Circulating Endothelin-1 Alters Critical Mechanisms Regulating Cerebral Microcirculation

Giuseppe Faraco, Ana Moraga, Jamie Moore, Joseph Anrather, Virginia M. Pickel, Costantino Iadecola

Abstract—Endothelin-1 (ET1) is a potent vasoconstrictor peptide implicated in the cerebrovascular alterations occurring in stroke, subarachnoid hemorrhage, and brain trauma. Brain or circulating levels of ET1 are elevated in these conditions and in risk factors for cerebrovascular diseases. Most studies on the cerebrovascular effects of ET1 have focused on vascular smooth muscle constriction, and little is known about the effect of the peptide on cerebrovascular regulation. We tested the hypothesis that ET1 increases cerebrovascular risk by disrupting critical mechanisms regulating cerebral blood flow. Male C57Bl6/J mice equipped with a cranial window were infused intravenously with vehicle or ET1, and somatosensory cortex blood flow was assessed by laser Doppler flowmetry. ET1 infusion increased mean arterial pressure and attenuated the blood flow increase produced by neural activity (whisker stimulation) or neocortical application of the endothelium-dependent vasodilator acetylcholine but not A23187. The cerebrovascular effects of ET1 were abrogated by the ETα receptor antagonist BQ123 and were not related to vascular oxidative stress. Rather, the dysfunction was dependent on Rho-associated protein kinase activity. Furthermore, in vitro studies demonstrated that ET1 suppresses endothelial nitric oxide (NO) production, assessed by its metabolite nitrite, an effect associated with Rho-associated protein kinase–dependent changes in the phosphorylation state of endothelial NO synthase. Collectively, these novel observations demonstrate that increased ET1 plasma levels alter key regulatory mechanisms of the cerebral circulation by modulating endothelial NO synthase phosphorylation and NO production through Rho-associated protein kinase. The ET1-induced cerebrovascular dysfunction may increase cerebrovascular risk by lowering cerebrovascular reserves and increasing the vulnerability of the brain to cerebral ischemia. (Hypertension. 2013;62:759-766.) • Online Data Supplement

Key Words: endothelin-1 ■ nitric oxide synthase ■ Rho-associated kinases

Endothelin-1 (ET1), a 21-aa peptide produced mainly by endothelial cells, is one of the most potent vasoconstrictors known. ET1 acts on 2 distinct receptors (ETα and ETβ), both coupled to phospholipase C via a GTP-binding protein. ETα receptors (ETαR) are expressed on vascular smooth muscle cells (VSMC) and, possibly, brain endothelial cells, and their activation leads to vasoconstriction. ETβ receptors are mainly located on endothelial cells and induce vasodilatation by promoting nitric oxide (NO) release.

ET1 has been implicated in a wide variety of neurological diseases, and several studies in animal models have documented that ETαR are involved in the cerebral blood flow (CBF) reduction observed in cerebral ischemia, traumatic brain injury, and subarachnoid hemorrhage. Furthermore, increased plasma levels of ET1 have been observed in risk factors for stroke, such as hypertension, diabetes mellitus, obstructive sleep apnea, and cardiac failure. The deleterious effects of ET1 have been attributed predominantly to its potent vascular actions. However, most studies have focused on the contractile effects of ET1 on VSMC, and little is known about the effect of ET1 on the mechanisms regulating the cerebral circulation.

The brain is uniquely dependent on a well-regulated delivery of blood flow matched to its changing energy needs dictated by neural activity. Complex cerebrovascular control mechanisms assure that the brain is adequately perfused at all times. For example, neural activity increases CBF (functional hyperemia) to support the increased energy requirements, whereas endothelial cells regulate the distribution of CBF within cerebrovascular networks by releasing vasoactive factors. Alterations in these regulatory mechanisms have been implicated in a growing numbers of neurological conditions, ranging from ischemic stroke to neurodegenerative diseases. ET1 is involved in the cerebrovascular dysfunction induced by angiotensin II (AngII) slow-pressor hypertension, chronic intermittent hypoxia, diabetes mellitus, multiple sclerosis, and amyloid-β, a peptide involved in the pathogenesis of Alzheimer disease. However, it remains to be established whether ET1 can reproduce the cerebrovascular alterations observed in these models.
In this study, we investigated the effect of ET1 on the critical regulatory mechanisms of the brain circulation. Using a combination of in vivo and in vitro approaches, we found that elevations in circulating ET1 comparable with those observed in cerebrovascular conditions profoundly alter the regulation of CBF by neural activity and endothelial cells. Surprisingly, these effects, mediated by endothelial ET\textsubscript{R}, are not associated with a reduction in resting CBF and are not caused by oxidative stress. Rather, the dysfunction depends on endothelial Rho-associated protein kinase (ROCK) activity, which, in turn, leads to suppression of NO production by modulating the phosphorylation state of endothelial NO synthase (eNOS). These new findings demonstrate that circulating ET1 is able to impair vital regulatory mechanisms of the cerebral circulation. Such impairment in neurovascular regulation could render the brain more susceptible to injury by compromising the balance between energy demands and blood flow delivery.

**Methods**

**Materials and Methods** pertaining to reactive oxygen species (ROS) detection, immunofluorescence, electron microscopy, cell cultures, Western blotting, and biochemical measurements are described in the online-only Data Supplement.

**General Surgical Procedures**

All the procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College. Studies were conducted in 3-month-old C57Bl/6 male mice (weight, 25–30 g; The Jackson Laboratory). Mice were anesthetized with isoflurane (2% maintenance), intubated, and artificially ventilated (SAR-830; CWE Inc). Mean arterial pressure (MAP), rectal temperature, and blood gases were monitored and controlled. After surgery, anesthesia was maintained with urethane (750 mg/kg IP) and chloralose (50 mg/kg IP).

**Monitoring of CBF**

CBF was monitored with a laser Doppler probe (Periflux System 5010, Perimed AB) in a cranial window overlying the somatosensory cortex bathed with a modified Ringer’s solution. CBF was expressed as percentage increases relative to the resting level.

**Experimental Protocol**

After stabilization of MAP and blood gases (Table S2 in the online-only Data Supplement), baseline CBF responses were tested with hyperemia induced by ET1 infusion. However, neocortical application of cerebrovascular dysfunction. Therefore, we examined whether ET1 in endothelial cells, we used immunogold electron microscopy and demonstrated that the receptor was present in the membrane and the cytoplasm of cerebral endothelial cells (Figure 2F).

**Data Analysis**

Data are expressed as mean±SEM. Two-group comparisons were evaluated using the Student t test. Multiple comparisons were considered statistically significant if P<0.05.

**Results**

**ET1 Infusion Does Not Affect Resting CBF or the Blood–Brain Barrier But Impairs Functional Hyperemia and CBF Response to ACh**

ET1 infusion (20, 35, 50 pmol/kg per minute) increased MAP dose dependently, reaching a stable increase after 15 to 20 minutes (Figure 1A). ET1 infusion did not reduce resting CBF but attenuated the increase in CBF produced by whisker stimulation and ACh at 35 and 50 pmol/kg per minute (Figure 2C and 2D). Unlike the MAP increase, the effect on CBF responses was already maximal at 35 pmol/kg per minute. CBF responses to A23187 or adenosine were not suppressed (Figure 1E and 1F). Similarly, ET1 did not affect the increase in CBF induced by the NO donor SNAP (Figure S1A). To determine whether the effects of ET1 on CBF were secondary to entry of the peptide into the brain as a result of the opening of the blood–brain barrier (BBB), we assessed the integrity of the BBB after 45 minutes of ET1 infusion. ET1 (35 pmol/kg per minute) did not increase the BBB permeability to Evans blue (Figure S1B), but the anticipated increase was observed after focal cerebral ischemia (Figure S1B), attesting to the validity of the method. In agreement with the BBB results, ET1 infusion increases ET1 levels in plasma (vehicle: 1.9±0.3; ET1: 15.3±3.9 pg/mL; P<0.05; n=5/group) and lung (vehicle: 245.4±90.5; ET1: 920.8±228.2 pg/mL; P<0.05; n=5/group) but not in brain (vehicle: 5.7±0.5; ET1: 5.0±0.4 pg/mg; P<0.05; n=5/group).

**Cerebrovascular Effects of ET1 Are Mediated by ET\textsubscript{R}**

Next, we examined the role of ET\textsubscript{R} and ET\textsubscript{A} receptor in the cerebrovascular effects of ET1. Neocortical superfusion with the ET\textsubscript{A} receptor antagonist BQ123 (1 μmol/L) reversed the attenuation in CBF responses produced by ET1 (35 pmol/kg per minute), whereas the ET\textsubscript{A} receptor antagonist BQ788 (100 nmol/L) did not (Figure 2B). Neither antagonist influenced resting CBF or baseline CBF responses (Table S1 and Figure S2A and S2B). Then, we used immunocytochemistry to examine the localization of ET\textsubscript{R} in pial arterioles (diameter, 80–100 μm) of the somatosensory cortex. As anticipated,1 patchy ET\textsubscript{R} immunoreactivity was observed deep in the vessel wall consistent with smooth muscle cell localization (Figure 2E). However, ET\textsubscript{R} immunoreactivity was also observed in cerebral endothelial cells and endothelial cell cultures (Figure 2E–2G). To provide further evidence for ET\textsubscript{R} in endothelial cells, we used immunogold electron microscopy and demonstrated that the receptor was present in the membrane and the cytoplasm of cerebral endothelial cells (Figure 2F).

**ROS Are Not Involved in the Cerebrovascular Effects of Circulating ET1**

ET1 induces vascular oxidative stress, a well-known cause of cerebrovascular dysfunction. Therefore, we examined whether ROS are involved in the cerebrovascular dysfunction induced by ET1 infusion. However, neocortical application...
of the ROS scavenger MnTBAP (100 μmol/L), effective in suppressing AngII-induced cerebrovascular dysfunction in this model,\textsuperscript{26} did not abrogate the cerebrovascular effects of ET1 (Figure 3A and 3B). To confirm that ET1 did not induce oxidative stress, we assessed ROS production using hydroethidine fluoromicrography. Consistent with the findings with MnTBAP, ET1 infusion did not induce statistically significant increases in ROS (Figure 3C). However, infusion of AngII (0.25 μg/kg per minute; 200 μL/h) markedly increased somatosensory cortex ROS production (Figure 3C).

ROCK Is Involved in the Cerebrovascular Effects of Circulating ET1

ROCK has been implicated in the vascular effects of ET1.\textsuperscript{27,28} Therefore, we used the ROCK inhibitor Y27632 to test the hypothesis that this enzyme is involved in the cerebrovascular dysfunction induced by circulating ET1. Y27632 superfusion did not affect resting CBF or baseline cerebrovascular responses (Table S1 and Figure S2C) but counteracted the ET1-induced attenuation of the CBF response to whisker stimulation or ACh (Figure 4A and 4B). In contrast, Y27632 did not rescue the cerebrovascular dysfunction produced by systemic administration of AngII (Figure 4C and 4D), highlighting key differences in the mechanisms of the acute cerebrovascular effects of ET1 and AngII, the latter mediated by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-derived ROS in this model.\textsuperscript{25}

ET1 Attenuates Acetylcholine-Induced Production of Nitrite in Brain Endothelial Cell Cultures, an Effect Dependent on ET\textsubscript{A}R and ROCK

The finding that ET1 attenuates the increase in CBF induced by ACh, an eNOS-dependent response, suggests that ET1 may affect the production of NO by endothelial cells. To address this issue, we used cultures of brain endothelial cells. As shown in Figure 6A, ACh increased nitrite levels, an effect blocked by the NOS inhibitor L-\textsuperscript{N\textgreek{g}enitroarginine (L-NNA) (100 μmol/L), confirming that ACh induces eNOS activation in these cells. The increase in nitrite production induced by ACh was attenuated by ET1, an effect counteracted by BQ123 but not BQ788 (Figure 5B), implicating ET\textsubscript{A}R. Western blotting demonstrated ET\textsubscript{A}R expression in the culture (Figure 2G).
Our in vivo data implicate ROCK activation in the cerebrovascular effects of ET1. Therefore, we examined whether the ET1-induced attenuation of the nitrites produced by ACh in endothelial cultures was also dependent on ROCK activity. As anticipated, the ROCK inhibitor Y27632 (1 μmol/L) counteracted the effects of ET1 on ACh-induced nitrite production (Figure 5B), suggesting that endothelial ROCK is involved in the effects of ET1 on NO production in vitro.

ET1 Suppresses NO Production by Altering eNOS Phosphorylation Through ROCK

eNOS phosphorylation is a key regulator of eNOS activity.29 Thr495 phosphorylation is associated with reductions and Ser1177 phosphorylation with increases in eNOS catalytic activity.30 Therefore, we examined whether the effects of ET1 on eNOS activity are related to eNOS phosphorylation. In agreement with previous reports,30 ACh elicited
a relatively rapid (1–5 minutes) and transient increase in p-Ser<sup>1177</sup> eNOS, followed by a delayed and slow rise in p-Thr<sup>495</sup> eNOS (Figure 5C, 5E, and 5F). ET1 suppressed ACh-induced eNOS activation by reducing p-Ser<sup>1177</sup> and enhancing p-Thr<sup>495</sup> (Figure 5D–5F), an effect prevented by BQ123 (Figure 6A and 6B). ROCK can inhibit eNOS by increasing p-Thr<sup>495</sup> and reducing p-Ser<sup>1177</sup>.<sup>31–33</sup> Therefore, we examined the effect of the ROCK inhibitor Y27632 on the attenuation of ACh-induced eNOS phosphorylation by ET1. As illustrated in Figure 6C and 6D, Y27632 prevented the effects of ET1 on ACh-induced eNOS phosphorylation, implicating ROCK in the mechanisms of ACh-induced eNOS phosphorylation.

### Discussion

**Novel Findings of the Study**

This study has several novel aspects. First, we demonstrated that ET1 impairs the regulation of the cerebral circulation by endothelial cells and neural activity. Most studies on the vascular actions of ET1 in brain and other organs have focused on the ability of this peptide to constrict arteries and reduce flow, and little was known about the effects of this peptide on the dynamic regulation of organ blood flow.<sup>12,13</sup> Second, we found that the endothelial dysfunction was not mediated by vascular oxidative stress but was dependent on activation of endothelial ROCK, leading to changes in the phosphorylation state of eNOS. Third, we found that the effect of ET1 on endothelial NO production was mediated by endothelial ET<sub>A</sub>R, which we localized to endothelial cells both in

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Y27632 rescues the endothelin-1 (ET1)–induced attenuation of the cerebral blood flow (CBF) response to whisker stimulation (A) or ACh (B), whereas Y27632 does not rescue the dysfunction induced by angiotensin II (AngII; C and D). *P<0.05 from respective vehicle group; n= 5 to 8/group.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** Endothelin-1 (ET1) reduces ACh-induced nitrite production in brain endothelial cells (A). BQ123 and Y27632 but not BQ788 prevent the effect of ET1 on ACh-induced nitrite production (B). ACh increases phosphorylated endothelial nitric oxide synthase (p-eNOS; Ser<sup>1177</sup>) followed by an increase in p-eNOS (Thr<sup>495</sup>; C). ET1 attenuates the p-Ser<sup>1177</sup> and increases the p-Thr<sup>495</sup> induced by ACh (D). E and F. Optical densities of p-Ser<sup>1177</sup> and p-Thr<sup>495</sup> bands were measured, and changes in phosphorylation levels were expressed relative to vehicle-treated cells. *P<0.05 from vehicle; †P<0.05 from ACh; n=8 to 10/group.
vivo and in vitro. These observations demonstrate for the first time that increases in ET1 plasma levels are associated with profound alterations of vital mechanisms regulating the cerebral circulation, mediated by a previously unrecognized effect of ET1 on endothelial ROCK activity and eNOS phosphorylation.

Cerebrovascular Effects of Circulating ET1 Are Mediated by ETAR

The cerebrovascular dysfunction induced by ET1 was dependent on ETAR. Using immunocytochemistry to determine the cellular localization of ETAR, we found that ETAR immunoreactivity is present both in VSMC and in brain endothelial cells. The endothelial localization of ETAR was also confirmed by immunogold electron microscopy and Western blotting in brain endothelial cell cultures. ETAR are abundant in VSMC, but the present results cannot be attributed to these receptors because ET1 did not reduce resting CBF and did not impair the CBF response evoked by smooth muscle relaxants. Therefore, our data suggest that endothelial ETAR are the target of plasma ET1 and mediate the deleterious vascular effects of the peptide.

Circulating ET1 Does Not Alter Resting CBF

In some vascular districts, ET1 has been reported to produce vasodilatation at low concentrations, an effect attributed to ETB receptor, and vasoconstriction at higher concentrations via ETAR. We found that circulating ET1 did not affect resting CBF. This finding is in agreement with previous observations demonstrating that circulating ET1 does not reduce brain blood flow except in areas outside the BBB, such as choroid plexus and dura mater. Considering that ET1 in our model did not breach the BBB in the somatosensory cortex, the data support the hypothesis that ET1 needs to cross the BBB to reduce CBF. Therefore, circulating ET1 could induce a more severe disruption of cerebrovascular function, involving also VSMC, if the BBB is disrupted.

Selectivity of the Cerebrovascular Effects of Circulating ET1

The cerebrovascular dysfunction induced by ET1 did not result from an indiscriminate impairment of cerebrovascular reactivity. Rather, only CBF responses evoked by neural activity and ACh were attenuated, whereas those evoked by A23187, adenosine, and SNAP were preserved. These findings have important mechanistic implications for the cerebrovascular effects of circulating ET1. First, they suggest that ET1 does not impair the ability of smooth muscle cells to relax, but only the capacity of neural activity and selected endothelial agonists to increase CBF. Second, the fact that the CBF increase produced by ACh, a response mediated by eNOS, was attenuated, whereas the response to A23187, which is mediated by cyclooxygenase, was not affected suggests that ET1 interferes only with NO-mediated endothelial responses. Finally, the finding that ET1 did not inhibit the response to the NO donor SNAP suggests that ET1 does not act by impairing the ability of NO-cGMP to relax vascular smooth muscles.

Based on these observations, we hypothesized that ET1 impairs the ability of endothelial cells to generate NO. This hypothesis was supported by the observation that ET1 suppresses ACh-induced NO production in endothelial cell cultures. Consistent with the results in vivo, the effect was dependent on ETAR and ROCK activity. Because ROCK can inhibit eNOS activity by modulating its activatory (Ser1177) and inhibitory (Thr495) phosphorylation sites, we investigated the effect of ET1 on eNOS phosphorylation. We found that ET1 leads to Thr495 phosphorylation and Ser1177 dephosphorylation, resulting in a reduction in eNOS phosphorylation. Although ET1 could also inhibit the NO response to ACh by downregulating endothelial muscarinic receptors, the fact that ET1 does not inhibit muscarinic receptor signaling argues against this possibility.
Mechanisms of the Effects of ET1 on Functional Hyperemia

We also found that ET1 attenuates the increase in CBF produced by neural activity. ET1 could attenuate functional hyperemia by suppressing neuronal NOS activity, which plays a role in the CBF increase. Because we did not detect an increase in ET1 in the brain after intravenous infusion, we doubt that the peptide crossed the BBB. Although small increases in brain ET1 below the sensitivity of the assay cannot be ruled out, a direct neuronal action of the peptide is unlikely. Furthermore, the fact that ET1 did not alter resting CBF, which is closely linked to basal neural activity, supports the hypothesis that ET1 did not act by suppressing neural activity. Another possibility is that plasma ET1 exerts its effects via the circumventricular organs, structures outside the BBB that could affect cerebrovascular function broadly through neurohumoral mechanisms. Indeed, we recently reported that chronic administration of AngII alters neurovascular coupling by acting on the subfornical organ, one of the circumventricular organs, and inducing cerebrovascular oxidative stress. However, this possibility seems unlikely because the effects of circulating ET1 on functional hyperemia are not mediated by ROS. Therefore, additional studies are required to elucidate how ET1 affects neurovascular coupling.

ROS Do Not Contribute to the Cerebrovascular Effects of Cirulating ET1

ROS play a major role in cerebrovascular dysfunction induced by different agents, including AngII and β-amyloid. ET1 has also been reported to induce oxidative stress in blood vessels, endothelial cells, and VSMC, effects attributed to mitochondrial ROS, eNOS uncoupling, or activation of the superoxide-producing enzyme NADPH oxidase. In contrast, we found that circulating ET1 does not increase ROS in the neocortex and that an ROS scavenger is not able to reverse the cerebrovascular dysfunction, suggesting that ROS do not play a primary role in the cerebrovascular dysfunction. The reasons for this discrepancy remain unclear, but the difference could be because of the fact that the ET1 concentrations previously used were greater than those of the present experiments. However, increases in endogenous ET1 in the wall of cerebral blood vessels have been reported to contribute to the cerebrovascular oxidative stress induced by administration of subpressor doses of AngII, chronic intermittent hypoxia, or diabetes mellitus. Therefore, the present study, in which ET1 had access mainly to the endothelium, may indicate that the cellular localization of the peptide and its receptors may be critical for inducing vascular oxidative stress. Alternatively, the ability of ET1 to increase ROS may be context dependent, requiring the presence of other mediators and longer exposure times.

Role of ET1 in Conditions Associated With Cerebrovascular Dysfunction

ET1 has been implicated in the pathogenesis of several conditions associated with cerebrovascular dysfunction and damage, including subarachnoid hemorrhage, ischemic stroke, and brain trauma. As noted above, these effects have been attributed to the vasoconstrictor effects of ET1 or to its ability to promote ROS production. The present results provide evidence that ET1 can also alter cerebrovascular regulation, independently of vasoconstriction or vascular oxidative stress. Our observations are relevant to conditions in which circulating levels of ET1 are elevated, such as risk factors for ischemic stroke. Although in these conditions ET1 is likely to act in concert with other vascular mechanisms, the data suggest that ET1 is sufficient to induce cerebrovascular dysfunction. The dependence of the cerebrovascular effect of circulating ET1 on ETAR and ROCK strengthens the argument for targeting ETAR and ROCK for therapeutic purposes.

Perspectives

We have demonstrated that elevations in circulating levels of ET1, comparable with those observed in cerebrovascular conditions, suppress functional hyperemia and impair selected endothelium-dependent responses, critical factors in CBF regulation. The effect is mediated by endothelial ETAR and is independent of vascular oxidative stress, instead requiring activation of endothelial ROCK. In vitro studies suggest that endothelial ROCK acts by modulating eNOS phosphorylation and attenuating eNOS activity. These novel observations, collectively, suggest that circulating levels of ET1 are sufficient to alter cerebrovascular regulation, which, alone or in combination with other pathogenic mechanisms, may play a role in the cerebrovascular dysfunction associated with acute and chronic brain injury.

Sources of Funding

This work was supported by National Institutes of Health grant HL96571.

Disclosures

None.

References

Novelty and Significance

What Is New?

- Endothelin-1 (ET1) alters critical cerebrovascular regulatory mechanisms.
- The effect is mediated by endothelial ET<sub>R</sub> receptor and Rho-associated protein kinase.
- ET1 suppresses nitric oxide production by altering endothelial nitric oxide synthase phosphorylation status.

What Is Relevant?

- Elevated ET1 plasma levels have been observed in risk factors for stroke.
- ET1-induced cerebrovascular dysfunction may increase the susceptibility of the brain to ischemic injury by reducing cerebrovascular reserves.

- ET<sub>1</sub> receptor antagonists and Rho-associated protein kinase inhibitor are potentially useful to reverse the cerebrovascular abnormalities.

Summary

ET1 alters cerebrovascular function and might be involved in the increased susceptibility to ischemic injury observed in risk factors for stroke. The involvement of ET<sub>1</sub> receptor and Rho-associated protein kinase suggests new therapeutic approaches to counteract the deleterious cerebrovascular effects of ET-1.
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_Hypertension._ 2013;62:759-766; originally published online August 19, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.01761

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Circulating endothelin-1 alters critical mechanisms regulating the cerebral microcirculation

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EXPANDED MATERIALS AND METHODS

General Surgical Procedures
All of the procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College. Studies were conducted in 3-month–old C57Bl/6 male mice (weight: 25 to 30 g) (The Jackson Laboratory). Mice were anesthetized with isoflurane (2% maintenance) intubated and artificially ventilated (SAR-830, CWE Inc). Mean arterial pressure (MAP), rectal temperature, and blood gases were monitored and controlled. After surgery, anesthesia was maintained with urethane (750 mg/kg ip) and chloralose (50 mg/kg ip).

Monitoring of CBF
CBF was monitored with a laser-Doppler probe (Periflux System 5010, Perimed AB) in a cranial window overlying the somatosensory cortex bathed with a modified Ringer’s solution. CBF was expressed as percentage increases relative to the resting level.

Experimental Protocol
After stabilization of MAP and blood gases (Table S2), baseline CBF responses were tested with i.v. infusion of vehicle (saline; rate: 200µL/h). Functional hyperemia was tested by repetitive deflection of the facial whiskers (3-5 Hz; 60 sec) contralateral to the window and recording the corresponding CBF changes. To test endothelial-dependent responses, ACh (10µmol/L) or A23187 (5µmol/L) were topically applied to the window and the CBF changes recorded. Agents were used at concentrations determined in dose response studies not to be supramaximal, and were applied to the window until a stable increase of CBF was reached (usually 3-5 min). In the mouse cerebral microcirculation ACh acts by releasing NO from eNOS, whereas A23187 acts through cyclooxygenase reaction products. Smooth muscle reactivity was examined using adenosine (400µmol/L). After obtaining baseline CBF responses, ET1 (Sigma) was administered intravenously (20, 35 and 50pmol/Kg/min; rate: 150-200µL/h) and CBF responses tested again 20 min later when a stable MAP was reached. In some experiments the effect of BQ123, BQ788, Y27632, or MnTBAP was studied. The cranial window was superfused with Ringer’s solution and the CBF increases evoked by whisker stimulation, acetylcholine and adenosine were tested. Next, ET-1 i.v. infusion was started and the superfusion solution was switched to Ringer containing BQ123, BQ788, Y27682 or MnTBAP and CBF responses were tested again 30 mins later. In experiments in which smooth muscle reactivity was studied, the CBF increases evoked by the nitric oxide donor S-nitroso-D-penicillamine (SNAP; 10-50 µmol/L; Sigma) was tested before and 30 minuter after ET-1 i.v. infusion was started.

Pharmacological Agents
The ETₐR antagonist BQ123, the ET₈R antagonist BQ788 and the ROCK inhibitor Y27632 (Tocris), or the ROS scavenger MnTBAP (Calbiochem) were dissolved in Ringer solution and superfused on the somatosensory cortex. BQ123 and BQ788 have been used extensively, and their selectivity and specificity at the concentrations used...
has been verified\textsuperscript{5,6}. Y27632 is relatively selective for ROCK at 1\textmu mol/L concentration\textsuperscript{7} and has been used in cerebrovascular studies\textsuperscript{8,9}.

**Immunofluorescence and immunoperoxidase labeling**

Coronal brain sections were cut through the somatosensory cortex using a cryostat and incubated with primary antibodies against ET\textsubscript{A}R and the endothelial marker CD31. After incubation with a FITC- and a Cy5-conjugated secondary antibody, sections were mounted on slides and examined using a Leica confocal microscope. The specificity of the antibody was tested as previously described\textsuperscript{10}. For electron microscopic analysis of ET\textsubscript{A}R distribution, sections through the somatosensory cortex of acrolein-fixed mouse brain were processed for immunoperoxidase labeling. For this, acrolein-fixed sections through this brain region were incubated with the ET\textsubscript{A}R antibody followed by the avidin-biotin complex (Vectastain Elite Kit, Vector Lab.). The bound peroxidase was visualized by incubation in diaminobenzidine and hydrogen peroxide. This tissue was subsequently postfixed in osmium tetroxide and embedded in plastic prior to cutting ultrathin sections that were examined with an electron microscope.

**ROS Detection**

ROS production was assessed by hydroethidine (HE) microfluorography\textsuperscript{1}. Ringer solution containing HE (2\textmu mol/L) was superfused and, 15 minutes later, the intravenous infusion of ET1 (20, 35, 50pmol/Kg/min) or vehicle was started. Mice were killed 45 minutes later, coronal brain sections were cut through cortex underlying the cranial window, and ROS dependent fluorescence was quantified as described previously\textsuperscript{1}.

**Blood brain barrier permeability measurement**

Evans Blue (EB) (200\textmu L of a 2\% solution)\textsuperscript{11} was administered i.v. 15 min prior to ET1. After 60 min of EB circulation, mice were sacrificed, brains removed and EB concentration determined fluorometrically in cerebral cortex homogenates\textsuperscript{11}. Mice in which EB was administered 6 hrs after transient middle cerebral artery occlusion served as positive controls\textsuperscript{12}.

**Endothelin Assay**

Mice were killed after 45 minutes of intravenous ET1 infusion. Plasma, lung and brain samples were immediately collected and ET1 concentration (pg/mg or pg/ml) was determined using an enzyme immunoassay kit as previously described\textsuperscript{1}.

**Brain endothelial cell cultures and nitrite measurement**

Immortalized murine brain microvascular endothelial cells (bEnd.3) were obtained from ATCC and maintained in D-MEM supplemented with 10\% (v/v) heat inactivated FBS, 100 units/ml penicillin, 100 \mu g/ml streptomycin and 4 mM L-glutamine. Cells were split 1:4 to 1:8 upon reaching confluence and cultured in 6-well plates. Prior to the experiments, bEnd.3 cells were grown overnight in FBS-free culture medium supplemented with 0.1\% (w/v) BSA. Cells were washed with Hepes buffer, pre-treated for 45 min with ET1 (10-100 nmol/L). Nitrite, stable NO metabolites reflecting NO production by endothelial cells, was measured after 45 min of exposure to ACh (20 \mu mol/L) or vehicle at 37°C. In some experiments, cells were exposed to the NOS...
inhibitor L-NNA (100 µmol/L) prior to testing responses to ACh. Nitrites levels were measured fluorimetrically using a flurimetric kit (Calbiochem). eNOS activity was expressed as the amount of nitrite/mg of protein. Experiments were repeated five times.

**Western Blotting**

Cells were lysed in RIPA buffer (Roche Applied Sciences), and equal volumes were mixed with SDS sample buffer, boiled, and analyzed on 10% SDS polyacrylamide gels. Proteins were transferred to PVDF membranes (Millipore), blocked with 5% milk in TBS/0.1% Tween-20 (TBST), and incubated with polyclonal anti-ET\textsubscript{A}R (AbCam), anti-phospho-eNOS (Ser\textsuperscript{1177}), anti-phospho-eNOS (Thr\textsuperscript{495}) and anti-eNOS (Cell Signaling). Membranes were washed in TBST, incubated with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and protein bands were visualized with Chemiluminescence Reagent (Santa Cruz Biotechnology) on a Kodak Image Station 2000R.
Reference List


Table S1. Mean arterial pressure (MAP), resting CBF and CBF increases evoked by adenosine in mice treated with BQ123, BQ788, MnTBAP or Y7632

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAP (mmHg)</th>
<th>Resting CBF (PU)</th>
<th>Adenosine (% CBF increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>ET1</td>
<td>Vehicle</td>
</tr>
<tr>
<td>BQ123</td>
<td>74.77±3.45</td>
<td>102.0±3.36*</td>
<td>124±7</td>
</tr>
<tr>
<td>BQ788</td>
<td>76.55±3.92</td>
<td>103.9±4.82*</td>
<td>136±10</td>
</tr>
<tr>
<td>MnTBAP</td>
<td>74.62±3.50</td>
<td>96.7±3.87*</td>
<td>129±8</td>
</tr>
<tr>
<td>Y7632</td>
<td>72.63±4.73</td>
<td>106.4±6.22*</td>
<td>125±9</td>
</tr>
</tbody>
</table>

*p<0.05, Analysis of variance and Tukey’s test.

Table S2. Blood gases and pH of mice studied

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pO2</th>
<th>pCO2</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Vehicle</td>
<td>145.0±8</td>
<td>128.5±15</td>
<td>29.3±6</td>
</tr>
<tr>
<td>ET1 20pmol/Kg/min</td>
<td>144.7±12</td>
<td>124.5±19</td>
<td>25.3±3</td>
</tr>
<tr>
<td>ET1 35pmol/Kg/min</td>
<td>133.4±13</td>
<td>126.5±17</td>
<td>27.0±4</td>
</tr>
<tr>
<td>ET1 50pmol/Kg/min</td>
<td>133.8±18</td>
<td>115.7±30</td>
<td>30.1±7</td>
</tr>
<tr>
<td>BQ123 (ET1)</td>
<td>137.8±6</td>
<td>130.7±5</td>
<td>24.6±5</td>
</tr>
<tr>
<td>BQ788 (ET1)</td>
<td>146.2±11</td>
<td>137.2±9</td>
<td>27.1±5</td>
</tr>
<tr>
<td>MnTBAP (ET1)</td>
<td>134.8±9</td>
<td>123.9±18</td>
<td>31.6±4</td>
</tr>
<tr>
<td>Y27632 (ET1)</td>
<td>137.8±15</td>
<td>131.9±12</td>
<td>27.5±4</td>
</tr>
<tr>
<td>SNAP (ET1)</td>
<td>133.4±18</td>
<td>114.5±13</td>
<td>28.5±4</td>
</tr>
<tr>
<td>Y27632 (ANGII)</td>
<td>149.4±13</td>
<td>149.5±8</td>
<td>25.5±1</td>
</tr>
</tbody>
</table>
Figure S1. (A) ET1 does not attenuate CBF response to NO donor SNAP. (B) ET1 does not alter the BBB permeability to Evans Blue, but tMCAo does (*p<0.05 from vehicle, Analysis of variance and Tukey’s test; n=5/group).

Figure S2. BQ123 (A), BQ788 (B) and Y27632 (C) have no effect on CBF responses to whisker stimulation, ACh or Adenosine.