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Interaction of mechanisms involving epoxyeicosatrienoic acids, adenosine receptors, and metabotropic glutamate receptors in neurovascular coupling in rat whisker barrel cortex

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Abstract

Adenosine, astrocyte metabotropic glutamate receptors (mGluRs), and epoxyeicosatrienoic acids (EETs) have been implicated in neurovascular coupling. Although A_{2A} and A_{2B} receptors mediate cerebral vasodilation to adenosine, the role of each receptor in the cerebral blood flow (CBF) response to neural activation remains to be fully elucidated. In addition, adenosine can amplify astrocyte calcium, which may increase arachidonic acid metabolites such as EETs. The interaction of these pathways was investigated by determining if combined treatment with antagonists exerted an additive inhibitory effect on the CBF response. During whisker stimulation of anesthetized rats, the increase in cortical CBF was reduced by approximately half after individual administration of A_{2B}, mGluR and EET antagonists and EET synthesis inhibitors. Combining treatment of either a mGluR antagonist, an EET antagonist, or an EET synthesis inhibitor with an A_{2B} receptor antagonist did not produce an additional decrement in the CBF response. Likewise, the CBF response also remained reduced by ~50% when an EET antagonist was combined with an mGluR antagonist or an mGluR antagonist plus an A_{2B} receptor antagonist. In contrast, A_{2A} and A_3 receptor antagonists had no effect on the CBF response to whisker stimulation. We conclude that (1) adenosine A_{2B} receptors, rather than A_{2A} or A_3 receptors, play a significant role in coupling cortical CBF to neuronal activity, and (2) the adenosine A_{2B} receptor, mGluR, and EETs signaling pathways are not functionally additive, consistent with the possibility of astrocytic mGluR and adenosine A_{2B} receptor linkage to the synthesis and release of vasodilatory EETs.

Keywords

cerebral circulation; epoxygenase; functional activation; nitric oxide; vibrissae

Introduction

The coupling of increased cerebral blood flow (CBF) to increased neuronal activity is of fundamental physiologic importance. Signaling pathways involved in the functional hyperemic response include neuronally derived nitric oxide (NO), cyclooxygenase (COX) activity, adenosine, and the epoxyeicosatrienoic acid (EET) products of cytochrome *P*450 metabolism

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of arachidonic acid (Koehler *et al*, 2006). In the cerebral cortex, NO is viewed as a modulator, rather than mediator, of the hyperemic response (Lindauer *et al*, 1999). Cyclooxygenase-2, which is constitutively expressed in selected cortical neurons (Wang *et al*, 2005), has been implicated as a mediator of the CBF response to whisker stimulation in mouse (Niwa *et al*, 2000). Cyclooxygenase-1 has been implicated in cortical vasodilation in response to increased Ca^{2+} in mouse astrocyte end-feet (Takano *et al*, 2006), although COX-1 gene deletion or inhibition does not reduce the CBF response to vibrissal stimulation (Niwa *et al*, 2001). In addition, astrocytes in rats express cytochrome *P*450 2C11 (Alkayed *et al*, 1996; Peng *et al*, 2004), which possesses expoxygenase activity and releases EETs in response to glutamate (Alkayed *et al*, 1997). Inhibition of EET synthesis in rats attenuates the CBF response to vibrissal (Peng *et al*, 2002) and forepaw stimulation (Peng *et al*, 2004).

One hypothesis is that release of the neurotransmitter glutamate stimulates metabotropic glutamate receptors (mGluRs) on astrocytes to increase Ca^{2+}, mobilize arachidonic acid, and stimulate production and release of COX products (Zonta *et al*, 2003) and EETs from astrocytes (Alkayed *et al*, 1997; Gebremedhin *et al*, 2003; Metea and Newman, 2006), which then mediate vasodilation (Gebremedhin *et al*, 1992; Takano *et al*, 2006).

A role for adenosine is supported by the observations that adenosine deaminase and the nonselective adenosine antagonist theophylline attenuated the CBF response to whisker stimulation by approximately 40% in the rat (Dirnagl *et al*, 1994) and that theophylline attenuated pial arteriolar dilation in response to sciatic nerve stimulation (Ko *et al*, 1990). However, the role of specific adenosine receptors in the CBF response is unclear. Dilation of cerebral arterioles to exogenous adenosine is attenuated by the selective A_{2A} antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol (ZM-241385) and by the selective A_{2B} antagonist alloxazine (Ngai *et al*, 2001; Shin *et al*, 2000). Moreover, ZM-241385 inhibits pial arteriolar dilation to sciatic nerve stimulation and to glutamate (Iliff *et al*, 2003; Meno *et al*, 2001), and ZM-241385 and alloxazine each attenuate pial arteriolar dilation to activation of AMPA receptors (Ohata *et al*, 2006). Whether A_{2A} and A_{2B} receptors contribute to whisker stimulation-evoked CBF response, which largely depends on dilation of intraparenchymal arterioles, has not been demonstrated. Moreover, various adenosine receptors are functional in astrocytes (Fields and Burnstock, 2006), and A_{2B} receptors appear to be particularly important in amplifying ATP-dependent increases in intracellular Ca^{2+} (Alloisio *et al*, 2004; Jimenez *et al*, 1999; Pilitsis and Kimelberg, 1998). Thus, it is possible that adenosine may act upstream from vascular smooth muscle to increase astrocyte Ca^{2+}, which could act in concert with mGluR-mediated Ca^{2+} increase to promote vasodilation that is dependent on arachidonic acid metabolites. In addition, expression of A_3 receptors has been described in cerebral vessels (Di Tullio *et al*, 2004), but their function has not been well delineated (Ngai *et al*, 2001).

Because adenosine antagonists, mGluR antagonists, and epoxygenase inhibitors do not completely block the CBF response to cortical activation, these mechanisms may act by parallel, additive pathways. The purpose of this study was to determine (1) whether adenosine A_{2A}, A_{2B}, and A_3 receptors contribute to the increase in CBF during whisker stimulation, (2) whether the attenuating effect of inhibiting the cytochrome *P*450 epoxygenase pathway on the CBF response to cortical activation depends on adenosine receptor or mGluR activation, and (3) whether inhibiting adenosine receptor and mGluR results in additive inhibition of the CBF response. The specific hypotheses tested were as follows: (1) the CBF response to whisker stimulation is attenuated by the A_{2A} antagonists ZM-241385 and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4, 3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH-58261), the A_{2B} antagonists alloxazine and 8-[4-[((4-cyanophenyl)-carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine (MRS-1754), and A_3 antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191); (2)

blocking the cytochrome *P*450 epoxygenase pathway produces additional attenuation of the CBF response in the presence of A_{2A}, A_{2B}, and mGluR antagonists; and (3) blocking the particular adenosine receptor that contributes to the CBF response produces additional inhibition of the CBF response in the presence of an mGluR antagonist.

Previous work used the EETs synthesis inhibitor *N*-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) or miconazole to reduce the CBF response to functional activation (Peng *et al*, 2002, 2004). Because preformed, stored EETs can be released from the phospholipid membrane (Shivachar *et al*, 1995), the EET antagonists 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) and 14,15-epoxyeicosa-5(Z)-enoic methylsulfonylimide (14,15-EEZE-mSI) were also used to confirm the role of EETs in this study (Gauthier *et al*, 2002, 2003) and to test the interaction between EETs and adenosine pathways.

Methods

Surgical Procedures

All procedures were approved by the Johns Hopkins University Animal Care and Use Committee. The surgical preparation and experimental design are similar to that described previously (Peng *et al*, 2002). Male Wistar rats (230 to 300 g) were anesthetized with halothane and mechanically ventilated through a tracheostomy with approximately 30% O_2 and 1.5% halothane. A femoral artery was catheterized for arterial blood pressure measurement and for analysis of pH and partial pressure of CO_2 (P_{CO_2}) and O_2 (P_{O_2}). Rectal temperature was maintained near 37°C. The scalp and muscle over the parietal and temporal bone were retracted. The bone was thinned by drilling a 5-mm region overlying the whisker barrel sensory cortex (2 to 3 mm posterior and 7 mm lateral from bregma). Cortical perfusion in this region was measured with a laser-Doppler flow (LDF) probe (Perimed, North Royalton, OH) kept in a fixed position over the thinned skull with a stereotactic head holder. For local superfusion of drugs over the cortical surface, a PE-10 catheter was finely tapered to a diameter of ~120 μm and the tip was gently placed under the dura mater at a site 3 mm dorsal and posterior to the flow probe site. Artificial cerebrospinal fluid (CSF) was superfused and passively drained from a small hole in the skull and dura located approximately 2 to 3 mm ventral and anterior to the recording site. Halothane was discontinued, and a post-surgical sedation level of anesthesia was maintained for the duration of the experiment by administration of warmed α-chloralose (25 to 33 mg/kg, intraperitoneal, plus 12 to 15 mg/kg/h). This dose prevented spontaneous muscle movement and arterial hypertension.

Experimental Protocol

Subarachnoid superfusion of artificial CSF was initiated 1 h after discontinuing halothane and continued throughout the experiment at a rate of 5 μL/min. The LDF response to whisker stimulation commenced at least 1 h after starting the CSF superfusion. Whisker stimulation was achieved by mechanical displacement of multiple whiskers inserted through a screen mesh connected to a solenoid-driven piston (Peng *et al*, 2002). After recording LDF for a 60-sec baseline period for each stimulation trial, the whiskers were displaced at 5 Hz for 60 secs. The LDF was averaged over 1-sec intervals. The percentage change in LDF was calculated from the preceding 60-sec baseline period and was averaged on a second-by-second basis over three trials spaced 2 to 3 mins apart. After obtaining the records of three trials with CSF superfusion at the end of the first hour of the protocol, an antagonist/inhibitor was administered. Three trials of whisker stimulation were repeated after 1 h of superfusion of the drug. After the second hour of the protocol, either the antagonist/inhibitor superfusion continued for another hour, or a second drug was administered while the first drug continued to be superfused. After the third hour of the protocol, three trials of whisker stimulation were repeated.

To evaluate the time course and steady-state concentration of superfused drugs in the CSF, [^{3}H]-ZM-241385 (2 μCi/mL) was infused in CSF in four rats with subarachnoid superfusion. A well was constructed with acrylic cement around the outflow hole. Outflow CSF samples were collected at 5-min intervals over a 1-h period, and the concentration of radioactivity was analyzed as a percentage of the inflow concentration. Results were compared with CSF outflow from a standard closed cranial window (~200 μL volume) superfused with [^{3}H]-ZM-241385 at a rate of 200 μL/min in four rats.

Drug treatments included 1 mg/kg, intravenous, plus superfusion of 1 μmol/L ZM-241385 (vehicle = 0.01% dimethylsulfoxide (DMSO) in CSF; Tocris Cookson Inc., Ellisville, MO); 0.1 mg/kg + 0.1 mg/kg/h, intravenous, SCH-58261 (vehicle = 10 μL/kg DMSO diluted 1:100 in saline; Sigma Chemical Co., St Louis, MO); 1 mg/kg, intravenous, plus superfusion of 1 μmol/L alloxazine (vehicle = 0.1 mol/L NaOH diluted 1:10,000 in CSF; Sigma); 1 mg/kg, intravenous, plus superfusion of 1 μmol/L MRS-1754 (vehicle = 210 μL/kg DMSO diluted 6:100 in saline intravenous, and 0.1% DMSO in CSF; Sigma); superfusion of 1 μmol/L MRS-1191 (vehicle = 0.005% DMSO in CSF; Sigma); 1 mmol/L superfusion of the NO synthase inhibitor N$^{\omega}$-nitro-L-arginine (L-NNA; vehicle = CSF; Sigma); superfusion of 100 μmol/L of the Group I mGluR antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA; vehicle = CSF; Sigma); superfusion of 20 μmol/L miconazole (vehicle = 0.5% ethanol in CSF; Sigma); superfusion of 20 μmol/L MS-PPOH (vehicle = 0.1% ethanol in CSF); superfusion of 30 μmol/L 14,15-EEZE (vehicle = 0.1% ethanol in CSF); and superfusion of 30 μmol/L 14,15-EEZE-mSI (vehicle = 0.1% ethanol in CSF). The latter three compounds were synthesized by JR Falck. The concentration of the DMSO and ethanol vehicles used in these experiments did not affect the LDF response to whisker stimulation. The LDF response to whisker stimulation was examined in 18 groups of rats that received these drugs alone or in combination. An additional two groups received lower doses of alloxazine to obtain a dose–response relationship.

To determine if drugs that reduced the evoked LDF response were capable of reducing evoked potentials, somatosensory evoked potentials were examined. Electrical stimulation of the forepaw was used to maximize the evoked potential and to provide a precise triggering time for gating the averaging of evoked potentials, as described previously (Peng *et al*, 2002). Through subcutaneous electrodes, the foreleg was stimulated with 150-μs pulses of 2-mA current at a rate of 2.9 pulses/sec. The time-gated average of 128 repetitions of evoked potentials was recorded with a silver ball electrode placed over the dura near the forelimb primary sensory cortex. The amplitude between the first positive and first negative waves was measured in triplicate after 1 h of CSF superfusion and again after 1 h of superfusion of 30 μmol/L 14,15-EEZE, superfusion of 100 μmol/L AIDA, 1 mg/kg, intravenous, plus superfusion of 1 μmol/L ZM-241385, or 1 mg/kg, intravenous, plus superfusion of 1 μmol/L alloxazine. The percentage change in the primary wave of the evoked potential amplitude after drug administration was compared with the percentage change in a time-control group that was superfused with CSF for the second hour.

To determine the efficacy and selectivity of ZM-241385 and alloxazine in this model, the LDF responses to an A_{2A} agonist 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropranoic acid (CGS-21680; Tocris) and an A_2 agonist 5′-*N*-ethylcarboxamido-adenosine (NECA; Sigma) were tested. Although highly selective A_{2B} agonists are not currently available, alloxazine has been shown to be more potent than ZM-241385 in inhibiting pial arteriolar dilation to NECA (Shin *et al*, 2000). In one experiment, three groups were treated with vehicle (0.01% DMSO in CSF), ZM-241385, or alloxazine starting 1 h before superfusion of 0.3 μmol/L CGS-21680 for 1 h. In a second experiment, three groups were treated with vehicle (0.03% DMSO), ZM-241385, or alloxazine starting 1 h before superfusion of 10 μmol/L NECA for 1 h. Superfusion of the antagonist continued during the superfusion of the agonist.

Isolated Middle Cerebral Arteries

Rat middle cerebral arterial segments (8 to 10 mm in length and 200 to 250 μmol/L outer diameter) were isolated, placed in a perfusion chamber, cannulated at both ends with glass micropipettes, and secured in place with 8-0 polyethylene suture using a stereo microscope. Side branches of the arteries were tied off with 10-0 polyethylene suture. The arterial segments were perfused and superfused with physiologic salt solution (Gebremedhin *et al*, 1992) aerated with a 21% O_2–5% CO_2 gas mixture (balance N_2) and were maintained at 37°C and pH 7.4. A bolus of air was passed through the lumen to cause damage to the endothelium of the arterial segments. The inflow cannula was connected in series with a volume reservoir and a pressure transducer to allow continuous monitoring of transmural pressure. Internal diameter of the arteries was measured using a videomicroscopy system composed of a television camera and a videomicrometer, as described previously (Gebremedhin *et al*, 1992). After an equilibration period of 15 mins, the cannulated arteries were pressurized to 80 mm Hg, and maintained at this pressure throughout the course of the experiment. After an additional 15 mins equilibration, the vessels were preconstricted with 30 μmol/L serotonin to produce additional tone for measuring the relaxation response to EETs and acetylcholine. The relaxation response to 1 μmol/L acetylcholine was determined in attempt to judge the integrity of the endothelium. Arterial segments that constricted to serotonin and failed to dilate in response to acetylcholine were studied. After repeated washout and a further 30 mins equilibration of the arterial segments, the effects of 100 and 300 nmol/L 14,15-EET before and after treatment of the arterial segments with the EETs antagonist 14,15-EEZE-mSI (10 μmol/L) on the internal arterial diameter was determined.

Statistical Analysis

The percentage change in LDF was averaged over the 60-sec period of whisker stimulation. For each rat, an average percentage response was obtained from three trials for each hourly intervention. Within each group, the average percentage responses at 1, 2, and 3 h were tested for an effect of treatment by repeated measures analysis of variance. If the *F*-value was significant, differences in individual mean values at each time point were compared by paired *t*-test with Bonferroni correction. To determine if drug treatment altered baseline LDF without whisker stimulation, the percentage change in baseline LDF from the initial value at 1 h was calculated in each drug-treatment group and compared with the percentage change in baseline LDF in the time-control group by *t*-test. All values are presented as means ±s.d. The level of significance was set at $P < 0.05$.

Results

To determine the degree to which endogenous CSF dilutes drugs infused at a rate of 5 μL/min into the subarachnoid space, radiolabeled ZM-241385 was infused. The concentration of radioactivity in the outflow from the drainage hole on the opposite side of the LDF monitoring site reached 80±3% of the inflow concentration by the 10 to 15-min collection period and 95 ±6% by the 55 to 60-min period (Figure 1). Superfusion of a closed cranial window at a rate of 200 μL/min resulted in an outflow concentration equivalent to the inflow concentration by the 10 to 15-min collection period. Over the 1-h period, the area under the curve of the outflow concentration with subarachnoid superfusion was 84% of that of the cranial window outflow concentration. A 1-h period of subarachnoid superfusion was used to permit penetration of drugs into tissue sensitive to flowmetry by the LDF probe (Irikura *et al*, 1994).

Within each of the experimental groups subjected to whisker stimulation trials at 1-h intervals, mean arterial blood pressure and $PaCO_2$ remained stable in the physiologic range (Table 1). Arterial pH was in the range of 7.35 to 7.45, PaO_2 was maintained at 130 to 150 mm Hg, arterial

hemoglobin concentration was in the range of 10 to 13 g/dL, and rectal temperature was in the range of 36.5 to 37.5°C.

In the absence of whisker stimulation, baseline LDF remained unchanged at the second and third hours of CSF superfusion of cortex in the time-control group (Table 2). Stoppage of CSF superfusion did not change baseline LDF. As expected, the group superfused with L-NNA exhibited a decrease in baseline LDF. Except for a 29% increase in baseline LDF after 2 h of superfusion with 14,15-EEZE, none of the other antagonists and inhibitors produced a significant change in baseline LDF, compared with the time-control group.

The percentage change in LDF averaged over three trials of 60 secs of whisker stimulation did not change during 3 h of CSF superfusion in the time-control group (Figure 2A; $n = 12$). Treatment with the adenosine A_{2A} antagonist ZM-241385 (1 mg/kg, intavenous, plus 1-μmol/L superfusion) did not change the LDF response to whisker stimulation over a 2-h superfusion period, compared with the control response during CSF superfusion (Figure 2B; $n = 6$). However, administration of the adenosine A_{2B} antagonist alloxazine produced a dose-dependent reduction of the LDF response to whisker stimulation. At a dose of 0.1 mg/kg, intravenous, plus 0.1 μmol/L superfusion, the response was not significantly attenuated (111 ±7% of CSF baseline response; $n = 2$). At a dose of 0.3 mg/kg, intravenous, plus 0.3-μmol/L superfusion, the response was marginally reduced ($P < 0.10$) to 86±17% of the CSF baseline response ($n = 7$). At a dose of 1 mg/kg, intravenous, plus 1-μmol/L superfusion, the response was significantly reduced to 58±25% of the CSF baseline response ($n = 6$; Figure 2C). The latter dose was used in other groups with combined treatments. Higher doses of alloxazine were not tested because of concern of nonselectivity and because this dose antagonizes dilation of pial arterioles to exogenous adenosine (Shin *et al*, 2000) and dilation to activation of AMPA receptors (Ohata *et al*, 2006).

Pial arteriolar dilation mediated by adenosine A_{2B} receptors is thought to be associated with increased NO synthase activity (Shin *et al*, 2000). Superfusion of 1 mmol/L L-NNA attenuated the LDF response to whisker stimulation (Figure 2D; $n = 6$), in agreement with others (Lindauer *et al*, 1999). Administration of alloxazine with L-NNA resulted in no additional decrease in the LDF response to whisker stimulation.

To determine if the doses of ZM-241385 and alloxazine were adequate for blocking the LDF responses to adenosine receptor activation, LDF responses to the adenosine receptor agonists CGS-21680 and NECA were evaluated. Superfusion of 0.3 μmol/L CGS-21680 increased LDF by 64±10% by 35 mins and the increase remained stable through 60 mins of superfusion (Figure 3A). Treatment with 1 mg/kg of ZM-241385 plus 1 μmol/L superfusion starting 1 h before CGS-21680 superfusion nearly blocked the increase in LDF seen at 35 mins of CGS-21680 superfusion (5±7%), and the response remained suppressed through 60 mins. In contrast, treatment with 1 mg/kg of alloxazine plus 1 μmol/L superfusion had no significant effect on the LDF response to CGS-21680 (58±13% at 35 mins). Superfusion of 10 μmol/L NECA increased LDF by 26±6% by 20 mins and the increase remained stable through 60 mins of superfusion (Figure 3B). Treatment with ZM-241385 had no effect on the increase in LDF (24 ±1% at 20 mins). However, treatment with alloxazine markedly blunted the response to NECA throughout the 60-min superfusion period (4 ± 4% at 20 mins).

Another A_{2A} antagonist SCH-58261 was tested to assure that the lack of effect on the whisker stimulation response was not specific for ZM-241385. Moreover, SCH-58261 was administered only systemically without placement of a subarachnoid catheter to assure that catheter placement was not responsible for the lack of effect by ZM-241385. A dose of 0.1 mg/kg + 0.1 mg/kg/h, intravenous, which is one order of magnitude greater than the dose found to be neuroprotective in rats (Melani *et al*, 2006), did not change the LDF response to whisker

stimulation (Figure 4A; $n = 6$). To confirm the inhibitory effect of alloxazine, the chemically distinct A_{2B} antagonist MRS-1754 was tested. A dose of 1 mg/kg, intravenous, plus 1 μmol/L superfusion of MRS-1754 significantly attenuated the LDF response to whisker stimulation (Figure 3B; $n = 7$). However, the A_3 antagonist MRS-1191 (1 μmol/L superfusion) had no significant effect on the LDF response (Figure 4C; $n = 5$). Systemic administration of MRS-1191 was not used because of effects on blood pressure.

Previous work has demonstrated that the EET synthesis inhibitors MS-PPOH and miconazole at doses of 20 μmol/L reduced the LDF response to whisker stimulation (Peng *et al*, 2002) and electrical stimulation of the forepaw (Peng *et al*, 2004). This inhibitory effect was confirmed in this study. Superfusion of 20 μmol/L MS-PPOH (Figure 5A; $n = 6$) or miconazole (Figure 5B; $n = 6$) for 1 h decreased the LDF response to whisker stimulation to 62±22% and to 54 ±18% of the baseline response, respectively. No further reductions in the response occurred after the second hour of superfusion. The EETs antagonist 14,15-EEZE-mSI, at a dose of 10 μmol/L, has been reported to inhibit relaxation of coronary artery rings to 14,15-EET (Gauthier *et al*, 2003). In isolated rat middle cerebral artery, 10 μmol/L of 14,15-EEZE-mSI was found to reduce dilation to 100 and 300 nmol/L 14,15-EET (Figure 5C; $n = 4$). For *in vivo* experiments, a slightly higher dose of 30 μmol/L was used to help ensure adequate penetration into cerebral cortex. Superfusion of cortex with 30 μmol/L 14,15-EEZE-mSI for 1 h decreased the LDF response to whisker stimulation to 63±23% of the CSF response. The response after the second hour of superfusion was not significantly different from the first hour's response (Figure 5D; $n = 7$). Likewise, 14,15-EEZE (30 μmol/L) reduced the response to 55±20% of the baseline response, with no further change after the second hour of superfusion (Figure 5E; $n = 6$). Because 14,15-EEZE can antagonize the action of all four regioisomers (Gauthier *et al*, 2002), this antagonist was used in further experiments.

Knowing that the second hour of superfusion of alloxazine, 14,15-EEZE, or MS-PPOH alone did not produce a further attenuation of the response, in additional groups studied, a second drug was added 1 h after the start of superfusion of the first drug to determine if combined drug treatment would produce additional attenuation of the response. In the presence of alloxazine, addition of 14,15-EEZE to the superfusate did not produce greater attenuation of the LDF response, compared with alloxazine alone (Figure 6; $n = 8$). Moreover, in the presence of MS-PPOH, administration of alloxazine did not produce additional attenuation of the response (Figure 7; $n = 6$). In the presence of ZM-214385, which did not attenuate the response by itself, addition of 14,15-EEZE to the superfusate did reduce the LDF response (Figure 8; $n = 6$) by an amount similar to 14,15-EEZE alone (Figure 5E). The time course of the mean and s.d. of the percentage changes in LDF from a 60-sec baseline period are shown in Figures 6–8 on a second-by-second basis for each group of rats. During the control response, LDF began to increase at 1 to 2 secs from the onset of whisker stimulation and reached over 80% of the steady-state response within 5 secs. Alloxazine, MS-PPOH, and 14,15-EEZE reduced the steady-state response, but did not delay the initial increase in LDF.

Superfusion of 100 μmol/L of the mGluR antagonist AIDA reduced the LDF response to 53 ±18% of the control response, with no further reduction during the second hour of superfusion (45±10% of control response; $n = 6$). In the presence of AIDA, addition of alloxazine did not produce a further reduction of the steady-state LDF response or a substantial delay in the response (Figure 9; $n = 6$). However, addition of 14,15-EEZE to AIDA did produce a small decrement in the 60-sec average response compared with AIDA alone (Figure 10; $n = 6$), although the attenuation by AIDA alone was less than in the previous group with AIDA alone (Figure 9). Moreover, the combination of AIDA plus 14,15-EEZE reduced the response to 59 ±17% of the baseline response (Figure 10), and this attenuation was not significantly different from the reduction to 52±12% of the baseline response seen with the combination of AIDA plus alloxazine (Figure 9).

When AIDA and alloxazine were administered simultaneously, the LDF response was decreased to 59±14% of the control response (Figure 11; $n = 6$). Addition of 14,15-EEZE did not produce a further decrease in the response.

Previous work has demonstrated that MS-PPOH and miconazole did not reduce the amplitude of somatosensory evoked potentials over cerebral cortex during electrical stimulation of the forepaw (Peng *et al*, 2002). As a percent of the baseline response, the amplitude of the primary cortical evoked potential with contralateral forepaw stimulation was 121±44% in a time-control group ($n = 6$), 114±25% in a group superfused with 30 μmol/L 14,15-EEZE ($n = 5$), 89±27% in a group superfused with 100 μmol/L AIDA ($n = 6$), 94±21% in a group administered 1 mg/kg, intravenous, plus 1 μmol/L superfused ZM-241385 ($n = 5$), and 83±28% in a group administered 1 mg/kg, intravenous, plus 1 μmol/L superfused alloxazine ($n = 10$). The evoked potential amplitude after drug treatment was not significantly different from the baseline response, and analysis of variance did not indicate a significant difference among groups after drug treatment. The lack of effect of AIDA is consistent with other findings showing that the suppressed LDF response to forepaw stimulation with other Group I mGluR antagonists is not the result of suppression of evoked potentials (Zonta *et al*, 2003).

Discussion

The major findings of this study are that (1) adenosine A_{2B} rather than A_{2A} or A_3 receptors play a significant role in coupling cortical blood flow to neuronal activity evoked by whisker stimulation; (2) EET antagonists inhibit the blood flow response, further supporting the role of EETs in neurovascular coupling; (3) the effect of an antagonist of A_{2B} receptors on the evoked flow response is not additive with the effect of an EET antagonist or synthesis inhibitor; (4) the effect of an antagonist of A_{2B} receptors is not additive with the effect of a mGluR antagonist on the flow response; and (5) combining A_{2B}, mGluR, and EET antagonists does not completely block the blood flow response to whisker stimulation.

Adenosine Receptors

During functional activation elicited by sciatic nerve stimulation, pial arteriolar dilation is attenuated by the nonselective adenosine antagonists theophylline (Ko *et al*, 1990) and caffeine (Meno *et al*, 2005). The selective A_{2A} receptor antagonist ZM-241385 was also effective at 1 mg/kg, although not with topical application at 1 μmol/L (Meno *et al*, 2001). In addition, pial arteriolar dilation to topical glutamate was inhibited by ZM-241385 at CSF concentrations of 0.1 and 1 μmol/L or at an intravenous dose of 1 mg/kg (Iliff *et al*, 2003). Likewise, topical ZM-241385 at 1 μmol/L attenutated pial arteriolar dilation AMPA with no further attenuation at 10 μmol/L (Ohata *et al*, 2006). In this study, a combination of 1 mg/kg intravenous dose and 1 μmol/L continuous superfusion was used to assure adequate availability of the drug in the vasculature and parenchyma. Thus, the lack of effect of ZM-241385 on the LDF response to whisker stimulation in this study was unexpected.

To address the possibility that subarachnoid superfusion diluted the drug, the outflow of radiolabeled ZM-241385 was measured. Over a 1-h period, the outflow concentration averaged 84% of the concentration in a standard cranial window. Thus, the concentration in the CSF with an inflow concentration of 1 μmol/L should have been well above the 0.1 μmol/L concentration shown to inhibit pial arteriolar responses to glutamate. Furthermore, the outflow concentration was 95% of the inflow concentration by 1 h, and extending the superfusion period for an additional hour did not inhibit the LDF response to whisker stimulation (Figure 2B). Other drugs that did inhibit the LDF response with 1 h of superfusion displayed no further inhibition with an additional hour of superfusion, thereby indicating adequate time for penetration into the underlying tissue. Hence, the dose and duration of ZM-241385 superfusion should have been adequate to discern an effect.

Another possibility is that the tissue monitored by LDF over a presumed 1-mm depth is not sensitive to A_{2A} receptor activation or that the antagonist is ineffective in blocking A_{2A} receptors in the underlying tissue. However, superfusion of the A_{2A} agonist CGS-21680 increased LDF, and the increase was nearly completely blocked by 1 mg/kg, intravenous, plus 1 μmol/L superfusion of ZM-241385. Alloxazine had no effect on this response. Thus, the dose and routes of administration of ZM-241385 were effective in inhibiting a selective A_{2A} vascular response. Although high doses of ZM-241385 can also inhibit A_{2B} receptors, the difference between the effects of ZM-241385 and alloxazine on the LDF response infers that ZM-241385 was not exerting an effect on A_{2B} receptors at the doses employed in this study. In support of ZM-241385 selectivity for A_{2A} receptors, ZM-241385 had no effect on the LDF response to 10 μmol/L NECA, a dose at which alloxazine largely suppressed the LDF response. A greater potency of alloxazine than ZM-241385 for inhibiting vasodilation to NECA has also been reported for pial arterioles (Shin *et al*, 2000).

Lastly, the lack of effect of ZM-241385 on the LDF response to whisker stimulation was supported by the lack of effect of another A_{2A} antagonist SCH-58261. Curiously, others reported that topical ZM-241385 was ineffective in blocking pial arteriolar dilation to sciatic nerve stimulation (Meno *et al*, 2001) at doses that blocked the dilation to glutamate (Iliff *et al*, 2003). This incongruity raises the possibility that the positive effect of systemically administered ZM-241385 in inhibiting the pial arteriolar responses to sciatic nerve stimulation (Meno *et al*, 2001) may be due to an indirect peripheral effect of systemic administration. However, our observation that systemic administration of SCH-58261 without CSF superfusion exerted no inhibitory effect on the LDF response indicates that a nonspecific effect of A_{2A} antagonism in other tissues is unlikely to have a major effect on neurovascular coupling. Together, our results indicate that A_{2A} receptors are not required for coupling cortical blood flow to neuronal activation. Any pial arteriolar dilation that is partially dependent on A_{2A} receptors during functional activation may help maintain blood flow in non-activated cortical areas surrounding the primary activated regions.

Another possibility for the lack of effect of A_{2A} antagonists is that intraparenchymal arterioles do not have functional A_{2A} receptors. However, penetrating arterioles in rat brain dilate in response to an A_{2A} receptor agonist, and dilation to adenosine is attenuated by ZM-241385 (Ngai *et al*, 2001). It is interesting that dilation of penetrating arterioles to adenosine is greater than dilation to an A_{2A} agonist and that dilation to adenosine is not reduced by an A_1 or A_3 antagonist and is only partially reduced by ZM-241385. These findings indirectly imply that part of the dilation of intraparenchymal arterioles to adenosine is mediated by another receptor such as the A_{2B} receptor. In pial arterioles, the A_{2B} receptor antagonist alloxazine also attenuated dilation in response to adenosine (Shin *et al*, 2000). A physiologic role of both A_{2A} and A_{2B} receptors has been implicated by the findings that both ZM-241385 and alloxazine attenuate pial arteriolar dilation to hypotension (Shin *et al*, 2000) and to AMPA receptor activation (Ohata *et al*, 2006). The present results, showing that alloxazine and MRS-1754, but not A_{2A} antagonists, are effective in reducing a physiologic vascular response, appear unique. Because A_{2A} receptors have a higher affinity for adenosine than A_{2B} receptors, the differential activation of A_{2B} receptors is best explained by localized increases in adenosine restricted to the vicinity of A_{2B} receptors.

Neurons release ATP as a co-neurotransmitter, and astrocytes release ATP during propagation of Ca^{2+} waves. Extracellular ATP is metabolized to adenosine by ecto-ATPase and ecto-5′-nucleotidase (Fields and Burnstock, 2006), and ecto-ATPase appears to be localized near astrocyte hemichannels, where ATP is released (Joseph *et al*, 2003). Astrocytes express various adenosine receptor subtypes (Fields and Burnstock, 2006). Activation of A_{2B} receptors on astrocytes has been noted to increase intracellular Ca^{2+} (Newman, 2005; Pilitsis and Kimelberg, 1998) and to potentiate Ca^{2+} waves in astrocytes evoked by ATP (Alloisio *et al*,

2004; Jimenez *et al*, 1999). Therefore, one explanation for the selective effect of A_{2B} antagonists in the present experiment is that activation of A_{2B} receptors on astrocytes or on vascular smooth muscle near astrocyte foot processes releasing ATP is important in the communication between neurons and arterioles.

Our observation that MRS-1191 had no effect on the LDF response to whisker stimulation is consistent with data that MRS-1191 does not inhibit dilation of intraparenchymal aterioles to adenosine (Ngai *et al*, 2001). Thus, the functional role of A_3 receptors purported to be expressed in cerebral vessels (Di Tullio *et al*, 2004) remains unclear. An A_1 receptor antagonist had no effect on intraparenchymal dilation to adenosine (Ngai *et al*, 2001) or on pial arteriolar dilation to glutamate (Iliff *et al*, 2003), but increased the dilatory response to sciatic nerve stimulation (Meno *et al*, 2001). The primary role of A_1 receptors presumably is in presynaptic modulation of glutamate release. Because of the difficulty in dissociating whether changes in vascular responses are due to changes in neuronal activation or neurovascular coupling, an A_1 receptor antagonist was not tested in this study.

Adenosine Receptors and Metabotropic Glutamate Receptor

Increases in astrocyte Ca^{2+} triggered by neuronal activation is thought to be initiated by glutamate acting on Group 1 mGluR on astrocytes (Filosa *et al*, 2004; Zonta *et al*, 2003). The group I mGluR antagonists LY367385 and MPEP have been shown to reduce the LDF response to electrical forepaw stimulation (Zonta *et al*, 2003). The current results, which show an attenuation of the LDF response to whisker stimulation by the group I mGluR antagonist AIDA, are consistent with this previous study. The new finding that alloxazine does not produce additional attenuation of the response, compared with AIDA alone, is consistent with the possibility of a sequential mechanism in which A_{2B} receptor activation promotes the Ca^{2+} signaling that is initiated within astrocytes by mGluR activation. An alternative possibility that is also consistent with the data is that mGluR-induced increased Ca^{2+} causes release of ATP at astrocyte end-feet (Simard *et al*, 2003), where ATP might be converted into adenosine and act on vascular A_{2B} receptors.

Epoxyeicosatrienoic Acids

In isolated retina without blood perfusion, PPOH was found to markedly decrease arteriolar dilation evoked by natural light stimulus or by release of caged Ca^{2+} or IP_3 in astrocytes, whereas indomethacin and aspirin were ineffective (Metea and Newman, 2006). The presently observed decrease in the LDF response with MS-PPOH and miconazole confirms previous work with these distinct inhibitors of EET synthesis (Peng *et al*, 2002, 2004). Decreases in the LDF response to whisker stimulation with the EET antagonists 14,15-EEZE and 14,15-EEZE-mSI further strengthen the role of EETs in coupling the neurovascular response to physiologic activation. Because the decrease in the LDF response with the antagonists was approximately the same as with the synthesis inhibitors, one might conclude that EET signaling depends on *de novo* synthesis rather than on release of preformed stores of EETs. However, it is also possible that preformed stores of EETs became depleted during the 1-h superfusion period of the synthesis inhibitors.

The ability of 14,15-EEZE-mSI to block relaxation by 14,15-EET in rat middle cerebral artery is consistent with work in bovine coronary artery (Gauthier *et al*, 2003). Among the four regioisomers of EETs, 14,15-EEZE-mSI appears to be more selective for 14,15-EET and 5,6-EET (Gauthier *et al*, 2003), whereas 14,15-EEZE inhibits relaxation to all four regioisomers (Gauthier *et al*, 2002). The substantial inhibition of the LDF response to whisker stimulation with 14,15-EEZE-mSI is consistent with results showing that 14,15-EET is a major regioisomer synthesized in astrocytes (Alkayed *et al*, 1996; Amruthesh *et al*, 1993) and is either metabolized by epoxide hydrolase or incorporated into the phospholipid membrane (Shivachar *et al*,

1995). Because of its broad spectrum potency, 14,15-EEZE, rather than 14,15-EEZE-mSI, was chosen for testing interactions with adenosine and mGluR pathways. Although 14,15-EEZE produced a small increase in LDF after the second hour of superfusion by a mechanism that is not clear, superfusion was restricted to 1 h in groups in which 14,15-EEZE was combined with other antagonists. Baseline LDF was unchanged in these other groups.

Epoxyeicosatrienoic Acids, Adenosine Receptors, and Metabotropic Glutamate Receptor

Superfused 14,15-EEZE retained its ability to inhibit the LDF response to whisker stimulation in the presence of ZM-241385, but lost its inhibitory effect in the presence of alloxazine and combined alloxazine and AIDA. In addition, alloxazine produced no further inhibition of the LDF response in the presence of MS-PPOH. Although 14,15-EEZE did produce an additional inhibition of the response in the presence of AIDA, this additional inhibitory effect was small in magnitude and the overall attenuation with combined AIDA and 14,15-EEZE was comparable to that seen with EEZE alone or with AIDA alone in other groups. Thus, the effect of blocking EETs depends on the ability to activate mGluR and adenosine A_{2B} receptors. These results are consistent with an EET signaling pathway acting sequentially with mGluR and A_{2B} receptors, rather than by parallel, independent pathways. Glutamate stimulation of astrocytes causes release of EETs (Alkayed *et al*, 1997), which could then hyperpolarize cerebrovascular smooth muscle by opening Ca^{2+}-sensitive K^+ channels (Alkayed *et al*, 1996; Gebremedhin *et al*, 1992). In astrocytes, EETs can serve as a Ca^{2+} influx factor (Rzigalinski *et al*, 1999) and, during stimulation of mGluR, promote opening of Ca^{2+}-sensitive K^+ channels, which will help maintain a hyperpolarized state favorable for Ca^{2+} influx (Gebremedhin *et al*, 2003). Therefore, EETs could act by multiple mechanisms to sustain mGluR- and A_{2B}-evoked Ca^{2+} signaling within astrocytes and astrocyte-vascular coupling.

Combined treatment with AIDA, alloxazine, and 14,15-EEZE did not fully block the LDF response to whisker stimulation. Moreover, none of the drugs substantially delayed the onset of the LDF response. These results suggest that either (1) other pathways normally contribute to about half of the blood flow response with a rapid time constant, or (2) the mGluR, A_{2B}, and EET pathways normally contribute to more than half of the response, but new, back-up mechanisms with equally fast time constants are recruited when the primary mechanism is inhibited. In a previous study (Peng *et al*, 2002), indomethacin did not significantly reduce the LDF response to whisker stimulation in the rat, and MS-PPOH attenuated, but did not completely block the response in the presence of indomethacin (Peng *et al*, 2002). Metabolites of COX were not examined in this study, but could be responsible for the remaining response. COX-1 has been identified in perivascular astrocytes in mouse cortex, and use of a high dose of a COX-1 inhibitor was found to decrease arteriolar dilation to increased astrocytic Ca^{2+} (Takano *et al*, 2006). However, a lower dose of the COX-1 inhibitor was not effective in reducing the LDF response to whisker stimulation, and COX-1 null mice had a normal LDF response (Niwa *et al*, 2001). Moreover, stimulation of COX-1 in astrocytes is presumed to be dependent on mGluR stimulation of Ca^{2+} and subsequent activation of phospholipase A_2 (Zonta *et al*, 2003). Thus, inhibition of mGluR may be expected to impede this COX-1-dependent pathway, and COX-1 may not contribute to the residual LDF response. Alternatively, COX-2 metabolites derived from neurons might bypass astrocyte signaling and directly dilate vascular smooth muscle (Niwa *et al*, 2000; Wang *et al*, 2005) and thereby be responsible for the remaining LDF response.

Adenosine and Nitric Oxide

Cortical activation leads to a rapid increase in NO, but the increase lasts only 2 secs (Buerk *et al*, 2003) and thus does not temporally correlate with the rapid decrease in LDF during the off-response. The transient increase in NO may permit vasodilation by other mediators, rather than act as a primary mediator of the cortical flow response to activation (Lindauer *et al*, 1999).

Combining L-NNA with MS-PPOH or miconazole did not eliminate the LDF response to forepaw stimulation (Peng *et al*, 2004). Combining L-NNA and theophylline was reported to inhibit the LDF response to whisker stimulation by 58%, which was slightly greater than the 41% inhibition with theophylline alone (Dirnagl *et al*, 1994). Application of adenosine or an A_{2B} agonist on the cortical surface was found to produce vasodilation that was partially dependent on NO and cGMP (Dirnagl *et al*, 1994; Shin *et al*, 2000). In this study, alloxazine did not produce additional inhibition or complete blockage of the LDF response in the presence of L-NNA. This finding is consistent with the possibility that the adenosine A_{2B} mechanism involved in functional hyperemia depends on NO. However, our data do not distinguish whether adenosine acting on vascular smooth muscle A_{2B} receptors requires NO as a mediator or permissive enabler of vasodilation, or if adenosine acting on astrocyte A_{2B} receptors contributes to release of vasoactive arachidonic acid metabolites that, in turn, require NO as a permissive enabler of vasodilation. The lack of complete blockage of the LDF response when L-NNA is combined with theophylline, alloxazine, MS-PPOH, or miconazole suggests that the residual response presently seen after AIDA, alloxazine, and 14,15-EEZE is unlikely to be attributed to NO.

Anesthesia

Although the use of anesthesia is known to decrease the magnitude of the evoked CBF response (Nakao *et al*, 2001) and could influence the interaction of signaling pathways, this study demonstrates a proof of principle that the mGluR, A_{2B}, and EETs pathways are capable of regulating the CBF response to functional activation in a non-additive fashion. With chloralose anesthesia and the use of cranial windows, others reported an average increase in LDF during whisker stimulation in the rat in the range of 15 to 20% (Dirnagl *et al*, 1994; Irikura *et al*, 1994; Lindauer *et al*, 1999). In this study with chloralose anesthesia and subarachnoid superfusion, the average response for each group generally fell into this range, although the LDF response displayed some variability among groups. However, the response was more reproducible within the same animal, and paired analysis permitted detection of moderate changes in the response.

In summary, this study demonstrates involvement of adenosine A_{2B} receptors in the coupling of cortical blood flow to neural activation. Further evidence supports previous work that implicates EETs and mGluR in the neurovascular coupling. The lack of additive inhibitory effects on the CBF response by combined antagonists is consistent with the mGluR, A_{2B}, and EET signaling in sequential pathways, possibly located in astrocytes.

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References

Alkayed NJ, Birks EK, Narayanan J, Petrie KA, Kohler-Cabot AE, Harder DR. Role of P-450 arachidonic acid expoygenase in the response of cerebral blood flow to glutamate in rats. Stroke 1997;28:1066–72. [PubMed: 9158651]

Alkayed NJ, Narayanan J, Gebremedhin D, Medhora M, Roman RJ, Harder DR. Molecular characterization of an arachidonic acid epoxygenase in rat brain astrocytes. Stroke 1996;27:971–9. [PubMed: 8623121]

Alloisio S, Cugnoli C, Ferroni S, Nobile M. Differential modulation of ATP-induced calcium signalling by A1 and A2 adenosine receptors in cultured cortical astrocytes. Br J Pharmacol 2004;141:935–42. [PubMed: 14993103]

Amruthesh SC, Boerschel MF, McKinney JS, Willoughby KA, Ellis EF. Metabolism of arachidonic acid to epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and prostaglandins in cultured rat hippocampal astrocytes. J Neurochem 1993;61:150–9. [PubMed: 8515261]

Buerk DG, Ances BM, Greenberg JH, Detre JA. Temporal dynamics of brain tissue nitric oxide during functional forepaw stimulation in rats. Neuroimage 2003;18:1–9. [PubMed: 12507439]

Di Tullio MA, Tayebati SK, Amenta F. Identification of adenosine A1 and A3 receptor subtypes in rat pial and intracerebral arteries. Neurosci Lett 2004;366:48–52. [PubMed: 15265588]

Dirnagl U, Niwa K, Lindauer U, Villringer A. Coupling of cerebral blood flow to neuronal activation: role of adenosine and nitric oxide. Am J Physiol Heart Circ Physiol 1994;267:H296–301.

Fields RD, Burnstock G. Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci 2006;7:423–36. [PubMed: 16715052]

Filosa JA, Bonev AD, Nelson MT. Calcium dynamics in cortical astrocytes and arterioles during neurovascular coupling. Circ Res 2004;95:e73–81. [PubMed: 15499024]

Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR, Campbell WB. 14,15-Epoxyeicosa-5(Z)-enoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyper-polarization and relaxation in coronary arteries. Circ Res 2002;90:1028–36. [PubMed: 12016270]

Gauthier KM, Jagadeesh SG, Falck JR, Campbell WB. 14,15-epoxyeicosa-5(Z)-enoic-mSI: a 14,15- and 5,6-EET antagonist in bovine coronary arteries. Hypertension 2003;42:555–61. [PubMed: 12953017]

Gebremedhin D, Ma Y-H, Falck JR, Roman RJ, VanRollins M, Harder DR. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. Am J Physiol Heart Circ Physiol 1992;263:H519–25.

Gebremedhin D, Yamaura K, Zhang C, Bylund J, Koehler RC, Harder DR. Metabotropic glutamate receptor activation enhances the activities of two types of Ca^{2+}-activated K^{+} channels in rat hippocampal astrocytes. J Neurosci 2003;23:1678–87. [PubMed: 12629172]

Iliff JJ, D'Ambrosio R, Ngai AC, Winn HR. Adenosine receptors mediate glutamate-evoked arteriolar dilation in the rat cerebral cortex. Am J Physiol Heart Circ Physiol 2003;284:H1631–7. [PubMed: 12543638]

Irikura K, Maynard KI, Moskowitz MA. Importance of nitric oxide synthase inhibition to the attenuated vascular responses induced by topical L-nitroarginine during vibrissal stimulation. J Cereb Blood Flow Metab 1994;14:45–8. [PubMed: 7505281]

Jimenez AI, Castro E, Mirabet M, Franco R, Delicado EG, Miras-Portugal MT. Potentiation of ATP calcium responses by A2B receptor stimulation and other signals coupled to Gs proteins in type-1 cerebellar astrocytes. Glia 1999;26:119–28. [PubMed: 10384877]

Joseph SM, Buchakjian MR, Dubyak GR. Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. J Biol Chem 2003;278:23331–42. [PubMed: 12684505]

Ko KR, Ngai AC, Winn HR. Role of adenosine in regulation of regional cerebral blood flow in sensory cortex. Am J Physiol Heart Circ Physiol 1990;259:H1703–8.

Koehler RC, Gebremedhin D, Harder DR. Role of astrocytes in cerebrovascular regulation. J Appl Physiol 2006;100:307–17. [PubMed: 16357084]

Lindauer U, Megow D, Matsuda H, Dirnagl U. Nitric oxide: a modulator, but not a mediator, of neurovascular coupling in rat somatosensory cortex. Am J Physiol Heart Circ Physiol 1999;277:H799–811.

Melani A, Gianfriddo M, Vannucchi MG, Cipriani S, Baraldi PG, Giovannini MG, Pedata F. The selective A2A receptor antagonist SCH 58261 protects from neurological deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia. Brain Res 2006;1073–1074:470–80.

Meno JR, Crum AV, Winn HR. Effect of adenosine receptor blockade on pial arteriolar dilation during sciatic nerve stimulation. Am J Physiol Heart Circ Physiol 2001;281:H2018–27. [PubMed: 11668063]

Meno JR, Nguyen TS, Jensen EM, Alexander WG, Groysman L, Kung DK, Ngai AC, Britz GW, Winn HR. Effect of caffeine on cerebral blood flow response to somatosensory stimulation. J Cereb Blood Flow Metab 2005;25:775–84. [PubMed: 15703695]

Metea MR, Newman EA. Glial cells dilate and constrict blood vessels: a mechanism of neurovascular coupling. J Neurosci 2006;26:2862–70. [PubMed: 16540563]

Nakao Y, Itoh Y, Kuang TY, Cook M, Jehle J, Sokoloff L. Effects of anesthesia on functional activation of cerebral blood flow and metabolism. Proc Natl Acad Sci USA 2001;98:7593–8. [PubMed: 11390971]

Newman EA. Calcium increases in retinal glial cells evoked by light-induced neuronal activity. J Neurosci 2005;25:5502–10. [PubMed: 15944378]

Ngai AC, Coyne EF, Meno JR, West GA, Winn HR. Receptor subtypes mediating adenosine-induced dilation of cerebral arterioles. Am J Physiol Heart Circ Physiol 2001;280:H2329–35. [PubMed: 11299238]

Niwa K, Araki E, Morham SG, Ross ME, Iadecola C. Cyclooxygenase-2 contributes to functional hyperemia in whisker-barrel cortex. J Neurosci 2000;20:763–70. [PubMed: 10632605]

Niwa K, Haensel C, Ross ME, Iadecola C. Cyclooxygenase-1 participates in selected vasodilator responses of the cerebral circulation. Circ Res 2001;88:600–8. [PubMed: 11282894]

Ohata H, Cao S, Koehler RC. Contribution of adenosine A2A and A2B receptors and heme oxygenase to AMPA-induced dilation of pial arterioles in rats. Am J Physiol Regul Integr Comp Physiol 2006;291:R728–35. [PubMed: 16601261]

Peng X, Carhuapoma JR, Bhardwaj A, Alkayed NJ, Falck JR, Harder DR, Traystman RJ, Koehler RC. Suppression of cortical functional hyperemia to vibrissal stimulation in the rat by epoxygenase inhibitors. Am J Physiol Heart Circ Physiol 2002;283:H2029–37. [PubMed: 12384482]

Peng X, Zhang C, Alkayed NJ, Harder DR, Koehler RC. Dependency of cortical functional hyperemia to forepaw stimulation on epoxygenase and nitric oxide synthase activities in rats. J Cereb Blood Flow Metab 2004;24:509–17. [PubMed: 15129182]

Pilitsis JG, Kimelberg HK. Adenosine receptor mediated stimulation of intracellular calcium in acutely isolated astrocytes. Brain Res 1998;798:294–303. [PubMed: 9666151]

Rzigalinski BA, Willoughby KA, Hoffman SW, Falck JR, Ellis EF. Calcium influx factor, further evidence it is 5,6-epoxyeicosatrienoic acid. J Biol Chem 1999;274:175–182. [PubMed: 9867827]

Shin HK, Shin YW, Hong KW. Role of adenosine A(2B) receptors in vasodilation of rat pial artery and cerebral blood flow autoregulation. Am J Physiol Heart Circ Physiol 2000;278:H339–44. [PubMed: 10666062]

Shivachar AC, Willoughby KA, Ellis EF. Effect of protein kinase C modulators on 14,15-epoxyeicosatrienoic acid incorporation into astroglial phospholipids. J Neurochem 1995;65:338–46. [PubMed: 7790878]

Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M. Signaling at the gliovascular interface. J Neurosci 2003;23:9254–62. [PubMed: 14534260]

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. Astrocyte-mediated control of cerebral blood flow. Nat Neurosci 2006;9:260–7. [PubMed: 16388306]

Wang H, Hitron IM, Iadecola C, Pickel VM. Synaptic and vascular associations of neurons containing cyclooxygenase-2 and nitric oxide synthase in rat somatosensory cortex. Cereb Cortex 2005;15:1250–60. [PubMed: 15616132]

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 2003;6:43–50. [PubMed: 12469126]

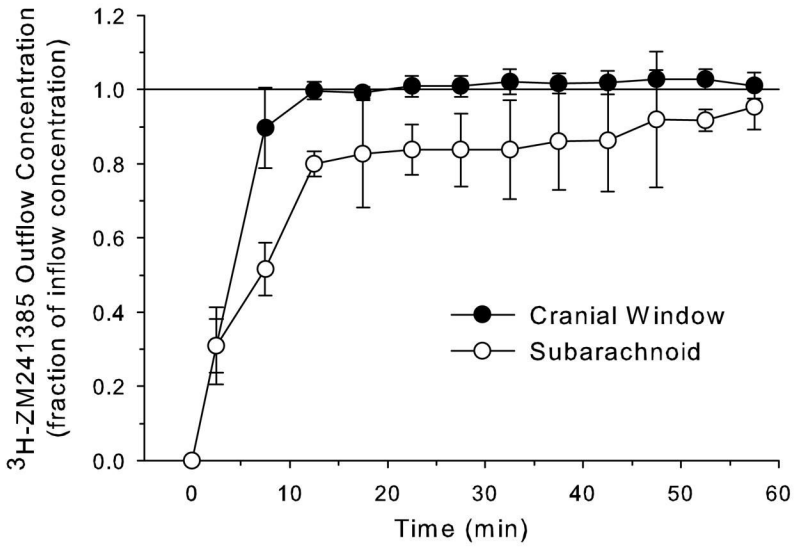

Figure 1.

Time course of [^{3}H]-ZM-241385 outflow concentration (±s.d.), as a fraction of the inflow concentration, in rats with subarachnoid superfusion at 5 μL/min (n = 4) or with a closed cranial window superfused at 200 μL/min (n = 4) for 1 h. The area under the curve for subarachnoid superfusion was 84% of that with cranial window superfusion.

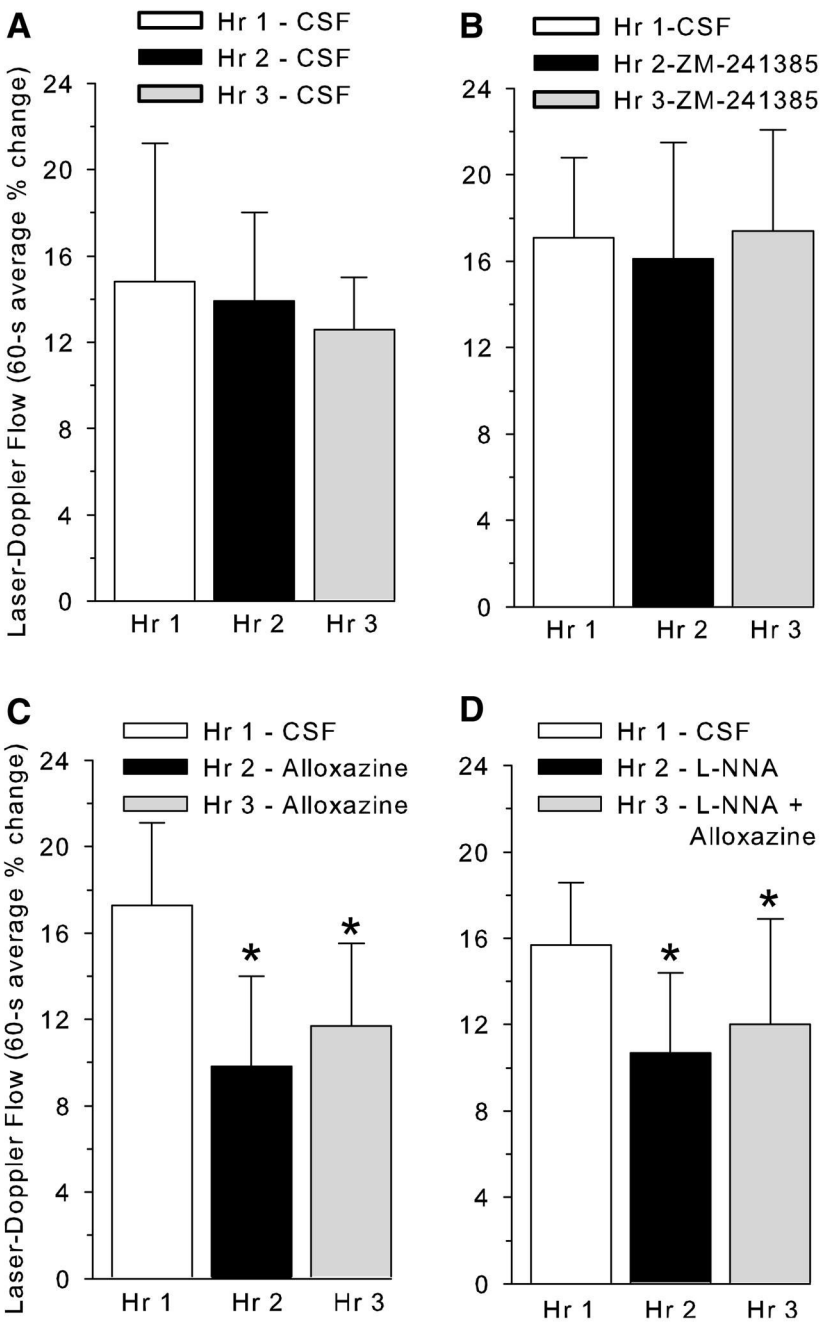

Figure 2.

Percentage change of cortical LDF (±s.d.) averaged over a 60-sec period of whisker stimulation at 1-h superfusion of CSF and (**A**) after an additional 2 h of continued CSF superfusion(n = 12) or (**B**) after an additional 2 h administration of the A_{2A} antagonist ZM-241385 (n = 6), or (**C**) the A_{2B} antagonist alloxazine (n = 6). In **D**, the NO synthase inhibitor L-NNA was superfused during the second and third hours and alloxazine administration was combined with L-NNA during the third hour (n = 6). *P < 0.05 from the 1-h control response.

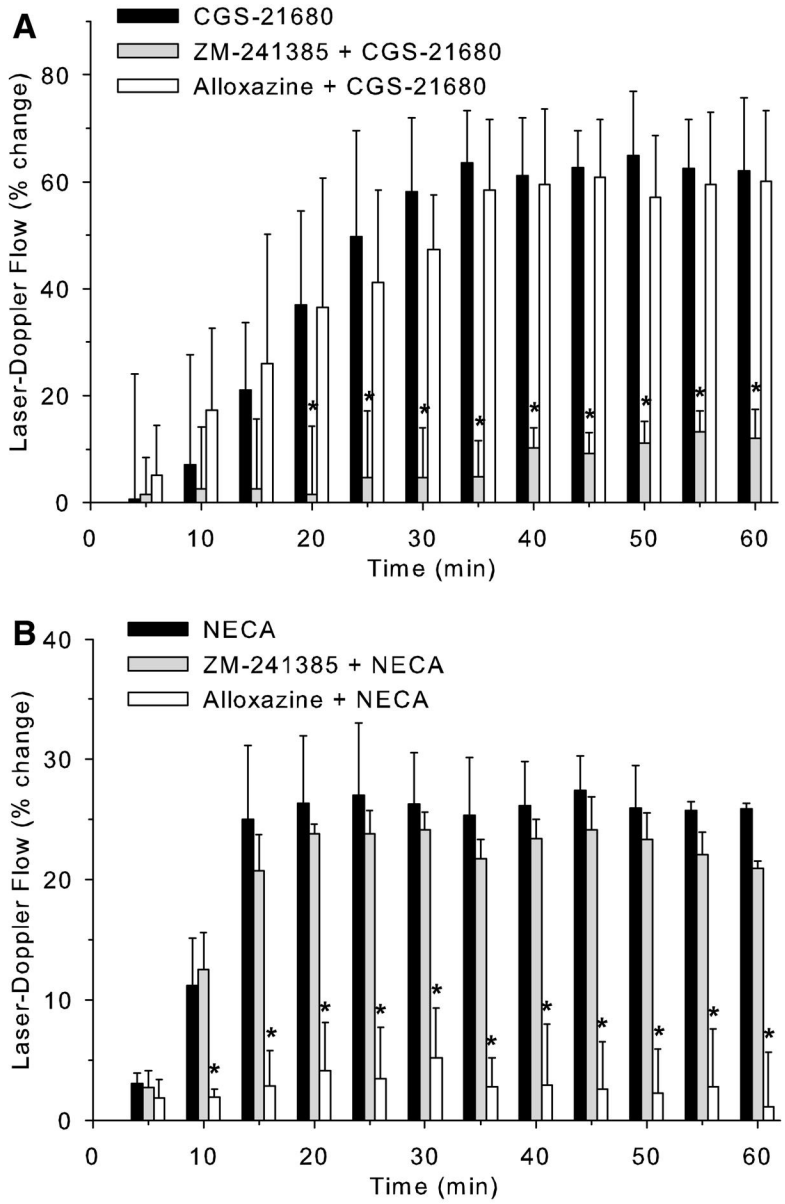

Figure 3.

Percentage change of cortical LDF (±s.d.) averaged over 5-min periods during 1 h of subarachnoid superfusion of (**A**) 0.3 μmol/L CGS-21680 or (**B**) 10 μmol/L NECA after 1 h pretreatment with vehicle (n = 4), 1 mg/kg, intravenous, plus 1 μmol/L CSF superfusion of ZM-241385 (n = 4), or 1 mg/kg, intravenous, plus 1 μmol/L CSF superfusion of alloxazine (n = 4). *P < 0.05 from vehicle group.

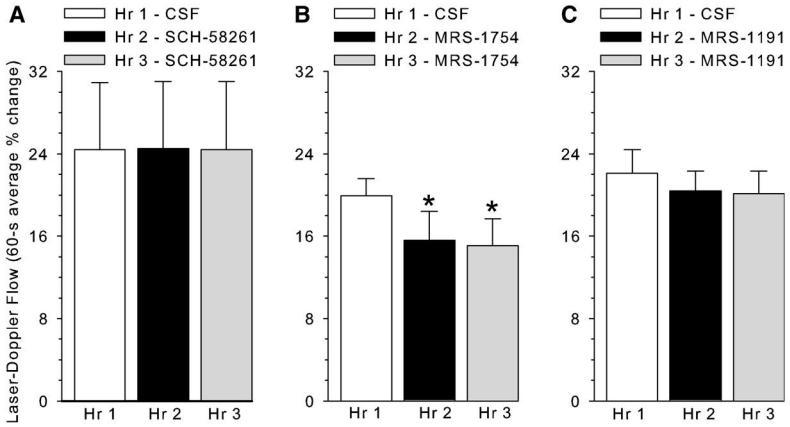

Figure 4.

Percentage change of cortical LDF (±s.d.) averaged over a 60-sec period of whisker stimulation at 1-h superfusion of CSF and (**A**) after an additional 2-h administration of the the A_{2A} antagonist SCH-58261(0.1 mg/kg + 0.1 mg/kg/h, intravenous; $n = 6$), (**B**) the A_{2B} antagonist MRS-1754 (1 mg/kg, intravenous, + 1 μmol/L superfusion; $n = 7$), or (**C**) the A_3 antagonist MRS-1191 (1 μmol/L superfusion; $n = 5$). *$P < 0.05$ from the 1-h control response.

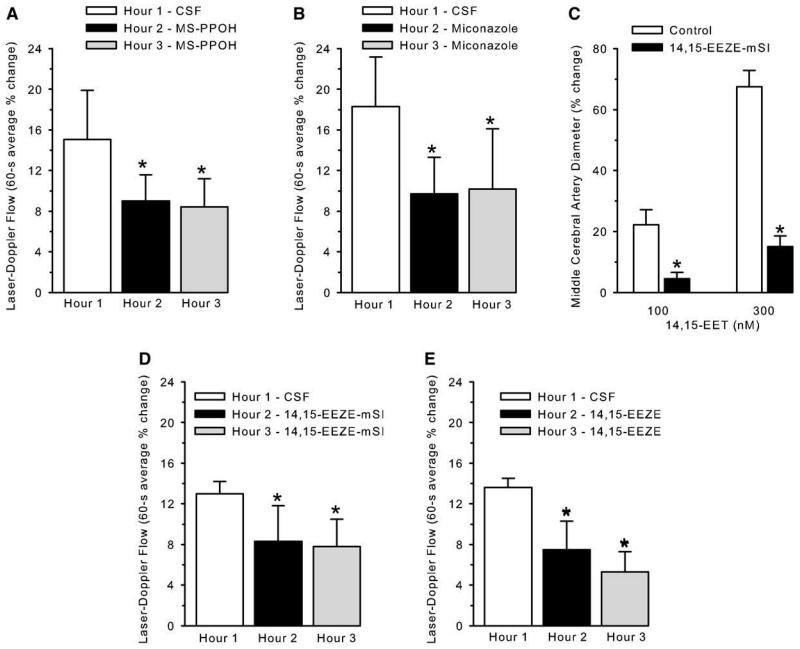

Figure 5.

Percentage change of cortical LDF (±s.d.) averaged over a 60-sec period of whisker stimulation at 1-h superfusion of CSF and (**A**) after an additional 2 h of superfusion with the EET synthesis inhibitors MS-PPOH ($n = 6$) and (**B**) miconazole ($n = 6$) or (**D**) with the EET antagonists 14,15-EEZE-mSI ($n = 7$) and (**E**) 14,15-EEZE ($n = 6$). *$P < 0.05$ from the 1-h control response. In (**C**), segments of rat middle cerebral arteries were pressurized to 80 mm Hg and exposed to 30 μmol/L serotonin to preconstrict the arteries by ~35%. Subsequent treatment with 100 and 300 nmol/L 14,15-EET produced dose-dependent dilation ($n = 4$) that was inhibited by 10 μmol/L 14,15-EEZE-mSI ($n = 4$). *$P < 0.05$ from control.

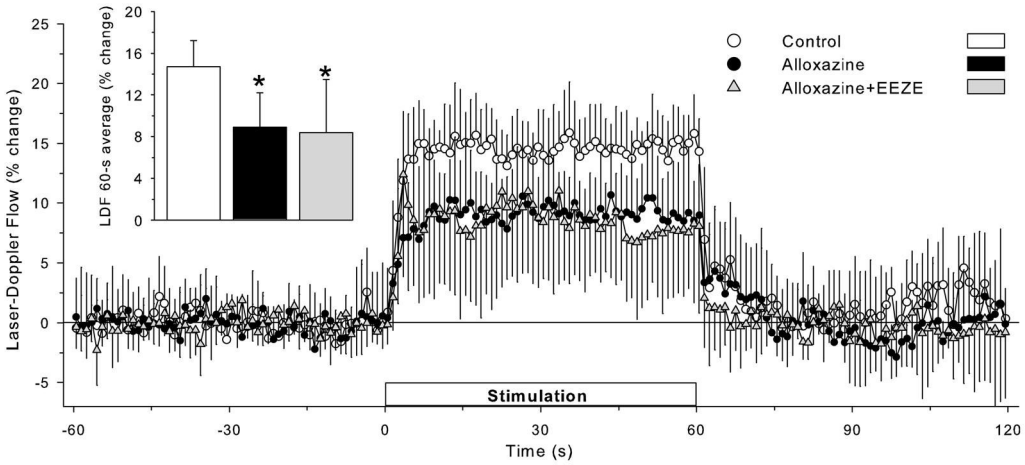

Figure 6.

Time course of 1-sec averages of cortical LDF (±s.d.; $n = 6$ rats), expressed as a percentage change from a 60-sec baseline recording, during 60 secs of whisker stimulation and 60 secs of recovery after 1-h superfusion of CSF, 1 h of alloxazine administration, and 1 h of combined alloxazine and 14,15-EEZE administration. Inset bar graph shows percentage change in LDF averaged over the 60-sec stimulation period. *$P < 0.05$ from control response.

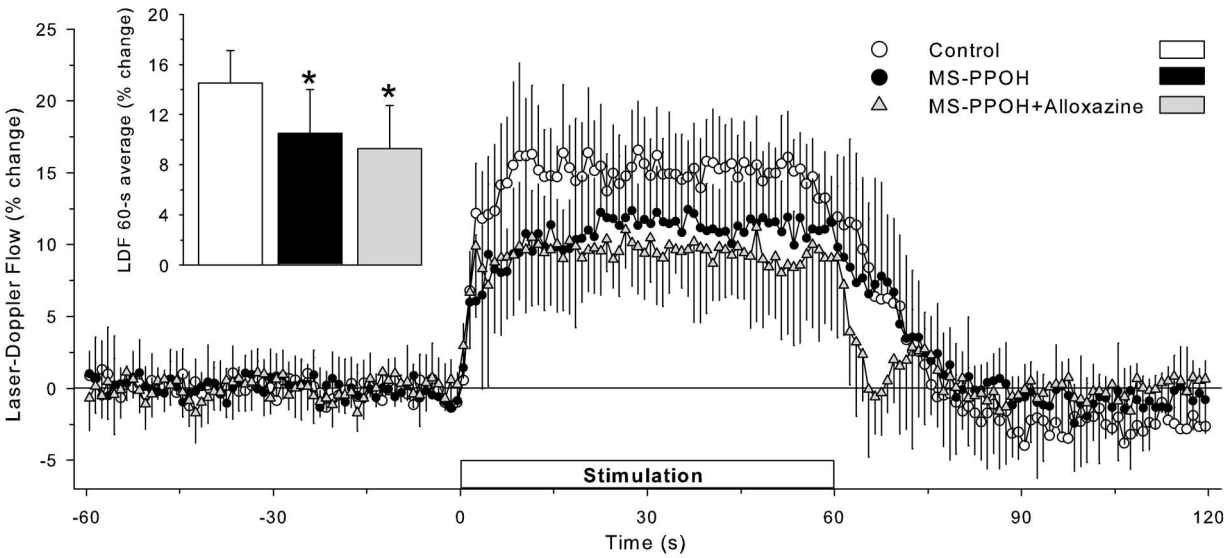

Figure 7.

Time course of 1-sec averages of cortical LDF (±s.d.; $n = 6$ rats), expressed as a percentage change from a 60-sec baseline recording, during 60 secs of whisker stimulation and 60 secs of recovery after 1-h superfusion of CSF, 1 h of MS-PPOH superfusion, and 1 h of combined MS-PPOH and alloxazine administration. Inset bar graph shows percentage change in LDF averaged over the 60-sec stimulation period. *$P < 0.05$ from control response.

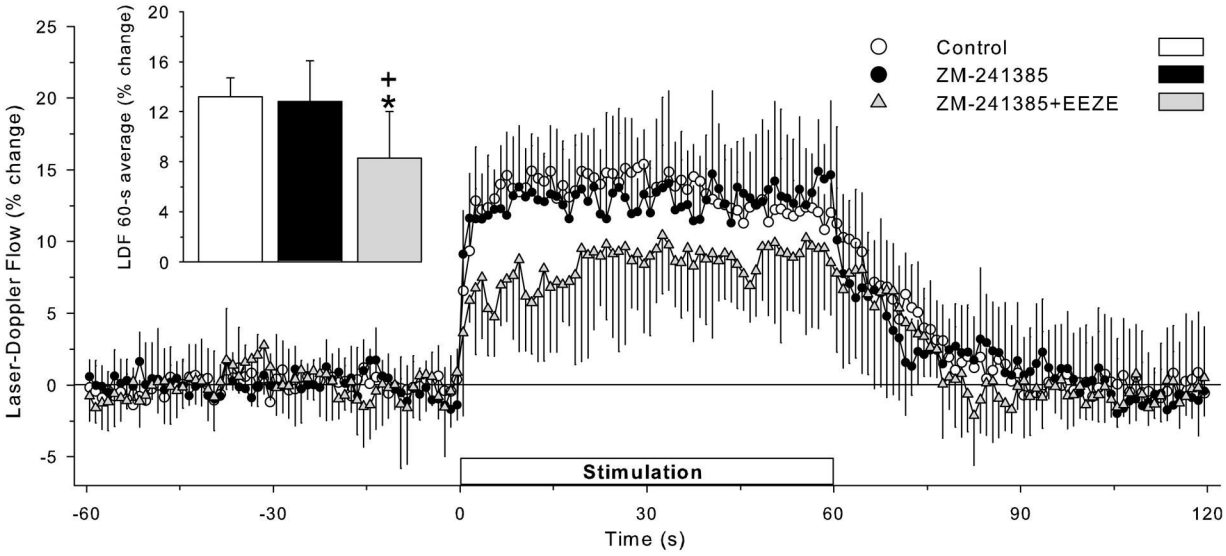

Figure 8.

Time course of 1-sec averages of cortical LDF (±s.d.; $n = 6$ rats), expressed as a percentage change from a 60-sec baseline recording, during 60 secs of whisker stimulation and 60 secs of recovery after 1-h superfusion of CSF, 1 h of ZM-241385 administration, and 1 h of combined ZM-241385 and 14,15-EEZE administration. Inset bar graph shows percentage change in LDF averaged over the 60-sec stimulation period. *$P < 0.05$ from control response; + $P < 0.05$ from ZM-241385 alone.

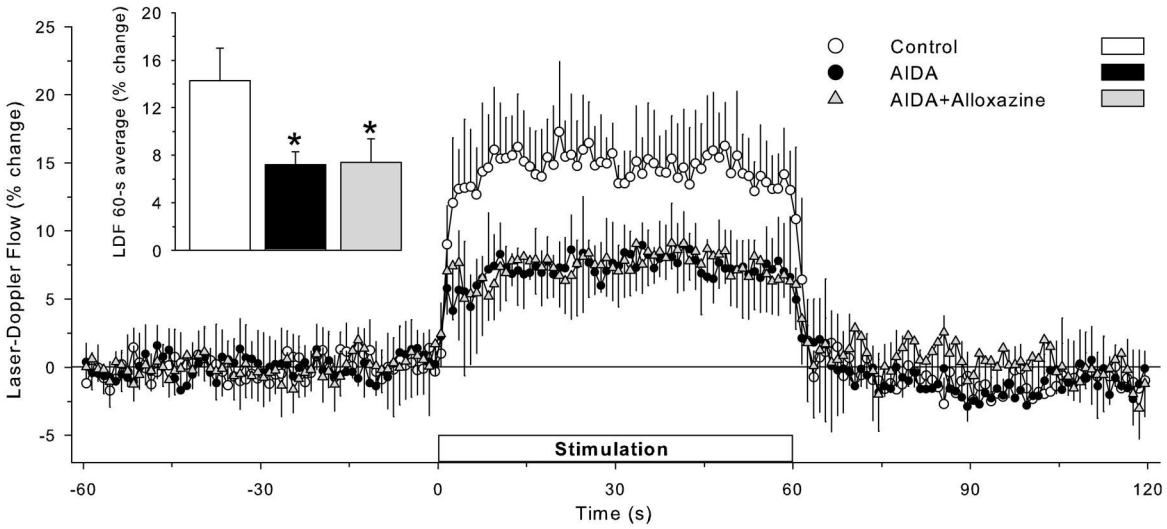

Figure 9.

Time course of 1-sec averages of cortical LDF (±s.d.; $n = 6$ rats), expressed as a percentage change from a 60-sec baseline recording, during 60 secs of whisker stimulation and 60 secs of recovery after 1-h superfusion of CSF, 1 h of AIDA superfusion, and 1 h of combined AIDA and alloxazine administration. Inset bar graph shows percentage change in LDF averaged over the 60-sec stimulation period. $*P < 0.05$ from control response.

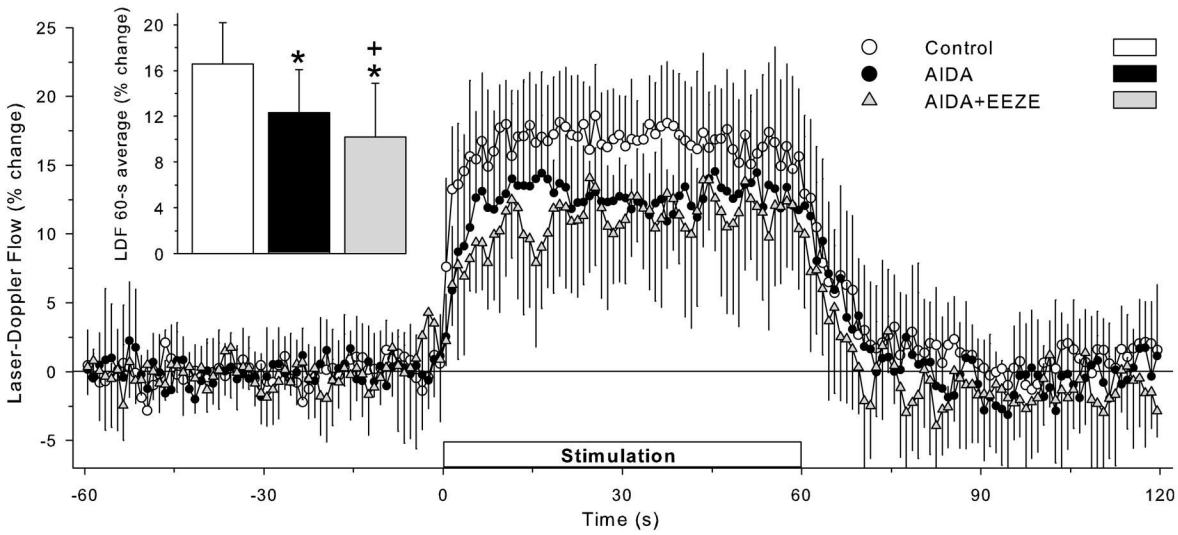

Figure 10.

Time course of 1-sec averages of cortical LDF (±s.d.; $n = 6$ rats), expressed as a percentage change from a 60-sec baseline recording, during 60 secs of whisker stimulation and 60 secs of recovery after 1-h superfusion of CSF, 1 h of AIDA superfusion, and 1 h of combined AIDA and 14,15-EEZE superfusion. Inset bar graph shows percentage change in LDF averaged over the 60-sec stimulation period. *$P < 0.05$ from control response; + $P < 0.05$ from AIDA alone.

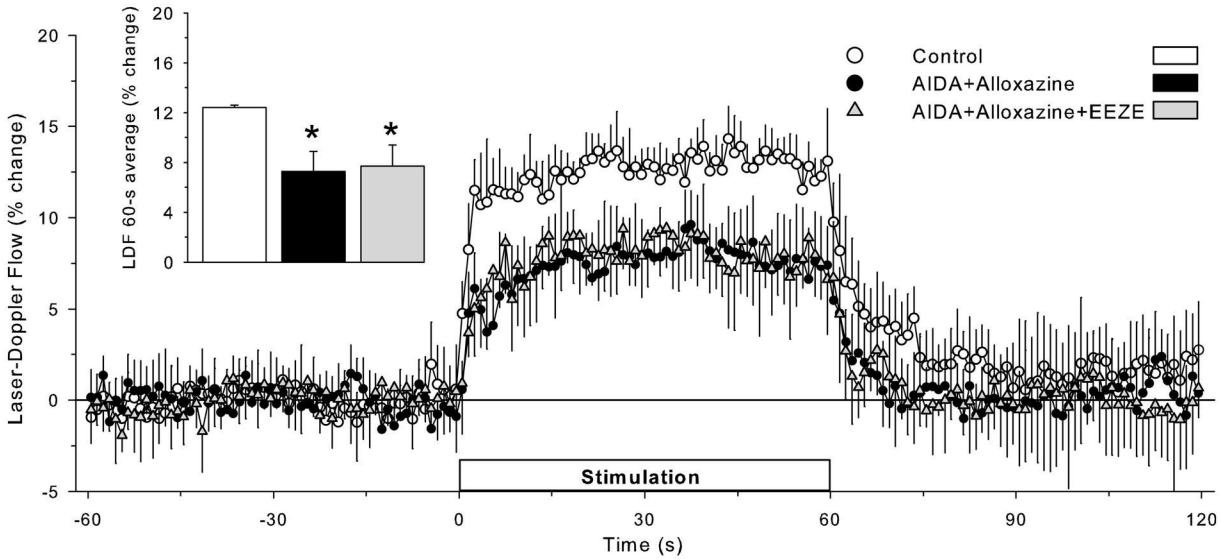

Figure 11.

Time course of 1-sec averages of cortical LDF (±s.d.; $n = 6$ rats), expressed as a percentage change from a 60-sec baseline recording, during 60 secs of whisker stimulation and 60 secs of recovery after 1-h superfusion of CSF, 1 h of AIDA plus alloxazine administration, and 1 h of combined AIDA, alloxazine and 14,15-EEZE administration. Inset bar graph shows percentage change in LDF averaged over the 60-sec stimulation period. $*P < 0.05$ from control response.

Table 1

Mean arterial blood pressure (MABP) and $PaCO_2$ at times of whisker stimulation

	MABP (mm Hg)			$PaCO_2$ (mm Hg)		
	1 h	2 h	3 h	1 h	2 h	3 h
Time control	108±18	110±20	109±13	39±1	38±1	38±1
ZM-241385/ZM-241385	117±12	109±10	106±13	37±2	38±2	39±1
SCH-58261/SCH-58261	97±6	109±12	108±11	37±2	37±2	38±1
MRS-1191/MRS-1191	101±9	100±10	100±10	37±2	38±2	38±2
MRS-1754/MRS-1754	96±9	95±8	97±9	39±1	36±1	36±1
Alloxazine/alloxazine	111±15	109±22	97±8	38±2	39±1	38±2
L-NNA/Alloxazine	108±7	112±13	113±9	37±1	39±1	39±1
MS-PPOH/MS-PPOH	111±13	111±13	113±8	39±2	38±1	38±1
Miconazole/miconazole	110±4	108±3	106±3	38±1	38±1	38±1
14,15-EEZE-mSI/14,15-EEZE-mSI	110±14	111±16	109±14	37±2	38±1	39±1
14,15-EEZE/14,15-EEZE	113±14	108±6	103±11	37±2	38±1	38±1
Alloxazine/14,15-EEZE	110±11	119±8	113±8	38±2	38±2	37±2
MS-PPOH/alloxazine	119±7	114±10	111±7	38±2	38±2	38±1
ZM-241385/14,15-EEZE	112±14	113±15	110±12	39±2	37±2	38±2
AIDA/AIDA	108±6	110±12	107±10	38±2	37±2	38±2
AIDA/alloxazine	101±8	99±5	94±9	37±2	38±1	38±2
AIDA/14,15-EEZE	106±9	118±12	116±8	38±2	38±2	38±1
AIDA+alloxazine/14,15-EEZE	116±18	114±10	106±4	38±2	37±2	38±2

AIDA, 1-aminoindan-1,5-dicarboxylic acid; L-NNA, Nù-nitro-L-arginine; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; 14,15-EEZE-mSI, 14,15-epoxyeicosa-5(Z)-enoic methylsulfonylimide; MRS-1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; MRS-1754, 8-[4-[((4-cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl) xanthine; MS-PPOH, *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide; SCH-58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; ZM-241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol.

Groups are defined by treatment at 2 h/combined treatment at 3 h.

Table 2

Percentage changes in baseline LDF without whisker stimulation at hours 2 and 3 with various treatments, compared to hour 1 with CSF superfusion

	2 h	3 h
Time control	2±9	1±14
ZM-241385/ZM-241385	10±7	4±19
SCH-58261/SCH-58261	−7±7	−2±8
MRS-1191/MRS-1191	−3±8	−4±10
MRS-1754/MRS-1754	−1±2	0±2
Alloxazine/alloxazine	12±14	8±18
L-NNA/alloxazine	−11±13*	−23±7*
MS-PPOH/MS-PPOH	1±12	−3±8
Miconazole/miconazole	−1±21	4±35
14,15-EEZE-mSI/14,15-EEZE-mSI	13±17	20±22
14,15-EEZE/14,15-EEZE	10±5	29±18*
Alloxazine/14,15-EEZE	7±12	8±8
MS-PPOH/alloxazine	9±11	16±22
ZM-241385/14,15-EEZE	6±29	7±30
AIDA/AIDA	6±17	4±16
AIDA/alloxazine	18±31	3±30
AIDA/14,15-EEZE	6±26	−4±26
AIDA+alloxazine/14,15-EEZE	5±9	3±16

AIDA, 1-aminoindan-1,5-dicarboxylic acid; L-NNA, Nù-nitro-L-arginine; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; 14,15-EEZE-mSI, 14,15-epoxyeicosa-5(Z)-enoic methylsulfonylimide; MRS-1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; MRS-1754, 8-[4-[((4-cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine; MS-PPOH, *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide; SCH-58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; ZM-241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol.

Groups are defined by treatment at 2 h/combined treatment at 3 h.

* $P < 0.05$ from time control group.