Blockade and Reversal of Endothelin-Induced Constriction in Pial Arteries From Human Brain

Lisa N. Pierre, PhD; Anthony P. Davenport, PhD

Background and Purpose—Substantial evidence now implicates endothelin (ET) in the pathophysiology of cerebrovascular disorders such as the delayed vasospasm associated with subarachnoid hemorrhage and ischemic stroke. We investigated the ET receptor subtypes mediating vasoconstriction in human pial arteries.

Methods—ET receptors on human pial and intracerebral arteries were visualized with the use of autoradiography, and the subtypes mediating vasoconstriction were identified by means of wire myography.

Results—ET-1 was more potent than ET-3 as a vasoconstrictor, indicating an ET\textsubscript{A} -mediated effect. Similarly, the selective ET\textsubscript{A} agonist sarafotoxin S6c had no effect on contractile action at concentrations up to 30 nmol/L. The nonpeptide ET\textsubscript{A} receptor antagonist PD156707 (3 to 30 nmol/L) caused a parallel rightward shift of the ET-1–induced response, yielding a pA\textsubscript{2} of 9.2. Consistent with these results, PD156707 (30 nmol/L) fully reversed an established constriction in pial arteries induced by 1 nmol/L ET-1, while the selective ET\textsubscript{B} receptor antagonist BQ788 (1 \textmu mol/L) had little effect. The calcium channel blocker nimodipine (0.3 to 3 \textmu mol/L) significantly attenuated the maximum response to ET-1 in a concentration-dependent manner without changing potency. In agreement with the functional data, specific binding of \[^{[125]}\text{I}\]PD151242 to ET\textsubscript{A} receptors was localized to the smooth muscle layer of pial and intracerebral blood vessels. In contrast, little or no \[^{[125]}\text{I}\]BQ3020 binding to ET\textsubscript{B} receptors was detected.

Conclusions—These data indicate an important role for ET\textsubscript{A} receptors in ET-1–induced constriction of human pial arteries and suggest that ET\textsubscript{A} receptor antagonists may provide additional dilatory benefit in cerebrovascular disorders associated with raised ET levels. (Stroke. 1999;30:638-643.)

Key Words: calcium channel blockers ■ cerebral arteries ■ endothelins ■ vasoconstriction

The endothelins\textsuperscript{1,2} (ET) are powerful vasoconstrictors that mediate their effects through 2 receptor subtypes, the ET\textsubscript{A} and ET\textsubscript{B} receptors.\textsuperscript{3,4} A potent and long-lasting constrictor action of ET has been described in both animal and human cerebral vasculature,\textsuperscript{5-9} leading to speculation that it may be involved in the genesis or maintenance of disorders such as the delayed vasospasm associated with subarachnoid hemorrhage (SAH) or ischemic damage after stroke. Studies attempting to correlate ET with cerebrovascular disease have described raised plasma ET levels after SAH and focal cerebral ischemia\textsuperscript{10,11} and in the cerebrospinal fluid after SAH.\textsuperscript{12} In addition, ET receptor antagonists have been shown to be beneficial in attenuating the ischemic damage and cerebral vasospasm in animal models of stroke and SAH, respectively.\textsuperscript{9,13,14} Although the effect of ET in human conduit cerebral vessels has been studied,\textsuperscript{5-7} limited information is available on the effects of ET in small cerebral arteries in humans. Given the therapeutic potential of ET receptor antagonists in cerebrovascular disease, it is of particular interest to determine which ET receptor subtype(s) mediate ET-induced vasoconstriction in these vessels.

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We have investigated the ET receptors present on small pial arteries supplying the cerebral cortex using autoradiographic techniques. In vitro pharmacology was used to determine which ET receptors mediate vasoconstriction and whether an established ET-induced constrictor response can be reversed by an ET receptor antagonist. We also investigated the effect of nimodipine, the current therapy for SAH, on responses to ET-1 in pial arteries. A preliminary account of these data has been presented previously.\textsuperscript{15,16}

Methods

Tissue Samples

With local ethical committee approval, macroscopically normal samples of human frontal and temporal cerebral cortex were obtained from 33 patients (19 male, 14 female; mean age, 44.5±3.3 years) after neurosurgery for the treatment of deep-seated gliomas or epilepsy. Cortex was obtained at the time of surgery and placed immediately into ice-cold Krebs’ solution. With the use of a dissecting microscope, sections of arachnoid were carefully removed, and small pial arteries were dissected from the surface of the
cortical samples. Cortex was frozen and stored at −70°C until used for sectioning.

**Autoradiography**

Slide-mounted cryostat sections (10 μm) of human cerebral cortex from 5 patients were preincubated with buffer (50 mmol/L HEPES, 5 mmol/L MgCl₂, 0.3% bovine serum albumin, pH 7.4) for 15 minutes. Adjacent sections were then incubated for 2 hours in buffer containing either [125I]PD151242 (0.1 nmol/L), to label ET₆ receptors, or [125I]BQ3020 (0.3 mmol/L), to label ET₅ receptors. With the use of the law of mass action and data from saturation binding studies, the concentrations of ligands used have been calculated to label ~30% of the respective receptor populations. Nonspecific binding was determined by incubating adjacent sections with [125I]PD151242 (0.1 nmol/L) or [125I]BQ3020 (0.3 mmol/L) together with the corresponding unlabeled ligand (1 μmol/L). Sections were apposed to Kodak NTB2 nuclear emulsion, and receptor binding was visualized under the microscope with dark field illumination. Adjacent sections were stained with hematoxylin and eosin to facilitate identification of vascular structures.

**In Vitro Pharmacology**

Rings of pial artery (1 to 2 mm in length) were threaded onto 40-μm-diameter stainless steel wires and mounted onto jaws within a wire myograph (model 500A; J.P. Trading) containing oxygenated modified Krebs' solution (composition [mmol/L]: NaCl 90, KCl 5, MgSO₄ 7, H₂O 0.5, NaHPO₄ 1, Na₂CO₃ 45, CaCl₂ 2.25, glucose 10, glutamate 5, Na pyruvate 5, fumarate 5, EDTA 0.04), pH 7.4, maintained at 37°C. Isometric tension measurements were made by force transducers mounted on the myograph jaws. Output was displayed digitally on the myograph and on a Graphite chart recorder (Linton Instrumentation). After a 1-hour equilibrium period, the vessels were stretched radially, and the relation of wall tension to internal circumference was determined. With the use of the Laplace relationship, the internal diameter at which the transmural pressure was 100 mm Hg (ie, as it would be, when relaxed, in vivo) could be estimated. The vessels were then set to 90% of this internal diameter since under these conditions maximal contractile force is obtained.

Vessels were stimulated twice with a potassium-rich solution (95 mmol/L) to assess contractile function. To test for a functional endothelium, vessels were contracted with the stable thromboxane mimetic U46619 (300 nmol/L), and on plateau of the response, endothelium, vessels were contracted with the stable thromboxane (95 mmol/L) to assess contractile function. To test for a functional endothelium, vessels were contracted with the stable thromboxane mimetic U46619 (300 nmol/L), and on plateau of the response, bradykinin (100 to 300 nmol/L) was administered. Relaxation in response to bradykinin was taken as demonstration of the presence of a functional endothelium. Subsequently, cumulative concentration-response curves were constructed to either ET-1 (1 pmol/L to 300 nmol/L), ET-3 (1 pmol/L to 700 nmol/L), or the selective ET₅ receptor agonist sarafotoxin S6c (1 pmol/L to 700 nmol/L). Responses were expressed as a percentage of the potassium-induced contraction. One curve was constructed per preparation. For the antagonist studies, concentration-response curves to ET-1 were constructed in the absence or presence of either the nonpeptide, selective ET₆ receptor antagonist PD156707 (3 to 30 nmol/L) or the calcium channel blocker nimodipine (0.3 to 3 μmol/L). Antagonists were added 30 minutes before the construction of the concentration-response curves to ET-1.

In some experiments, pial arteries were preconstricted with the half-maximal concentration of ET-1. Once the response had reached a plateau, PD156707 (30 nmol/L), BQ788 (1 μmol/L), or vehicle was added.

**Materials**

[125I]PD151242 and [125I]BQ3020 (both ~2000 Ci · mmol⁻¹) were from Amersham International plc; ET-1, ET-3, and S6c were from Peptide Institute; unlabeled BQ3020 ([Ala¹¹,¹⁵]Ac-ET-1₂₀₋₂₁) was synthesized by solid-phase t-butoxycarbonyloxy chemistry.

**Results**

The mean internal diameter of pial arteries was 361.7±11.7 μm. All arteries used had functional endothelium with a mean relaxation to bradykinin of 48.2±3.6% of the U46619-induced contraction. The arteries contracted in response to 95 mmol/L KCl with a mean absolute tension of 1.8±0.1 mN/mm.

ET-1 was a potent constrictor of human pial arteries, with an EC₅₀ of 1.2 nmol/L (n=26) (Table 1, Figure 1). While all

<p>| Table 1. Effects of ET-1, ET-3, and Sarafotoxin S6c on Human Pial Artery |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>E₅₀, nmol/L</th>
<th>E_max, % KCl</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>1.2 (0.8–1.6)</td>
<td>106.6±6.1</td>
</tr>
<tr>
<td>ET-3</td>
<td>65 (8.3–480)</td>
<td>77.0±18.2</td>
</tr>
<tr>
<td>S6c</td>
<td>Noresponse†</td>
<td>7</td>
</tr>
</tbody>
</table>

EC₅₀ values are geometric mean with 95% CIs. E_max values are arithmetic mean±SEM.

*Of the 12 arteries tested, only 7 responded to ET-3; the data are from the responders only.
†A small response was obtained in 1 of the 7 arteries tested.

Data Analysis

Concentration-response curves were analyzed with the curve-fitting package Fig. P. (Biosoft) to determine the EC₅₀ (the concentration required to produce 50% of the maximal response) for agonists. EC₅₀ values are given as geometric means with 95% CIs. Internal diameter and E_max values are arithmetic means with SEM. pA₂ values for PD156707 were determined by Schild regression. Significant differences between the Schild regression slope and unity and E_max values were tested with 2-tailed Student’s t test (P<0.05). For the PD156707, the E_max values for paired segments of pial artery were compared with a paired 2-tailed Student’s t test (P<0.05). EC₅₀ values were compared with the Mann-Whitney U test (P<0.05).

**FIGURE 1.** Responses to ET-1, ET-3, and the selective ET₅ agonist sarafotoxin S6c in human small pial arteries. Values are arithmetic mean±SEM of 7 to 26 individuals and are expressed as a percentage of the response to KCl. Only 7 of the 12 arteries tested responded to ET-3; data are from the responders only.
arteries responded to ET-1, only 7 of the 12 arteries tested responded to ET-3. In responding arteries, ET-3 was less potent than ET-1 as a constrictor, with a mean EC$_{50}$ of 65 nmol/L (range, 8.3 to 480 nmol/L). In addition, when vessels did respond to ET-3, the contractions were more variable than those obtained to ET-1, with maximal responses ranging from 22% to 150% of the initial KCl response. The selective ETA receptor agonist S6c was without effect in all but 1 artery tested (n=7) (Figure 1). In the artery that did respond, the E$_{max}$ was 20.7% of KCl.

The selective ETA receptor antagonist PD156707 (3 to 30 nmol/L) caused a parallel rightward shift of the concentration-response curves to ET-1 with no change in the maximal response. No portion of the ET-1 curve was resistant to the antagonist. PD156707 yielded a pA$_{2}$ of 9.16±0.11 (Figure 2). The slope of the Schild regression, 0.95±0.29, was not significantly different from unity (P>0.05; Student’s 2-tailed t test), indicating competitive antagonism.

The response induced by the EC$_{50}$ concentration of ET-1 (1 nmol/L) in pial arteries was maintained for >1 hour. PD156707 (30 nmol/L) elicited a full reversal of the established ET-1 response. In contrast, BQ788 (1 µmol/L) had little effect (Figure 3). The calcium channel blocker nifedipine (0.3 to 3 µmol/L) caused a significant, concentration-dependent decrease in the maximum response to ET-1 without any change in potency (n=4 to 6) (Table 2, Figure 4).

**Discussion**

Increasing evidence supports a role for ET in the pathogenesis of cerebrovascular disease. Raised ET levels have been reported in both the plasma and cerebrospinal fluid of patients after SAH,11,12,23 in the plasma of patients after ischemic stroke,10 and in experimental models of these conditions.24 ET receptor antagonists prevent13,25,26 and reverse14,27 cerebral vasospasm in animal models of SAH.

We have shown that ET is a potent constrictor of human small pial arteries with long-lasting effects. The values reported here (mean EC$_{50}$, 1.2 nmol/L) are similar to those described for human large cerebral arteries5–7,28 and in keeping with those determined in other human small vessels such as coronary29,30 and pulmonary arteries.31 After SAH, angiographic evidence clearly demonstrates constriction of conducting arteries such as the middle cerebral or basilar arteries. However, little is known about their smaller branches, the pial arteries, since these are below the level of detection when angiography or transcranial Doppler sonography is used, and they are difficult to monitor. Raised local ET levels may elicit constriction in both large arteries and small cerebral arteries. In support of this hypothesis, ET applied topically to animal pial arteries, in situ, mediates a potent and sustained constriction response.32 A recent study demonstrated the involvement of endogenous ET in the constriction of cortical pial arterioles in the preischemic area (penumbra) after middle cerebral artery occlusion in the cat.9 Importantly, PD156707 was effective in restoring cerebral perfusion to ischemic penumbra after intravenous administration in this animal model.
The exact origin of the increased ET production is unclear. However, substances associated with the subarachnoid clot, such as oxyhemoglobin, thrombin, and transforming growth factor-β, have been shown to induce ET-1 production in cultured vascular endothelial and smooth muscle cells.13,33–36 Both animal and human cerebral endothelial cells are known to secrete ET-1,37,38 and overexpression of ET-1 by the cerebral vasculature after SAH has been suggested. The hypoxia that follows both hemorrhagic and ischemic stroke may also trigger ET production.39 Moreover, while the blood vessel itself may be a source of ET, the peptide may also be produced by neuronal40 and glial41 cells, indicating a multitude of potential cellular sources within the brain.

Given the potential for beneficial dilatation of pial arteries with ET receptor antagonists, we investigated the ET receptor subtype(s) mediating ET-induced constriction in human small pial arteries. ET-1 was more potent than ET-3 as a constrictor, suggesting an ETA-mediated effect. This was supported by the lack of effect of the selective ETB receptor agonist S6c. Furthermore, the nonpeptide, selective ETA receptor antagonist PD15670720 caused a parallel rightward shift of the concentration-response curves to ET-1 without a change in the maximum response, suggesting competitive antagonism of the response. Interestingly, PD156707 was a more potent antagonist in pial artery, yielding a pA2 of 9.2 compared with values of 7.5 to 8.7 in rabbit femoral artery and human peripheral blood vessels.20,42 Thus, PD156707 may allow for selective targeting of cerebral arteries and minimization of unwanted peripheral vasodilatation.

ETA receptors were localized to the intracerebral arteries and arterioles when autoradiography was used, whereas little or no ETB binding was evident. Intracerebral arterioles have been shown to be particularly sensitive to ET-1 compared with other agonists43 and may therefore be especially susceptible to the effects of ET. These data suggest that the ETA receptor would mediate ET-1-induced contraction in these vessels.

Under some conditions, ET receptor antagonists may need to be able to reverse an established ET-induced constriction. ET-1 (1 nmol/L)–induced contractions were maintained for 1 hour in the pial arteries. Consistent with preincubation studies, this constriction was fully reversed by PD156707 (30 nmol/L); however, BQ788 (1 μmol/L) had little effect. These experiments demonstrate that ET-1–induced constriction can be effectively reversed in these arteries.

Cerebral vessels appear to be particularly dependent on extracellular calcium for the mediation of constriction in response to various stimuli. The current drug therapy for delayed cerebral vasospasm, nimodipine, has been shown to have some selectivity for calcium channels in cerebral rather than in peripheral vessels.44 Although the mediators of delayed cerebral vasospasm have yet to be fully elucidated, it

**Figure 4.** Effect of the calcium antagonist nimodipine (0.3, 1, 3 μmol/L) on response to ET-1 in human small pial arteries. Values are arithmetic mean ±SEM for 4 to 6 individuals and are expressed as a percentage of the response to KCl.

**Figure 5.** Representative microautoradiogram shows binding (visualized by the presence of silver grains) of [125I]PD151242 (ETA; 0.1 nmol/L) to a pial artery lying within the sulcus of a section of cerebral cortex (a) and an intracerebral artery penetrating the white matter (d). Binding of [125I]BQ3020 (ETB; 0.3 nmol/L) to the pial (b) and intracerebral (e) arteries is also shown. Histological hematoxylin and eosin staining is shown (c and f). Bar=200 μm. g indicates gray matter; s, sulcus; and w, white matter. Arrows indicate locations of vessels.

**TABLE 2.** Effect of Nimodipine (0.3–3 μmol/L) on Responses to ET-1

<table>
<thead>
<tr>
<th>Nimodipine, μmol/L</th>
<th>ET-1 Control</th>
<th>ET-1 + Nimodipine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50, nmol/L</td>
<td>Emax, % KCl</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5 (0.2–9.3)</td>
<td>102.3±8.7</td>
</tr>
<tr>
<td>1</td>
<td>1.0 (0.4–2.1)</td>
<td>85.9±8.5</td>
</tr>
<tr>
<td>3</td>
<td>1.5 (0.6–3.9)</td>
<td>87.6±10.7</td>
</tr>
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</table>

Comparisons of EC50 and Emax values in the absence and presence of nimodipine were made with paired artery segments from the same patient. EC50 values are geometric mean with 95% CIs. Emax values are arithmetic mean ±SEM.

*Not significantly different from control ET-1 (Mann-Whitney U test).
†P<0.005, ‡P<0.05 compared with ET-1 control (Student’s paired t test).

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Figure 6. Comparison of the effects of the calcium antagonist nimodipine (1 μmol/L) and the selective ET<sub>α</sub> receptor antagonist PD156707 (30 nmol/L) on responses to ET-1 in human small pial arteries. Values are arithmetic mean±SEM for 5 to 26 individuals and are expressed as a percentage of the response to KCl.

was anticipated that nimodipine would act as a physiological antagonist to reduce arterial spasm. There is increasing evidence to suggest that despite its ability to reduce the incidence of cerebral infarct and neuronal deficit, nimodipine does not reduce the spasm visualized by angiography. However, only the larger arteries are detected by angiography, and it is not clear whether nimodipine has some effect on the smaller pial arteries. Given the possible involvement of ET in cerebral vasospasm, we investigated the effect of nimodipine on responses to ET-1 in human small pial arteries. Nimodipine caused a reduction of the maximal response to ET-1 but did not affect its potency. Therefore, while nimodipine may partially attenuate the response to ET-1 in human pial arteries, ET<sub>α</sub> receptor antagonists are able to fully block the response over a given concentration range (Figure 6).

In conclusion, we have shown that human small pial artery smooth muscle expresses ET<sub>α</sub> receptors only and that these receptors mediate constrictor responses to ET. The response to ET can be effectively prevented or reversed with the use of an ET<sub>α</sub> receptor antagonist, and such compounds may provide additional therapeutic benefit in cerebrovascular disorders in humans.

Acknowledgments

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References

Endothelin-1 is a powerful endogenous vasoconstrictor substance produced by vascular endothelial cells. Although the exact role of endothelin-1 in the pathogenesis of cerebral vasospasm is still not completely understood, a number of studies demonstrated that endothelin antagonists might have beneficial effects on vasospasm in different experimental models of subarachnoid hemorrhage. Previous studies on isolated human cerebral arteries reported that endothelin-1 is a potent vasoconstrictor and that this effect is mediated by activation of endothelin A (ET_A) receptors. The importance of ET_A receptors is illustrated by the study by Pierre and Davenport of isolated human cerebral arteries. They used nonpeptide ETA receptor antagonists to demonstrate that ET_A receptors play a key role in the vasoconstrictor effect of endothelin-1. Furthermore, they also provided evidence that ET_A receptor antagonist may reverse vasoconstrictor effect of endothelin-1, suggesting that this compound may reverse vasospasm if induced by endothelin-1. Interestingly, a calcium antagonist, nimodipine, caused a concentration-dependent decrease in maximal contraction induced with endothelin-1 without change in endothelin-1 potency. This finding suggests that the vasoconstrictor effect of low concentrations of endothelin-1 (10^{-10}–10^{-9} M) may not be affected by nimodipine. If extrapolated to in vivo conditions, vasoconstriction of cerebral arteries to low concentrations of endothelin-1 would be resistant to vasoconstrictor effects of nimodipine.

The authors also used quantitative autoradiography to demonstrate that ET_A receptors are localized in arterial smooth muscle cells. Both functional and autoradiographic data presented suggest that in human cerebral arteries ET_B receptors are expressed at a very low level. This observation is at variance with the demonstrated ability of ET_B receptor activation to produce endothelin-dependent relaxation in human cerebral arteries. However, technical limitations of quantitative autoradiography may be responsible for the absence of ET_B signal in single layer of endothelial cells. Based on in vitro experiments reported in the literature and in the present study, it is anticipated that ET_A receptor antagonists may have a beneficial effect on narrowing of cerebral arteries induced with endothelin-1.

References


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