Endothelin rather than 20-HETE contributes to loss of pial arteriolar dilation during focal cerebral ischemia with and without polymeric hemoglobin transfusion

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Cao S, Wang LC, Kwansa H, Roman RJ, Harder DR, Koehler RC. Endothelin rather than 20-HETE contributes to loss of pial arteriolar dilation during focal cerebral ischemia with and without polymeric hemoglobin transfusion. Am J Physiol Regul Integr Comp Physiol 296: R1412–R1418, 2009. First published March 4, 2009; doi:10.1152/ajpregu.00003.2009.—Partial exchange transfusion with a cell-free hemoglobin (Hb) polymer during transient middle cerebral artery occlusion (MCAO) reduces infarct volume but fails to increase blood flow, as might be expected with the induced decrease in hematocrit. In ischemic brain, endothelin antagonists are known to produce vasodilation. In nonischemic brain, pial arterioles constrict after Hb exchange transfusion, and the constriction is blocked by an inhibitor of 20-HETE synthesis. We tested the hypothesis that a 20-HETE synthesis inhibitor and an endothelin A receptor antagonist increase pial arteriolar dilation after Hb exchange transfusion during MCAO. Pial arteriolar diameter was measured in the ischemic border region of the distal MCA border region through closed cranial windows in anesthetized rats subjected to the filament model of MCAO. During 2 h of MCAO, pial arteriolar dilation gradually subsided from 37 ± 3 to 7 ± 5% (±SE). Compared with residual dilation at 2 h of MCAO with vehicle superfusion (14 ± 3%), loss of dilation was not prevented by superfusion of a 20-HETE synthesis inhibitor (21 ± 5%), partial Hb exchange transfusion (7 ± 5%) that decreased hematocrit to 23%, or a combination of the two (5 ± 5%). However, loss of dilation was prevented by superfusion of an endothelin A receptor antagonist with (35 ± 4%) or without (32 ± 5%) Hb transfusion. Pial artery constriction during reperfusion was attenuated by HET0016 alone and by BQ610 with or without Hb transfusion. Systemic administration of the endothelin antagonist during prolonged MCAO increased blood flow in the border region. Thus loss of pial arteriolar dilation in the ischemic border region during prolonged MCAO depends on endothelin A receptor activation, and this effect was independent of the presence of cell-free Hb polymers in the plasma. In contrast to previous work in nonischemic brain, inhibition of oxygen-dependent 20-HETE synthesis does not significantly influence the pial arteriolar response to polymeric Hb exchange transfusion during focal ischemia.

To minimize peripheral extravasation, a compound containing a large polymer composed of many Hb tetramers was designed in which the polymerization agent was not retained. Unlike cross-linked tetramers (28, 33), this polymer, designated zero-link bovine Hb (ZL-HbBv), did not appear in renal lymph and did not produce peripheral vasoconstriction or arterial hypertension (13, 14). Exchange transfusion with ZL-HbBv after the onset of MCAO in mice reduced infarct volume (14). However, the distribution of intraischemic CBF was not improved despite the decrease in hematocrit. Collectively, these observations raise the possibility of arteriolar constriction that counteracts the decrease in blood viscosity and prevents a decrease cerebrovascular resistance.

In the absence of ischemia, partial exchange transfusion with ZL-HbBv resulted in constriction of pial arterioles, and this constriction appeared to offset the decrease in blood viscosity associated with a decrease in hematocrit because cerebrovascular resistance and CBF were unchanged (25). The constriction was reversed to dilation when plasma viscosity was increased, thereby suggesting that the constriction is a homeostatic response that prevents overoxygenation of the brain when blood viscosity is low. Interestingly, the constrictor response to ZL-HbBv exchange transfusion was not blocked by an inhibitor of NO synthase (22), and NO-dependent vasodilator responses to acetylcholine and ADP were preserved (21, 23). Thus, cell-free Hb in the plasma may not scavenge a physiologically greater amount of endothelially generated NO than does Hb in the red blood cell. However, the constrictor response to ZL-HbBv exchange transfusion was blocked by 20-hydroxyeicosatetraenoic acid (20-HETE) synthesis inhibitors, including N-hydroxy-N’-(4-n-butyl-2-methylphenyl)formamidine (HET0016) (22). Whereas 20-HETE promotes vasoconstric-
tion during increases in arterial pressure (5), the role of 20-HETE synthesis in limiting vasodilation during ischemia without Hb transfusion is unclear. Administration of HET0016 or other 20-HETE inhibitors did not increase laser-Doppler flux (LDF) recorded over the densely ischemic lateral cortex during MCAO, although LDF was improved by 3 h of reperfusion (4, 20, 26). However, if transfusion of cell-free Hb improves intraischemic oxygenation in arterioles, then O2-dependent synthesis of 20-HETE from arachidonic acid (6) may limit the degree of vasodilation during ischemia as it does without ischemia (22).

Several pieces of evidence suggest that vasodilatation during MCAO is not maximal and that additional dilatation is possible. For example, infusion of l-arginine can produce vasodilatation (16), and topical administration of ETα antagonists during MCAO can transiently dilate cat pial arterioles (19). However, little information exists on the natural time course of pial arteriolar dilatation during prolonged MCAO in the rat. In the present study, we focused on pial arterioles in the distal MCA region of parietal cortex near the anterior cerebral artery (ACA) watershed region. In this region, the direction of flow is typically reversed during MCAO because the region becomes supplied through anastomoses with the ACA. We determined the time course of changes in pial arteriolar diameter during 2 h of MCAO in the rat and whether exchange transfusion with ZL-HbBv during MCAO affected the time course. On the basis of the evidence that 20-HETE synthesis is involved in pial artery constriction after ZL-HbBv exchange transfusion and that ETα antagonists can dilate pial arteries during focal ischemia, we postulated that 20-HETE and ETα receptors may influence pial arterial dilatation after ZL-HbBv transfusion during MCAO. We tested the hypothesis that topical superfusion of the 20-HETE synthesis inhibitor HET0016 or the peptidergic ETα receptor antagonist BQ610 [homopiperidinyl-carbonyl-Leu-o-Trp (CHO)–o-Trp–ONH2] increases pial artery diameter during prolonged MCAO when the continuous superfusion was started before the ZL-HbBv transfusion. BQ610 has been used by others to reduce posts ischemic leukocyte adhesion (12).

**METHODS**

Surgical preparation. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee. In male Wistar rats (~300 g; Harlan Laboratories, Indianapolis, IN), anesthesia was induced with 5% isoflurane in O2-enriched air and maintained with 1.5–2% isoflurane during surgery and the experimental protocol. The right femoral artery was cannulated to monitor mean arterial blood pressure (MABP) and arterial blood gases; the left femoral artery was cannulated for transfusion. The rat was placed on a heating lamp throughout the surgical procedure and experiment. Pial arteriolar diameter was measured through the closed cranial window by intravital microscopy (Zeiss Axioshead System II microscope, Oberkochen, Germany) and a digital camera (Fast1394, Q Imaging, Surrey, BC, Canada) connected to a computer for image analysis (Metamorph software, Universal Imaging, Downingtown, PA). A LDF probe was placed on the right temporal bone that had been previously thinned by drilling a small hole lateral to the cranial window.

**Experimental protocol.** Forty-five minutes after the completion of surgery, the window was flushed over a 5-min period with aCSF, and baseline measurements of MABP, arteriolar diameter, arterial blood gases, and Hb concentration were obtained. MCAO was produced by advancing the filament to achieve a stable reduction in LDF by >60% over lateral cortex. The early change in arteriolar diameter was measured at 10 min of MCAO. Except for a time control group with no superfusion, the window was superfused with aCSF, vehicle, or drug at a rate of 200 μl/min for 10 min starting at 15 min of MCAO. The superfusion rate was decreased to 17 μl/min for the remainder of the experiment. At 25 min of MCAO, measurements of arteriolar diameter were repeated. In some groups, an exchange transfusion was performed starting at 35 min of MCAO at a rate of 0.5 ml/min for 15 min followed by a maintenance infusion of 1 ml/h for the remainder of the experiment. The solution contained ~6% ZL-HbBv and 5% human serum albumin to maintain oncotic pressure. Details of the production of ZL-HbBv have been described previously (13). Reperfusion was initiated at 120 min of MCAO by withdrawal of the filament. Arteriolar diameter measurements were repeated at 60, 75, 90, 105, and 120 min of MCAO and at 10 and 20 min of reperfusion. Arterial blood gases and Hb concentration measurements were repeated at 30, 90, and 120 min of MCAO. After 20 min of reperfusion, the heart was arrested by intravenous injection of KCl.

Drugs were applied by superfusion of the cranial window to ensure adequate delivery to the artery that was examined. Because the superfusion could possibly clear vasoactive agents produced during ischemia, comparisons first were made between groups of rats with no cranial window superfusion (n = 9) and superfusion with aCSF (n = 9). In the second experiment, the window was superfused with either 0.1% ethanol vehicle (n = 9) or 1 μmol/l of HET0016 (n = 8). In the third experiment, comparisons were made between groups transfused with ZL-HbBv after superfusion with either 0.1% ethanol (n = 9) or 1 μmol/l HET0016 (n = 8). In a fourth experiment, comparisons were made among groups superfused with 0.02% DMSO vehicle and no transfusion (n = 9), 3 μmol/l BQ610 and no transfusion (n = 8), or 3 μmol/l BQ610 and ZL-HbBv transfusion (n = 7).

To determine whether BQ610 was capable of increasing CBF in the ischemic border region, a separate group of five rats was studied without cranial window measurements of arteriolar diameter. LDF was measured in the border region (4 mm caudal and 3 mm lateral to bregma) and in the ischemic core (0 mm rostral and 10 mm lateral to bregma). The skull was thinned at both sites without exposing the dura mater, and LDF probes were held in a fixed position for the duration of MCAO. At 90 min of MCAO, 0.5 μmol/kg of BQ610 was injected intravenously and the percent change in LDF was measured. A time control group of five rats was also studied.

**Statistical analysis.** The percent change in diameter from the preischemic baseline was calculated for each arteriole (baseline diameter = 48 ± 17 μm). Statistical analysis was performed by using the
average percent change of four to seven pial arterioles per rat, with the sample size as the number of rats. On the basis of the 7% standard deviation of the initial change in diameter and an average sample size of eight, differences equivalent to 11% of baseline diameter could be detected between two groups with an estimated power of 80%. For each experiment, two-way ANOVA was performed. If a significant interaction occurred between treatment groups and time, then comparisons among groups were performed at individual time points with the Newman-Keuls multiple range test. Measurements of LDF before and after BQ610 administration were compared by paired *t*-test. A significance level of 0.05 was used in all tests.

**RESULTS**

MABP was relatively stable throughout the observation period in all groups (Table 1), and exchange transfusion with ZL-HbBv did not produce significant hypertension. In all groups, arterial P02 was kept above 100 Torr, arterial Pco2 was relatively constant at ~40 Torr, and arterial pH remained at ~7.40 throughout MCAO (Table 2).

Pial arterioles had increased in diameter by 30–40% at 10 min after MCAO (Fig. 1). However, this dilation subsided over the 2-h period of MCAO. Reperfusion resulted in a relative constriction. The gradual loss of dilation during sustained MCAO occurred whether or not the cranial window was superfused with aCSF starting at 15 min of MCAO.

Loss of dilation also occurred during prolonged MCAO in groups superfused with 0.1% ethanol (vehicle for HET0016) or HET0016 superfusion (Fig. 2). Although two-way ANOVA indicated a significant interaction of time and HET0016 superfusion, comparisons at individual time points revealed significant differences only during reperfusion. The constrictor response to reperfusion was attenuated by HET0016 superfusion.

Exchange transfusion with ZL-HbBv starting at 35 min of MCAO resulted in a decrease in hematocrit from 38 ± 1 to 23 ± 1% and in arterial Hb concentration from 12.3 ± 0.2 to 8.1 ± 0.3 g/dl. Transfusion did not significantly alter the time course of pial arteriolar diameter changes during MCAO or reperfusion (Fig. 3). Moreover, initiating HET0016 superfusion before exchange transfusion with ZL-HbBv did not prevent the decrease in diameter, and ANOVA indicated no significant interaction of HET0016 treatment with time in these two Hb-transfused groups (Fig. 4).

Table 1. Arterial blood pressure before and during MCAO in rats whose cortical surface was superfused with vehicle or drug via a cranial window starting at 15 min of MCAO

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCAO Duration, min</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>No superfusion</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>CSF superfusion</td>
<td>101 ± 1</td>
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<tr>
<td>0.1% ethanol superfusion</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>HET0016 superfusion</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>0.1% ethanol superfusion/Hb</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>transfusion</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>HET0016 superfusion/Hb transfusion</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>0.02% DMSO superfusion</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>BQ610 superfusion</td>
<td>101 ± 1</td>
</tr>
<tr>
<td>BQ610 superfusion/Hb transfusion</td>
<td>101 ± 1</td>
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</table>

Values are means ± SE. MCAO, middle cerebral artery occlusion; CSF, cerebrospinal fluid. Some groups of rats also underwent hemoglobin (Hb) exchange transfusion at 35–50 min.

As in other groups, loss of dilation occurred when the window was superfused with 0.02% DMSO, the vehicle for BQ610 (Fig. 5). In comparing this effect of time with that of the group superfused with CSF alone, two-way ANOVA indicated no significant interaction between CSF and 0.02% DMSO superfusion treatments over time (P < 0.90). However, the group superfused with BQ610 maintained significantly greater arteriolar diameter than did the vehicle group by 90 min of MCAO and thereafter. When ZL-HbBv was exchange transfused after BQ610 superfusion, diameter was sustained at the initial 10-min value throughout MCAO. The change in diameter from the preschismic baseline became significantly greater than that in the vehicle group by 75 min of MCAO and thereafter. In both groups superfused with BQ610, the extent of dilation at 2 h of MCAO was not different from the initial dilation before BQ610 superfusion had commenced.

To determine whether BQ610 administration can increase CBF during MCAO, the drug was injected intravenously at 90 min of MCAO. Delayed injection during MCAO permitted a paired statistical analysis of LDF within the same animal at a time when pial arterioles in the border region had exhibited decreased dilation and should be capable of additional dilation. The drug was administered systemically to provide delivery to both intraparenchymal and extraparenchymal blood vessels during LDF monitoring. Intravenous injection of BQ610 did not significantly change MABP (104 ± 2 to 107 ± 2 mmHg) but increased LDF in the cortical ischemic core and border region (Fig. 6). In a time control group, LDF was not significantly increased over the same time period.

**DISCUSSION**

This study revealed several major findings. First, pial arterioles in the distal MCA distribution area near the ACA border region exhibited a gradual loss of dilation during the course of the 2-h MCAO. Second, decreasing hematocrit by exchange transfusion with cell-free polymeric Hb after MCAO did not alter the loss of pial arteriolar dilation. Third, superfusion of a 20-HETE synthesis inhibitor did not increase pial diameter after Hb exchange transfusion during MCAO as it did in nonischemic pial arterioles (22). Fourth, the ETₐ receptor antagonist was equally effective at preventing the loss of dilation whether or not the rats were transfused with cell-free Hb during MCAO.

In a study by Patel et al. (19) that used chloralose-anesthetized cats with an open cranial window superfused with mineral oil, pial arteries in the penumbral region initially dilated after MCAO. However, by 30 min of MCAO, the responses became heterogeneous, with some animals displaying sustained arterial dilation and others showing constriction to diameters as much as 50% smaller than those of preschismic baseline. In our model with closed cranial windows in isoflurane-anesthetized rats, we observed initial pial arteriolar dilation that subsided after 10 min of MCAO. The decrease in dilation was more gradual than that seen in the study on cats and usually did not diminish to values significantly below preischemic baseline. Although differences in species, anesthetic, and methodology may account for quantitative differences between ours and this previous study, both studies are qualitatively consistent with the concept that vasodilation is submaximal during prolonged MCAO. Because the loss of...
vasodilation was observed in the group without continuous superfusion of the cranial window and in the three groups superfused with aCSF or each vehicle, the loss of vasodilation was a robust finding in our model, and the superfusion protocol did not appear to substantially dilute locally released vasoreactive agents.

Exchange transfusion of ZL-HbBv in nonischemic animals produced pial arteriolar constriction that offset the decrease in blood viscosity and resulted in no change in CBF (25). This constriction was blocked by acute superfusion with 1 μmol/l of HET0016 (22), the same concentration that we used here. Because 20-HETE synthesis is oxygen dependent (6, 10), the vasoconstrictor response to decreased blood viscosity and a plasma-based O₂ carrier was attributed to increased O₂-dependent 20-HETE synthesis (22). Previous work indicated that exchange transfusion with ZL-HbBv did not produce the increase in intracranial blood flow that would be expected with a decrease in hematocrit (14). Thus we postulated that the Hb-based O₂ carrier might improve oxygenation sufficiently to increase 20-HETE production and restrict pial arteriolar dilation during MCAO. However, we found that loss of pial arteriolar dilation during MCAO still occurred after ZL-HbBv exchange transfusion and that HET0016 superfusion started before the transfusion failed to block the loss of dilation. Thus 20-HETE synthesis did not appear to be a major factor that limited dilation when oxygenation was expected to be improved by ZL-HbBv.

Inhibition of 20-HETE synthesis has been found to decrease infarct size in a variety of MCAO models (4, 15, 17, 20). Because 20-HETE is produced by CYP 4A in cerebral vascular smooth muscle (4) and because 20-HETE is a cerebral vasodilator, tissue rescue by 20-HETE synthesis inhibitors might result from vasodilation. However, previous studies did not find a significant increase in LDF in lateral cortex where ischemia is dense (4, 20). An increase in intracerebral LDF was not detected over penumbral cortex after administration of a 20-HETE synthesis inhibitor, which retained neuroprotective properties when administered at reperfusion (26). The present finding that superfusion of HET0016 over the pial surface did not significantly attenuate the loss of pial arteriolar dilation during MCAO also supports the concept that protection by 20-HETE synthesis inhibition is not mediated by intracranial vasodilation. Protection could be mediated by a vascular mech-

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**Table 2. Arterial Po₂, Pco₂, and pH at baseline and at 120 min of MCAO in rats whose brains were superfused with vehicle or drug via a cranial window starting at 15 min of MCAO**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Po₂, Torr</th>
<th>Pco₂, Torr</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>120 min</td>
<td>Baseline</td>
</tr>
<tr>
<td>No superfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>CSF superfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>0.1% ethanol superfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>HET0016 superfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>0.02% DMSO superfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>BQ610 superfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>BQ610 superfusion/Hb transfusion</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
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</tbody>
</table>

Values are means ± SE. Some groups of rats also underwent Hb exchange transfusion at 35–50 min. Two-way ANOVA indicated a significant interaction between treatment and time (P = 0.033); *P < 0.05 from vehicle superfusion by Newman-Keuls multiple range test.
anism during reperfusion (20), consistent with greater dilation seen during early reperfusion in our experiment, or possibly by mitigating effects of 20-HETE on neurons and glia. Reoxygenation may increase O₂-dependent 20-HETE synthesis and constrain vasodilation during reperfusion. Maintaining pial arteries in a vasodilated state during reperfusion with HET0016 should help overcome the poor reflow phenomenon among capillaries during early reperfusion.

Loss of vasodilation during prolonged MCAO could be attributed to decreased release of vasodilator mediators, increased release of vasoconstrictors, decreased vasoactivity to released mediators, and decreased intraluminal distending pressure, which is known to fall to levels below 20 mmHg during MCAO (34). The observations in cats that perivascular microinjections of nonpeptidergic endothelin antagonists produce immediate, transient dilation of pial arterioles that either had remained dilated or had constricted below preischemic baseline implies that locally released endothelin contributed to submaximal dilation (19). On the basis of these observations, we continuously superfused an endothelin antagonist to demonstrate that pial arteriolar diameter could be maintained in a vasodilated state during prolonged MCAO. Superfusion with the peptidergic ETA antagonist BQ610 beginning at 15 min of MCAO prevented subsequent loss of dilation. Moreover, intravenous injection of BQ610 at 90 min of MCAO, a time at which pial arteriolar dilation had subsided, produced an increase in CBF in the ischemic border region. The increase in
CBF implies that loss of pial arteriolar dilation is associated with constrained CBF. Moreover, the potential effect on CBF could be greater than that observed because the low intraischemic CBF may have limited the delivery of BQ610. Collectively, these results are consistent with local release of endothelin as a major component of submaximal dilation in the ischemic border region and lend further support to other studies that have shown improved perfusion with endothelin antagonists (11, 18, 35). The improved pial artery diameter during reperfusion with BQ610 superfusion also suggests that endothelin antagonists could improve reflow. Because other studies have demonstrated that endothelin antagonist administration results in decreased infarct volume (11, 18, 32), the effect of BQ610 on infarct volume was not pursued in the present study.

Transfusion of cross-linked tetrameric Hb produces peripheral vasoconstriction that can be attenuated by NO synthase inhibition or an endothelin antagonist. Because the tetramers can extravasate, they are likely to scavenge NO and decrease NO-dependent inhibition of peripheral endothelin release. However, the large Hb polymers that we used here do not readily extravasate or produce peripheral vasoconstriction and thus appear less likely to stimulate endothelin release. We did not determine whether the ZL-HbBv augments endothelin release from the peripheral vasculature or ischemia-induced endothelin release from the cerebral vasculature. However, the observation that loss of pial arteriolar dilation was not significantly augmented by ZL-HbBv transfusion suggests that any augmentation of endothelin release by ZL-HbBv was not physiologically significant. Furthermore, BQ610 was equally potent in blocking the loss of dilation with and without ZL-HbBv transfusion. Without Hb transfusion, cerebral ischemia has been reported to increase plasma and cerebral tissue endothelin (2, 37), although the time course has not been well studied. We did not measure the time course of plasma endothelin during MCAO to determine whether the levels correlated with the time course of pial arterial diameter. However, the diameter difference between vehicle and BQ610 superfused groups increased progressively, which implies that the effect of endothelin also increased over time. Mean arterial pressure and blood gases were unchanged during MCAO and did not confound interpretation of the data.

We conclude that rescue of ischemic tissue by ZL-HbBv exchange transfusion is constrained by submaximal dilation mediated by endothelin rather than by 20-HETE synthesis. However, this constraint is not substantially greater than that without ZL-HbBv transfusion. Tissue rescue previously reported with ZL-HbBv transfusion is likely related to improved oxygenation within parenchyma rather than to extraparenchymal vasodilation.

**REFERENCES**


Loss of Pial Arteriolar Dilation during Focal Ischemia


