient to reduce baseline CBF but had no effect on the percent increase in CBF during DHPG superfusion. Thus a COX-1 metabolite does not appear to be essential for mediating the vasodilatory response to either neuronal activation or mGluR stimulation in vivo. Interestingly, a higher dose of 500 μM SC-560 inhibited arteriolar dilation evoked by photolysis of astrocyte caged Ca^{2+} in mouse cerebral cortex in vivo (41), whereas MS-PPOH was ineffective in inhibiting this response. In the present study, this high concentration of SC-560 significantly decreased the LDF response to DHPG at only one early time point, and the effect was variable at other time points. Although an increase in astrocytic Ca^{2+} is thought to play a key role in mediating mGluR signaling, the spatial and temporal characteristics of Ca^{2+} signaling evoked by physiological neuronal activation, photolysis of astrocytic caged Ca^{2+} , and prolonged exposure to a mGluR agonist are different and consequently may produce vasodilation by different mechanisms. Moreover, the relationship of the vascular response to Ca^{2+} in astrocyte endfeet is bimodal (15). Furthermore, SC-560 has been reported to lose its COX-1 selectivity and to inhibit COX-2 in the low nanomolar concentration range in some cell systems (6). Thus interpretation of the results with the high concentration of 500 μM SC-560 may be problematic.

Adenosine A_{2B} antagonists, including alloxazine, have been reported to attenuate the CBF response to neuronal activation, and combining alloxazine with a mGluR antagonist does not

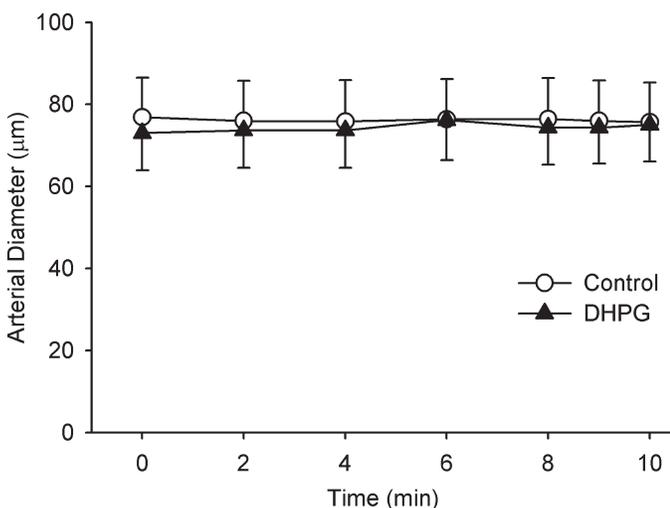


Fig. 9. Internal diameter of isolated, pressurized branches of rat middle cerebral artery (\pm SE; $n = 6$) during a 10-min time control period and after addition of 1 mM of DHPG to the external bathing solution. Two-way repeated-measures ANOVA indicated no significant effect of treatment or interaction of treatment with time.

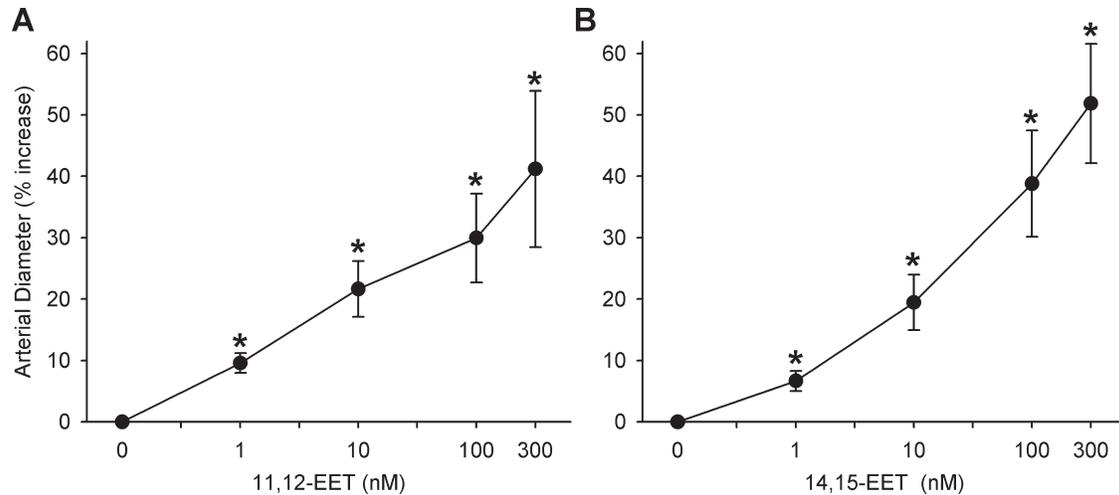


Fig. 10. Percent change of internal diameter of isolated, pressurized branches of rat middle cerebral artery (\pm SE; $n = 6$) that were precontracted with 50 μ M serotonin and then exposed to increasing concentrations of 1–300 nM of either 11,12-EET (A) or 14,15-EET (B). * $P < 0.01$, from baseline.

produce an additional reduction in the response (38). The lack of an additive effect suggests that stimulation of A_{2B} receptors participates in the mGluR-dependent component of the CBF response to neuronal activation. However, in the present study, alloxazine did not decrease the peak LDF response to DHPG, although the initial response was attenuated when the concentration of DHPG in the underlying cortical tissue presumably was still increasing. Activation of A_{2B} receptors on astrocytes can increase intracellular Ca^{2+} (27, 34) and may facilitate Ca^{2+} signaling during physiological neuronal activation. Our results imply that A_{2B} receptors may contribute to the increase in blood flow when the concentration of DHPG between the cortical surface and deep tissue is exerting a submaximal effect on LDF. However, during the peak response, the increase in astrocyte Ca^{2+} produced by DHPG may be sufficient to produce vasodilation without requiring facilitation by A_{2B} receptor stimulation.

Activation of nNOS also contributes to part of the hyperemic response evoked by neuronal activation (8, 22). Moreover, the mGluR agonist *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) is capable of increasing NOS activity in vivo (4). However, ACPD can act on both group I and group II mGluR, whereas DHPG appears more selective for group I mGluR (37). In the present study, DHPG is more likely to produce vasodilation directly through group I mGluR astrocyte pathways that do not require activation of nNOS and thus account for the lack of effect of 7-NI on the peak LDF response evoked by DHPG. However, 7-NI did attenuate the LDF response at 5–10 min of DHPG superfusion, and we cannot exclude an effect of DHPG acting on neuronal group I mGluR. Alternative explanations are that astrocyte activation might feed back on neuronal glutamate receptors (17) to activate nNOS or that a decrease in NO bioavailability after 7-NI administration enables cytochrome P450 synthesis of 20-HETE (23, 40), which then acts to blunt vasodilation at this early time point.

In adult rats, application of AMPA produces pial arterial dilation that is partly reduced by a heme oxygenase inhibitor (31). In piglet cortex, application of glutamate produces pial arterial dilation that is dependent on heme oxygenase activity (20, 36). Astrocytes appear to contribute to an increase in CO

in response to glutamate in immature piglets (20, 21). In the present study, CrMPIX moderately attenuated the LDF response to early superfusion of DHPG but did not affect the peak response. Thus it is possible that CO may contribute to the vasodilatory response when mGluR activation is submaximal but that CO is not necessary for the maximal flow response in mature rat brain. Differences in development, species, and arteriolar segments (pial arteries vs. intraparenchymal arterioles) need to be considered in evaluating the role of heme oxygenase in gliovascular coupling.

In cortical brain slices, application of ACPD produces 20-HETE-dependent constriction in arterioles with low basal tone and EET-dependent dilation in arterioles with high basal tone (5). Moreover, the 20-HETE-dependent constriction arising from photolysis of caged Ca^{2+} is thought to be caused by astrocytic phospholipase activity in astrocytes that releases arachidonic acid, which is the substrate for the synthesis of 20-HETE in vascular smooth muscle (26). In the present experiment, the 20-HETE synthesis inhibitor HET0016 was superfused at a concentration of 1 μ M, which is well above the IC_{50} of 35 nM for inhibiting 20-HETE synthesis in rat renal microsomes but well below the IC_{50} of 2.8 μ M for inhibiting EET synthesis and COX activity in rat renal microsomes (25) especially considering that HET0016 is avidly bound to proteins. HET0016 was found to produce a small increase in baseline CBF, but it did not affect the maximum increase in CBF produced by DHPG. This result suggests that 20-HETE synthesis does not initially oppose the EET-dependent dilation evoked by mGluR stimulation. However, HET0016 did significantly attenuate the CBF response during the first 5 min of DHPG superfusion. This observation implies that 20-HETE might serve as a vasodilator when the concentration of DHPG is beginning to increase in the cortical tissue. In this regard, 20-HETE has been shown to be avidly metabolized by COX (24) to 20-hydroxy-PGE₁, and 20-HETE has been reported to produce vasodilation of basilar artery by a COX-dependent mechanism (10). Thus one explanation for the initial response to HET0016 over the first 5 min of DHPG superfusion is that a COX metabolite of 20-HETE contributes to the vasodilatory response. In contrast, when mGluR activation is prolonged

beyond 30 min, the CBF response subsided and HET0016 prevented this decrement. This result implies that 20-HETE-dependent constriction acts to oppose the vasodilation seen following prolonged mGluR activation.

Administration of the sEH inhibitor *t*-AUCB did not affect the CBF response during the first 20 min of superfusion of DHPG. However, the CBF response continued to increase between 20–30 min, and the augmented CBF response did not wane beyond 30 min. This suggests that metabolism of EETs by sEH does not limit the CBF response to the initial activation of mGluR but that breakdown of EETs apparently becomes a limiting factor when mGluR activation is prolonged. Whether EET metabolism becomes a limiting factor with prolonged physiological activation remains to be determined.

Another consideration is that the attenuation of the vasodilator response to prolonged stimulation of mGluR may be due to the nonlinear vascular response to astrocyte Ca^{2+} . Whereas moderate increases in astrocyte Ca^{2+} are associated with vasodilation in mouse cortical slices, large increases in astrocyte Ca^{2+} are associated with vasoconstriction that is related to augmented release of K^+ from astrocyte K_{Ca} channels (15). Thus one explanation for the diminished CBF response beyond 30 min of DHPG superfusion is that prolonged mGluR stimulation produces a large astrocyte Ca^{2+} response and results in an extracellular K^+ concentration in the perivascular space that exceeds the K^+ concentration required for maximal vasodilatory response mediated by smooth muscle inward-rectifier K^+ channels. In support of this possibility, ACPD was found to decrease rather than increase CBF in mouse cortex when extracellular K^+ concentration was raised from 3 to 15 mM, and the corresponding increases and decreases in the CBF response to ACPD were both attenuated by an inhibitor of astrocyte K_{Ca} channels (15).

The EET and COX-2 inhibitors affected the CBF response to the mGluR agonist and not baseline flow, whereas the COX-1 and nNOS inhibitors reduced the baseline flow but did not substantially affect the response to the mGluR agonist. Thus EET and COX-2 metabolites appear more important for dynamic vascular responses to activation of parenchymal cells, whereas COX-1 metabolites and NO are important for steady-state inhibition of vascular tone. This raises the question of why the EET and COX-2 metabolites are not inhibiting vasomotor tone under baseline conditions when there is baseline electrical activity. Perhaps there is a threshold of activation that is required for astrocyte signaling and anesthesia lowers spontaneous activity below that threshold. In addition, the synthesis of EETs and 20-HETE is substrate limited, and arachidonic acid is rapidly reincorporated in membrane phospholipids. Thus one may need a stimulus, in this case an mGluR agonist, to release arachidonic acid and stimulate the production of these modulators of vasomotor tone.

In summary, activation of group I mGluR in rat cerebral cortex increases CBF in vivo, which is primarily dependent on the synthesis and release of EETs and partially dependent on the formation and release of COX-2 metabolites. With prolonged activation of mGluR, the vasodilator response fades apparently due to the breakdown of EETs by sEH and by accumulation of the vasoconstrictor 20-HETE.

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DISCLOSURES

B.D. Hammock and S. H. Hwang are authors of a patent on soluble epoxide hydrolase inhibitors and their biology; the patent is held by the University of California.

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