Relative contribution of cyclooxygenases, epoxyeicosatrienoic acids, and pH to the cerebral blood flow response to vibrissal stimulation

Xiaoguang Liu, Chunyuan Li, John R. Falck, David R. Harder, and Raymond C. Koehler

1Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, Maryland; 2Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas; and 3Cardiovascular Research Center and Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin

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Liu X, Li C, Falck JR, Harder DR, Koehler RC. Relative contribution of cyclooxygenases, epoxyeicosatrienoic acids, and pH to the cerebral blood flow response to vibrissal stimulation. Am J Physiol Heart Circ Physiol 302: H1075–H1085, 2012. First published December 23, 2011; doi:10.1152/ajpheart.00794.2011.—The increase in cerebral blood flow (CBF) during neuronal activation can be only partially attenuated by individual inhibitors of epoxyeicosatrienoic acids (EETs), cyclooxygenase-2, group I metabotropic glutamate receptors (mGluR), neuronal nitric oxide synthase (nNOS), N-methyl-D-aspartate receptors, or adenosine receptors. Some studies that used a high concentration (500 μM) of the cyclooxygenase-1 inhibitor SC-560 have implicated cyclooxygenase-1 in gliovascular coupling in certain model systems in the mouse. Here, we found that increasing the concentration of SC-560 from 25 μM to 500 μM over whisker barrel cortex in anesthetized rats attenuated the CBF response to whisker stimulation. However, exogenous prostaglandin E2 restored the response in the presence of 500 μM SC-560 but not in the presence of a cyclooxygenase-2 inhibitor, thereby suggesting a limited permissive role for cyclooxygenase-1. Furthermore, inhibition of the CBF response to whisker stimulation by an EET antagonist persisted in the presence of SC-560 or a cyclooxygenase-2 inhibitor, thereby indicating that the EET-dependent component of vasodilation did not require cyclooxygenase-1 or -2 activity. With combined inhibition of cyclooxygenase-1 and -2, mGluR, nNOS, EETs, N-methyl-D-aspartate receptors, and adenosine 2B receptors, the CBF response was reduced by 60%. We postulated that the inability to completely block the CBF response was due to tissue acidosis resulting from impaired clearance of metabolically produced CO2. We tested this idea by increasing the concentration of superfused bicarbonate from 25 to 60 mM and found a markedly reduced CBF response to hypercapnia. However, increasing bicarbonate had no effect on the initial or steady-state CBF response to whisker stimulation with or without combined inhibition. We conclude that the residual response after inhibition of several known vasodilatory mechanisms is not due to acidosis arising from impaired CO2 clearance when the CBF response is reduced. An unidentified mechanism apparently is responsible for the rapid, residual cortical vasodilation during vibrissal stimulation.

Relative contribution of cyclooxygenases, epoxyeicosatrienoic acids, and pH to the cerebral blood flow response to vibrissal stimulation.

METHODOLOGY

Multiple pathways have been implicated in coupling cerebral blood flow (CBF) to changes in neuronal activity (15). These include N-methyl-D-aspartate (NMDA) receptors (18, 29), neuronal nitric oxide (NO) synthase (nNOS) (6), astrocyte metabotropic glutamate receptors (mGluR) (18, 33, 39), epoxyeicosatrienoic acids (EETs) (18, 30, 31, 33), potassium channels (7, 11), adenosine (6, 28), cyclooxygenase-1 (COX-1) (35, 39), and cyclooxygenase-2 (COX-2) (2, 18, 23, 26, 34). However, questions remain about how these pathways are integrated and whether they account for all of the coupling mechanisms. Combinations of pharmacological inhibitors of multiple pathways generally do not produce additive effects in reducing the increase in CBF during neuronal activation and do not completely eliminate the response. For example, individual administration of inhibitors of mGluR, EETs, potassium channels, and adenosine receptors partially suppresses the vasodilatory response, whereas combined administration produces little additional suppression (28, 33). These observations are consistent with a sequential gliovascular signaling concept in which glutamate release during activation acts on astrocytic mGluR to stimulate release of ATP, which is subsequently hydrolyzed extracellularly to adenosine (37), and to stimulate release of EETs (1, 25), which can promote opening of potassium channels in astrocytes (10) and vascular smooth muscle (13) to produce vasodilation. Likewise, combining NOS inhibitors with adenosine (6) or EET (31) inhibitors or combining a NMDA receptor antagonist with an EET inhibitor (18) does not produce additive suppression of the CBF response or complete blockade of the response. Moreover, restoration of the CBF response after NOS inhibition by an NO donor suggests that NO acts in a permissive fashion (20), possibly by inhibiting synthesis of the vasoconstrictor 20-hydroxyeicosatetraenoic acid and thereby permitting vasodilation by other pathways (21).

Less work has been reported on the interaction of COX pathways with other signaling mechanisms. The nonspecific COX inhibitor indomethacin has been shown to individually inhibit the CBF response to activation (18) but not to completely block the response when combined with inhibitors of NOS, EETs, adenosine, and inward rectifier potassium channels (19). In the presence of indomethacin, a synthesis inhibitor of EET is still capable of reducing the CBF response to whisker stimulation (30). However, the interaction of COX with other pathways has not been well studied with the use of selective COX-1 and COX-2 inhibitors. A recent study reported no additive suppression of the CBF response to whisker stimulation with combined COX-2 and NMDA receptor inhibitors (18). Furthermore, whether a COX-1 or COX-2 metabolite mediates the CBF response or acts in a permissive fashion analogous to the role of NO has not been clearly discerned. Here, we examined whether an antagonist of EET was capable of reducing the CBF response in the presence of a selective COX-1 or COX-2 inhibitor. To investigate whether a COX-1 or COX-2 metabolite such as prostaglandin E2 (PGE2) acts in a permissive fashion, exogenous PGE2 was administered in the
presence of a selective COX-1 or COX-2 inhibitor. PGE2 was chosen because it can be released from astrocytes in response to glutamate (40). Whereas several studies have shown that COX-2 inhibitors decrease the CBF response to activation (2, 18, 23, 26, 34), some controversy persists on the role of COX-1. Studies using concentrations of 25–100 µM of the COX-1 inhibitor SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole] failed to reduce the CBF response to whisker stimulation in the mouse (27) or the rat (18) or to a mGluR agonist in the rat (22), whereas concentrations of 500 µM reduced vasodilation to photolysis of caged Ca2+ in astrocytes in vivo in mice (35) and to odorant stimulation in olfactory bulb glomeruli in mice (32). Thus, in the present study, we also examined the effects of SC-560 in both rats and mice and determined whether increasing the concentration to 500 µM would reveal an inhibitory effect on the CBF response to whisker stimulation.

Because the combination of COX inhibitors with other pathway inhibitors failed to completely block the CBF response, other unidentified mechanisms of neurovascular coupling may exist. One explanation for the incomplete block of the CBF response is a mismatch between metabolically produced CO2 and clearance of CO2 by CBF. Thus, when feedforward signaling mechanisms from neurons and astrocytes are blocked, additional metabolic feedback mechanisms may be recruited. Ordinarily, the increase in CBF during neuronal activation is proportional to an increase in glucose consumption (8), which, in turn, would be expected to generate a proportional increase in CO2 production. Thus proportional increases in CBF and CO2 production normally would be expected to result in little change in tissue Pco2. However, if inhibitors of vasodilation during neurovascular coupling do not reduce the glucose consumption response, then carbonic acidosis may develop and, because CO2 is a potent cerebral vasodilator, produce a residual CBF response to neuronal activation. Because vasodilation in response to increases in CO2 is known to be inversely related to the extracellular concentration of bicarbonate ions (16, 17), one strategy to address the role of CO2 is to manipulate the extracellular [HCO3−]. Thus increasing extracellular [HCO3−] would be expected to reduce the CBF response to neuronal activation if the response depends on an increase in tissue Pco2.

Five main hypotheses were tested in the present study. First, increasing the concentration of the COX-1 inhibitor SC-560 from 25 µM to 500 µM produces a significant reduction of the CBF response to whisker stimulation in rat and mouse. Second, administration of the EETs antagonist 14,15-EEZE [14,15-epoxyeicosa-5(Z)-enoic acid] produces additional inhibition of the CBF response to whisker stimulation in rats in the presence of the COX-1 inhibitor SC-560 or the COX-2 inhibitor NS-398 [N-[2-cyclohexyloxy]-4-nitrophenyl]-methanesulfonamide]. Third, reductions of the CBF response to whisker stimulation by the COX-1 and COX-2 inhibitor in rats can be reversed by concurrent administration of PGE2. Fourth, increasing extracellular [HCO3−] has no significant effect on the CBF response to whisker stimulation under control conditions when feedforward signaling mechanisms are intact. Fifth, when COX inhibitors are combined with inhibitors of other known feedforward pathways, increasing extracellular [HCO3−] now blocks the residual CBF response to whisker stimulation.
Table 1. Arterial PCO2, arterial blood pressure, and baseline laser-Doppler flow before stimulation in rats

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<th>Pco2, Torr</th>
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<tr>
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<tr>
<td>25 μM SC-560/14,15-EEZE</td>
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<td>500 μM SC-560/PGE2</td>
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<td>NS-398/PGE2</td>
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<tr>
<td>60 mM HCO3⁻/14,15-EEZE</td>
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<tr>
<td>MPEP + LY-367385/60 mM HCO3⁻</td>
<td>39 ± 1</td>
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<tr>
<td>MPEP + LY-367385 + 7-NI + NS-398/60 mM HCO3⁻</td>
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<td>Cocktail /60 mM HCO3⁻</td>
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Mean arterial blood pressure, mmHg

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<td>500 μM SC-560/14,15-EEZE</td>
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<td>93 ± 4</td>
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<td>500 μM SC-560/PGE2</td>
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<td>95 ± 4</td>
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<td>101 ± 3</td>
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<tr>
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<td>Cocktail /60 mM HCO3⁻</td>
<td>94 ± 2</td>
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Baseline laser-Doppler flow, % of 1-h baseline

<table>
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<tr>
<td>25 μM SC-560/14,15-EEZE</td>
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<td>MPEP + LY-367385/60 mM HCO3⁻</td>
<td>100 ± 1</td>
<td>92 ± 3*</td>
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<tr>
<td>MPEP + LY-367385 + 7-NI + NS-398/60 mM HCO3⁻</td>
<td>90 ± 1*</td>
<td>90 ± 1*</td>
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<tr>
<td>Cocktail /60 mM HCO3⁻</td>
<td>79 ± 3*</td>
<td>69 ± 3*</td>
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Values are means ± SE. Groups are defined by 2-h treatment/3-h treatment. MPEP, 2-methyl-6-(phenylethynyl)pyridine; 7-NI, 7-nitroindazole. *P < 0.05 from 1 h; †P < 0.05 from 2 h.
Whisker stimulation in rats produced a brisk increase in LDF over the whisker barrel cortex. After 60 s of stimulation, LDF rapidly returned to baseline (Fig. 1). Superfusion of the COX-1 inhibitor SC-560 at a concentration of 25 μM for 1 h decreased the baseline LDF (Table 1) but had no effect on the steady-state response to whisker stimulation when expressed as a percentage of the new baseline (Fig. 1). Addition of the EET antagonist 14,15-EEZE in the presence of SC-560 did not produce a further change in the baseline LDF but attenuated the steady-state response to whisker stimulation. The onset of the LDF response remained rapid in the presence of SC-560 alone or in combination with 14,15-EEZE.

In another set of rats, the concentration of superfused SC-560 was increased to 500 μM. The onset of the LDF response over the first 3 s of whisker stimulation remained unaffected (Fig. 2). With continued stimulation, the LDF response slightly subsided. The response averaged over the 60-s stimulation period was significantly reduced. Addition of 14,15-EEZE in the presence of the high concentration of SC-560 further reduced the steady-state response. Thus the EET-dependent component of vasodilation did not require COX-1 activity.

One possible explanation for the attenuated LDF response to the high concentration is that the full LDF response requires a minimal level of a vasodilatory metabolite, such as PGE2. To test this possibility, we superfused 5 μM PGE2 concurrently with 500 μM SC-560. In this set of rats, superfusion of SC-560 alone decreased baseline LDF (Table 1) and attenuated the increase in LDF during whisker stimulation (Fig. 3). Concurrent superfusion of PGE2 and SC-560 increased baseline LDF and increased the response to whisker stimulation compared with SC-560 alone. Moreover, the response was no longer significantly different from the control response.

To evaluate whether EET-dependent dilation required COX-2 activity, we tested the effect of the EET antagonist in
the presence of the COX-2 inhibitor NS-398 in another set of rats. Superfusion of 100 μM NS-398 did not affect baseline LDF (Table 1) but significantly reduced the LDF response to whisker stimulation (Fig. 4A). Addition of 14,15-EEZE in the presence of NS-398 did not change baseline LDF but further reduced the LDF response to whisker stimulation. To test whether a minimal amount of PGE2 is required for the LDF response to whisker stimulation in the presence of the COX-2 inhibitor, 5 μM PGE2 was superfused concurrently with 100 μM NS-398 in another set of rats. As in the previous set of rats, superfusion of NS-398 alone did not affect baseline LDF (Table 1) but significantly reduced the LDF response to whisker stimulation (Fig. 4A). However, in contrast with the effect of combining PGE2 with SC-560, combining PGE2 with NS-398 did not increase the LDF response to whisker stimulation.

In mice, SC-560 at a concentration of 500 μM has been shown to reduce arteriolar dilation to elevated Ca
tt, whereas SC-560 at a concentration of 25 μM had no effect on the LDF response to whisker stimulation (27). To test whether the higher concentration of SC-560 would more effectively inhibit the LDF response to whisker stimulation in mice, we superfused SC-560 over the cortical surface of mice at a concentration of 500 μM. The LDF response to 60 s of whisker stimulation was unchanged from the control response (Fig. 4C). In agreement with others (26), superfusion of 100 μM NS-398 in another set of mice was effective at reducing the LDF response to whisker stimulation (Fig. 4D).

None of the agents used in this study or combinations of other agents tested in previous studies (6, 11, 19, 31, 33) completely blocked the CBF response to activation. We speculated that the impaired CBF response might produce carbonic acidosis as a result of inadequate CO2 clearance. To test whether the residual response was related to enhanced carbonic acidosis, we increased the extracellular [HCO3-] to buffer any increase in CO2. Increasing [HCO3-] from 25 to 60 mM in the artificial CSF perfusate for 1 h in rats markedly reduced the LDF response to hypercapnia (Fig. 5). Thus the 1-h period of superfusion was sufficient for the increased [HCO3-] to permeate into the underlying tissue subtended by the LDF probe and to blunt the vascular response to carbonic acidosis.
response to whisker stimulation (Fig. 8). Increasing the super-

fusate and administration of 7-NI intraperitoneally de-

administration of MPEP, LY-367385, and NS-398 in the CSF

and the COX-2 and nNOS pathways was then evaluated.

additional decrement in the response.

mnia and hypercapnia after 1 h of subarachnoid superfusion with CSF containing

value; n = 5.

As expected, increasing [HCO$_3^-$] in the CSF perfusate to 60

mM for 1 h decreased baseline LDF (Table 1). However, the

increased [HCO$_3^-$] alone had no effect on the LDF response to

whisker stimulation from the new baseline (Fig. 6). Addition of

14,15-EEZE to the perfusate for an additional hour with

whisker stimulation from the new baseline (Fig. 6). Addition of

14,15-EEZE to the perfusate for an additional hour with

whisker stimulation after inhibition of mGluR, after combined

inhibition of mGluR, COX-2, nNOS, COX-1, EETs, NMDA

receptors, and adenosine A$_{2B}$ receptors. Thus carbonic acidosis

response further compared with that at the normal [HCO$_3^-$].

The on-response and off-response remained rapid in the presence of the combined inhibitors at elevated [HCO$_3^-$].

Other pathways implicated in the neurovascular coupling response include adenosine A$_{2B}$ receptors (33) and NMDA receptors (18, 29). In an attempt to further reduce the LDF response, the adenosine A$_{2B}$ receptor antagonist MRS-1754, the NMDA antagonist MK-801, the EET antagonist 14,15-EEZE, and the COX-1 inhibitor SC-560 (500 µM) were added with MPEP, LY-367385, and NS-398 for combined CSF superfusion for 1 h. In addition, 7-NI was administered intraperitoneally. This cocktail of eight drugs decreased baseline LDF (Table 1) and markedly inhibited the LDF response to whisker stimulation (Fig. 9). However, a residual response persisted. When the CSF [HCO$_3^-$] was then increased to 60 mM in the presence of the drug cocktail, baseline LDF was decreased further, but the LDF response to whisker stimulation was not decreased further. Again, the residual on-response and off-

response remained rapid in the presence of the drug cocktail at elevated [HCO$_3^-$].

**DISCUSSION**

This study presents several major findings. First, increasing the concentration of the COX-1 inhibitor SC-560 from 25 to 500 µM in rats moderately reduces the steady state cortical blood flow response to whisker stimulation in rats, but adding exogenous PGE$_2$ to increase baseline blood flow also restores the response, thereby suggesting a permissive role of COX-1 in rats. An obligatory role of a COX-1 metabolite was not apparent in mice. Second, the COX-2 inhibitor NS-398 effectively attenuates the blood flow response in both rats and mice, but exogenous PGE$_2$ does not restore the response in rats, thereby suggesting that COX-2 plays more of a mediatory role. Third, the EET antagonist reduces the blood flow response after either COX-1 or COX-2 inhibition, thereby indicating an independent effect of EETs. Fourth, elevating extracellular [HCO$_3^-$] to a level that inhibits hypercapnic vasodilation does not attenuate the blood flow response to whisker stimulation under control conditions. Fifth, elevating extracellular [HCO$_3^-$] does not result in further attenuation of the blood flow response to whisker stimulation after inhibition of mGluR, after combined inhibition of mGluR, COX-2, and nNOS, or after combined inhibition of mGluR, COX-2, nNOS, COX-1, EETs, NMDA receptors, and adenosine A$_{2B}$ Receptors. Thus carbonic acidosis is not a major contributor to the residual response after inhibition of several known signaling pathways for neurovascular coupling in rodent whisker barrel cortex.

The present results with whisker stimulation confirm the findings of others in mice and rats that COX-2 inhibitors have a greater effect than COX-1 inhibitors on the CBF response to activation in whisker barrel cortex (18, 26, 27). Inhibitors of COX-2 have also been shown to attenuate the CBF response in forelimb and hind limb cortex after peripheral electrical stimulation in rats (2, 23, 34). Thus COX-2 is the predominant isozyme contributing to functional hyperemia in various regions of primary sensory cortex in both rodent species. The present studies extend these findings by demonstrating that the EET antagonist 14,15-EEZE significantly attenuates the CBF response to whisker stimulation in the presence of a COX-1 or
COX-2 inhibitor. These results imply that COX metabolism of 5,6-EET, which is the only EET regioisomer that can be metabolized by COX, does not account for all of the EET-dependent vasodilation during neuronal activation. This conclusion is also consistent with previous work showing that an EET synthesis inhibitor was capable of reducing the CBF response to whisker stimulation in the presence of the non-isoform-specific COX inhibitor indomethacin (30). Furthermore, the current findings with whisker stimulation are congruent with recent work showing that superfusion of a group I mGluR agonist in vivo evoked an increase in CBF that was largely dependent on EETs, moderately dependent on COX-2 activity, and not significantly dependent on COX-1 activity (22).

Astrocytes in mouse cortex and rat hippocampus are reported to express COX-1 (12, 35), whereas astrocytes in mouse and rat cortex are reported to have little or undetectable constitutive expression of COX-2 (18, 35). Cultured astrocytes release PGE2 in response to external glutamate (40), and aspirin inhibits arteriolar dilation evoked by astrocyte activation in brain slices (39). Thus it has been assumed that a COX-1 metabolite mediates the dilation that occurs in response to astrocyte activation. In support of this concept, the COX-1 inhibitor SC-560 has been shown to inhibit arteriolar dilation in vivo to increased astrocyte Ca2+ in mouse cerebral cortex (35) and to sensory activation in olfactory glomeruli (32) and cochlear sensory organs (5). However, the concentration of SC-560 that was locally applied in those studies was 500 μM. In the present study, we showed that increasing the concentration of superfused SC-560 from 25 μM, which was shown previously to produce maximal inhibition of the vasodilation evoked by bradykinin (27), to 500 μM produced a small

Fig. 6. Time course of cortical laser-Doppler flow (±SE) in rats, expressed as percent change from a 60-s baseline recording, during and after 60 s of whisker stimulation at 1 h of subarachnoid superfusion with CSF containing normal [HCO3−] (25 mM), elevated [HCO3−] (60 mM), or 60 mM HCO3− + 30 μM 14,15-EEZE (A). Inset bar graph shows the percent change in flow during whisker stimulation averaged over the 60-s stimulation period (B). *P < 0.05 from CSF value; †P < 0.05 from 60 mM HCO3− value; n = 8.

Fig. 7. Time course of cortical laser-Doppler flow (±SE) in rats, expressed as percent change from a 60-s baseline recording, during and after 60 s of whisker stimulation at 1 h of subarachnoid superfusion with CSF containing normal [HCO3−] (25 mM), 100 μM 2-methyl-6-(phenylethynyl)pyridine (MPEP) + 300 μM LY-367385, or 100 μM MPEP + 300 μM LY-367385 + 60 mM HCO3− (A). Inset bar graph shows the percent change in flow during whisker stimulation averaged over the 60-s stimulation period (B). *P < 0.05 from CSF value; n = 7.
decrement in the steady-state CBF response to whisker stimulation in the rat, although no significant effect was detected in mice. However, adding exogenous PGE\textsubscript{2} to the superfusate at a concentration that increased baseline CBF resulted in a restoration of the CBF response to whisker stimulation. This result suggests that a COX-1 metabolite permits full expression of the steady-state hyperemic response but that a COX-1 metabolite does not necessarily mediate the response. This metabolite may be PGE\textsubscript{2}, PGI\textsubscript{2}, or other prostanoid dilators released from cells comprising the neurovascular unit.

Superfusion of 25 \mu M SC-560 decreased baseline LDF by 15 \pm 4\% but had no effect on the LDF response to whisker stimulation, whereas superfusion of 500 \mu M SC-560 decreased baseline LDF by 18 \pm 2\% but significantly reduced the response to whisker stimulation. Thus SC-560 exerts a differential sensitivity on baseline flow and activated flow. Although the difference between the 15\% and 18\% reduction in baseline LDF is small and not statistically different, inhibition of COX-1 at the 25 \mu M concentration may be submaximal. In isolated cochleae superfused with SC-560, increasing the concentration from 10 \mu M to 1,000 \mu M was found to produce nearly an additional 20\% inhibition of COX activity (5). Thus inhibition of COX-1 may need to be nearly 100\% to reveal this permissive effect of COX-1 to the neurovascular response in cerebral cortex of rat, whereas a significant decrease in tonic release of a COX-1 metabolite may require much less COX-1 inhibition. However, this permissive effect may not be present in all species since the high concentration of SC-560 had no effect on the LDF response to whisker stimulation in the mouse.

An alternative explanation for the 500 \mu M effect on the evoked LDF response is that the high concentration of SC-560...
NEUROVASCULAR COUPLING DEPENDENCY ON COX, EETs, AND pH

is also inhibiting COX-2 or other enzymes (3). However, if the reduction of the LDF response to whisker stimulation in the rat was simply due to inhibition of COX-2 by SC-560, then the addition of exogenous PGE2 might be expected to exert no effect on the activation response, as was the case with NS-398. The different effects of exogenous PGE2 in the presence of NS-398 versus the high concentration of SC-560 argues against a common target.

Because 14,15-EEZE superfusion in the presence of NS-398 produced additional inhibition of the CBF response, the epoxygenase and COX-2 signaling pathways are at least partly independent of each other. However, the CBF response was not completely blocked by combined inhibitors. The literature indicates that other combinations of inhibitors also failed to block the vascular response completely. These combinations included inhibitors of NOS + adenosine receptors (6, 33), NOS + EETs (21, 31), EETs + adenosine receptors (33), NOS + adenosine receptors + EETs (19), NOS + adenosine receptors + EETs + inward-rectifier K⁺ (Kᵢ) channels (19), Kᵢ channels + calcium-activated K⁺ (KᵥCa) channels (11), mGluR + adenosine receptors (33), mGluR + EETs (33), and mGluR + adenosine receptors + EETs (33), NMDA receptors + COX-2 (18), and NMDA receptors + EETs (18). One simple explanation for the inability of inhibitors of known signaling pathways in neurovascular coupling to completely eliminate the increase in CBF during neuronal activation is that metabolically based signals are recruited as a result of a mismatch between blood flow and metabolic demand when feed-forward signals from neurons and astrocytes are blocked.

We considered the possibility that an increase in tissue Pco₂ would occur when the increase in blood flow was impaired by inhibitors of these feed-forward signaling pathways. An increase in Pco₂ produces cerebral vasodilation by decreasing extracellular pH. Increasing extracellular [HCO₃⁻] will attenuate the change in extracellular pH for a given change in tissue Pco₂. Indeed, we found that increasing the CSF superfusate [HCO₃⁻] from 25 to 60 mM markedly attenuated the increase in LDF to an increase in arterial Pco₂. This finding is in agreement with earlier studies showing that increasing the CSF [HCO₃⁻] to 60 mM reduces pial arterial dilation (17) and the increase in CBF during hypercapnia (16). Moreover, this result indicated that the increase in [HCO₃⁻] was able to permeate sufficiently into the tissue sampled by the LDF probe. The decrease in baseline LDF after 1 h of superfusion with elevated [HCO₃⁻] also indicates the efficacy of permeation into the cortical tissue.

Whisker stimulation in the presence of increased extracellular [HCO₃⁻] produced the same percent increase in LDF from the new baseline LDF as that occurring with normal extracellular [HCO₃⁻]. Our interpretation of this finding is that tissue Pco₂ does not normally change significantly during neuronal activation. This interpretation is consistent with the lack of a large change in tissue pH arising from lactate accumulation (36). However, the umbrelliferone indicator technique used to measure tissue pH on cryostat sections of cortex after whisker stimulation in this study does not strictly control for postmortem changes in tissue Pco₂.

When group I mGluR antagonists were used to attenuate the LDF response to whisker stimulation, elevation of extracellular [HCO₃⁻] did not result in further attenuation of the LDF response to whisker stimulation. Thus inhibiting mGluR resulted in a residual LDF response that was not primarily attributable to tissue carbonic acidosis. Partial inhibition of the LDF response to cortical activation by mGluR antagonists is consistent with several reports in the literature (18, 33, 39). However, a recent study using 5 s of whisker stimulation did not show an inhibition of the flow response (4). Our results are not necessarily in conflict with this observation in that the rise time of the response over the first few seconds of stimulation was unabated. Thus mGluR may be more important for sustaining the CBF response to cortical activation. Although this same study failed to see an effect of the mGluR antagonists on the flow response to 24 s of whisker stimulation, the control flow response without antagonists spontaneously declined between 5 and 24 s. No such decline was evident in our study with 60 s of whisker stimulation. Perhaps differences in the methods of mechanical whisker stimulation may influence the contribution of mGluR to the steady-state flow response.

COX-2 and nNOS represent two other major signaling pathways that contribute to neurovascular coupling. When inhibitors of these two pathways were combined with group I mGluR antagonists, the LDF response to whisker stimulation was inhibited by 60%. However, the residual LDF response was unaffected by elevation of extracellular [HCO₃⁻]. By the addition of the inhibitors of EETs, COX-1, NMDA receptors, and adenosine A₂B receptors to the cocktail, the LDF response was not further suppressed and increasing extracellular [HCO₃⁻] did not substantially reduce the remaining 40% response. Thus carbonic acidosis is not responsible for the residual LDF response to whisker stimulation when several known signaling pathways are blocked.

It should be noted that we did not include inhibitors of KᵥCa and Kᵢ channels in the drug cocktail. Kᵢ activation on vascular smooth muscle depends on astrocyte release of K⁺ through KᵥCa channels (7), which depends on mGluR stimulation of EETs (10, 14). Addition of a Kᵢ antagonist to a cocktail of NOS, adenosine, and EET inhibitors does not produce a substantial additional diminution of the LDF response to whisker stimulation (19). Thus the residual LDF response in our experiments is unlikely to be attributable to activation of Kᵢ channels.

Interestingly, the residual LDF response remained rapid both in onset and offset after inhibition with the drug cocktail. Thus the unidentified residual mechanism of neurovascular coupling has a relatively rapid time constant. One mechanism that has been proposed is based on a sensing mechanism of the NADH redox state that results from the excess glycolysis relative to oxidative metabolism during cortical activation (24). One such sensing mechanism that has been proposed is that the associated increase in lactate will inhibit reuptake of PGE₂ and thereby prolong vasodilation (12). However, if the NADH redox state is responsible for the residual vasodilation, another sensing mechanism likely comes into play because the residual response persisted after combined COX-1 and COX-2 inhibition.

It is possible that the various inhibitors used in this study inhibited the increase in glucose consumption evoked by neuronal activation and consequently inhibited the increase in CO₂ production. Nevertheless, our conclusion that carbonic acidosis does not explain the residual LDF response after administration of the various inhibitors would still be valid in this case because the postulated mismatch between CO₂ production and
neural activity to vasodilation in the brain.

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