Control of Vascular Tone by Endogenous Endothelin-1 in Human Pial Arteries

Eric Thorin, PhD; Thanh-Dung Nguyen, BSc; Alain Bouthillier, MD

Background and Purpose—Endothelin-1 (ET) has been shown to be involved in human pathological conditions, but its physiological function remains to be elucidated. The aim of this work was to assess whether endothelium-derived ET was involved in the overall responsiveness of freshly isolated human pial arteries.

Methods—Samples of cerebral cortex, otherwise discarded, were obtained during tumor or epileptic lesion resections (n=10 donors). Arterial segments were isolated and mounted on a microvessel myograph.

Results—Inhibition of nitric oxide (NO) formation with Nω-nitro-L-arginine (L-NA, 100 μmol/L) increased basal tone by 7±1%Emax (n=5). This increase in tone was fully abolished in the presence of BQ123 (1 μmol/L; ETα receptor antagonist, P<.05) but potentiated by a subthreshold concentration of exogenous ET (1 nmol/L; 33±8%Emax; P<.05). In the presence of L-NA, serotonin (10 μmol/L)—induced tone was doubled compared with the control response (P<.05) but reduced by 90% in the presence of BQ123 (P<.05). In the absence of L-NA, BQ123 prevented serotonin-induced tone (n=3). Oxymetazoline, a selective α1-adrenergic receptor agonist, induced an endothelium-dependent relaxation of preconstricted human pial arteries. The relaxation was partially sensitive to NO synthase inhibition and fully prevented by the addition of ET, whereas substance P—induced relaxation was preserved. Glibenclamide (1 μmol/L), an inhibitor of ATP-sensitive K+ channels and tetraethylammonium (1 mmol/L), an inhibitor of Ca2+-activated K+ channels had no effect on oxymetazoline-induced relaxation.

Conclusions—The results of this study suggest first that ET is involved in the tonic response induced by NO synthase inhibition; second, part of the contractile response induced by serotonin is endothelium-dependent and sensitive to BQ123; and third, the data suggest that activation of α1-adrenergic receptors generated an endothelium-dependent relaxation that was selectively inhibited by exogenous ET. (Stroke. 1998;29:175-180.)

Key Words: endothelins • endothelium • nitric oxide synthase • pial arteries

Endothelin-1 is a potent endothelium-derived constricting factor first identified in the medium of cultured endothelial cells.1 It is a small peptide (21 amino acids) in which secretion is regulated by numerous factors. Most stimuli (such as α-thrombin,2 insulin,3 oxidized low-density lipoprotein,4 and hemodynamic shear stress5) regulate ET release at the level of gene transcription. The expression of preproET mRNA is regulated by mechanisms that involve receptor-mediated mobilization of Ca2+ and activation of protein kinase C in endothelial cells.6 Secretion of ET is also calcium-dependent. Serotonin7 and angiotensin II8,9 have an immediate effect on ET release in various resistance arteries. PreproET and ET are stored in intracellular vesicles of cultured bovine aortic endothelial cells,10 suggesting that this could be a target for some stimuli.11,12

The involvement of ET in the control of the cerebrovascular tone is uncertain. The reactivity of small arteries is regulated by numerous factors, including membrane potential,13 pressure, and shear stress14,15 as well as factors released by the endothelium.16-19 In cerebral arteries, all these factors are associated with the regulation of cerebral blood flow.20 A recent report showed that the diameter of canine cerebral arteries was enhanced 24 hours after in vivo injection of BQ123, an ETα receptor antagonist.21

However, it is accepted that ET is critical in pathological states only.22 ET-1 levels increase in patients with subarachnoid hemorrhage23 and coronary heart disease.24,25 Antagonism of ET receptors successfully prevented the appearance of cerebral vasospasms26,27 and the development of early atherosclerotic lesions28 in animal models. However, there is no direct demonstration of the involvement of ET in the regulation of human cerebrovascular tone in physiological conditions.

The purpose of the present study was to assess whether endothelium-derived ET was involved in the overall responsiveness of freshly isolated human pial arteries by comparing responses to agonists after selective inhibition of various endothelium-derived factors with responses obtained after endothelial denudation of human pial arteries. The results reported in this manuscript demonstrate that ET may regulate...
human cerebrovascular tone during agonist stimulation and in conditions in which NO production is blocked.

**Methods**

Human pial arteries (382 ± 21 µm, 69 rings), otherwise discarded, were obtained from 10 patients who were 14, 16, 29, 32, 35, 37, 44, and 46 years of age: 8 males, 2 females) during neurosurgical resection of brain tumor (2 male patients) or epileptic lesion. None of the patients were diabetic or hypertensive, and none had coronary heart disease. Only normal arteries (those not feeding the tumor or included in the epileptic lesion) were used. They were transported in the laboratory in ice-cold PSS containing indomethacin (10 µmol/L, inhibitor of cyclooxygenase) and of the following composition (mmol/L): NaCl 130, KCl 4.7, KH2PO4 1.18, MgSO4 1.17, NaHCO3 14.9, EDTA 0.026, glucose 10, and aerated with 12% O2/5% CO2/83% N2 (pH 7.4). Segments of 2 mm long were mounted on 30 µm tungsten wires in resistance artery myograph (IMF) and either studied 1 hour, 8 hours, or 16 hours after surgery. No significant changes in reactivity were observed after 8 or 16 hours of surgery as reported by others.13,17 At least 6 segments from a patient were used. Two segments were used per protocol, one being a control. After a 1-hour stabilization period, arterial segments were challenged with a 40 mmol/L KCl PSS with 15-minute wash-out periods until a stable contractile response, representing the optimal tension, was reached (usually between three and four challenges). Arterial segments were contracted with angiotensin II (0.1 µmol/L) or 40 mmol/L KCl PSS and acetylcholine (1 µmol/L) or substance P (0.1 µmol/L)-induced dilation tested.

The endothelium was removed mechanically by a human hair. The effectiveness of endothelium removal was confirmed by the absence of dilation induced by acetylcholine (1 µmol/L) and substance P (0.1 µmol/L) in preconstricted arteries.

To prepare K+-rich solutions, equimolar amounts of NaCl were replaced with KCl. The drugs used were acetylcholine hydrochloride, glibenclamide, indomethacin, L-NA, oxymetazoline hydrochloride, serotonin hydrochloride, tetracethylammonium (Sigma), ET, angiotensin II, substance P (Peninsula), and pinacidil (Research Biochemicals International). Stock solutions of drugs were dissolved in physiological saline except for indomethacin and pinacidil, which were dissolved in ethanol, and glibenclamide, which was dissolved in dimethyl sulfoxide. Solutions were prepared daily and kept on ice except for glibenclamide, which was kept at room temperature.

Results are expressed as mean ± SEM. In all experiments, n equals the number of patients. Vasoreactions are expressed as % of the maximal response (Emax) obtained in the presence of 127 mmol/L KCl PSS at the end of each individual experiment; vasoreactions are expressed as % inhibition of the preconstricting tone. Statistical differences between means were determined by ANOVA followed by Scheffé’s F test. In appropriate conditions, an unpaired Student’s t test was applied. A value of P < 0.05 was accepted as significant for differences between groups of data.

**Results**

In all patients, acetylcholine (1 µmol/L) and substance P (0.1 µmol/L) induced relaxation of 32 ± 9% and 82 ± 5%, respectively, of segments preconstricted with 40 mmol/L KCl PSS (64 ± 8% Emax). When arterial rings were preconstricted with angiotensin II (1 µmol/L; 19 ± 7% Emax, n = 3), acetylcholine-induced dilation induced a significantly greater relaxation (64 ± 6%, P < 0.05) compared with the level of relaxation obtained in depolarized conditions, whereas substance P induced a maximal relaxation.

After removal of the endothelium (n = 6), relaxations to acetylcholine (−9 ± 4%) and substance P (−15 ± 22%) were abolished (P < 0.05).

### Effect of NO Synthase Inhibition, BQ123, Exogenous ET, and Endothelial Denudation on Basal and Serotonin-Induced Tone

Preincubation (45 minutes) of the arterial rings in the presence of L-NA (inhibitor of NO synthase) significantly increased basal tone (7.4 ± 1.0% Emax, P < 0.05; n = 5). This constricting effect was abolished by endothelial denudation.

In intact arteries, the response induced by L-NA was abolished by BQ123 (ETα receptor antagonist; 0 ± 0% Emax, P > 0.05 versus L-NA alone), whereas BQ123 alone had no effect on basal tone. In contrast, L-NA–induced tone was potentiated (33 ± 8% Emax, P < 0.05 versus previous experimental conditions) by prior addition of exogenous ET at a concentration (1 nmol/L) that had no significant direct constricting effect.

Serotonin induced contraction of isolated pial arteries; all results are summarized in Table 1. In the presence of L-NA, serotonin-induced tone was 1.7-fold greater than in control conditions. The combined addition of L-NA (100 µmol/L) and BQ123 (1 µmol/L) reduced serotonin-induced tone by 90%. Furthermore, this contraction was not sustained (Fig 1, upper trace). In the presence of BQ123 alone, serotonin failed to induce a contractile response (n = 3, data not shown). In the presence of L-NA and exogenous ET (1 nmol/L), the contraction induced by serotonin was 1.6 times higher than the tone produced in the presence of L-NA alone. Finally, removal of the endothelium did not significantly potentiate the contraction induced by serotonin compared with the response obtained in control conditions. In denuded arteries, responses were sustained. However, the contraction was significantly lower than the response obtained in intact arteries in the presence of L-NA. L-NA or BQ123 did not affect the contraction induced by serotonin in the absence of endothelium (data not shown).

### Role of Endothelium-Derived Factors on the Relaxation Mediated by Oxymetazoline of Human Pial Arteries

Oxymetazoline (α₂-adrenoceptor agonist) had no direct contractile effect on arterial rings with or without endothelium. However, it induced a concentration-dependent relaxation of

### Constrictions Induced by Serotonin (10 µmol/L; %Emax) Before (Control) or After Inhibition of NO Formation with L-NA (100 µmol/L) Alone or Combined With Either BQ123 (1 µmol/L) or Exogenous ET (1 nmol/L)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-NA</th>
<th>L-NA+BQ123</th>
<th>L-NA+ET</th>
<th>−Endo</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Emax</td>
<td>27±8</td>
<td>45±5*</td>
<td>5±1*</td>
<td>74±4*</td>
<td>31±4†</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

−Endo indicates experiments performed in the absence of endothelium. Results are expressed as mean ± SEM. n, number of donors.

*P < 0.05 vs all groups; †P > 0.05 versus L-NA.
human pial arterial rings preconstricted with serotonin (Fig 1, lower trace); this response was endothelium dependent (Fig 2).

In the presence of L-NA, the relaxation induced by oxymetazoline was significantly decreased but not abolished (Fig 2).

Since BQ123 antagonized the preconstricting tone induced by serotonin (see above), it was not possible to obtain a concentration-dependent relaxation of arterial segments pretreated with L-NA. But the combined addition of L-NA and exogenous ET potentiated responses to serotonin; the addition of cumulative concentrations of oxymetazoline caused no relaxation (Fig 3), whereas substance P (0.1 μmol/L) still produced a significant relaxation (88±1%), although it slightly decreased compared with the response obtained in the absence of exogenous ET (103±2%; n=3, P<.05).

To investigate the possible involvement of EDHF, which would mediate α-adrenergic receptor–dependent relaxation by activating smooth muscle K⁺ conductance, we studied the effect of TEA (1 mmol/L), an inhibitor of Ca²⁺-activated K⁺ (Kca) channels, and glibenclamide (1 μmol/L), an inhibitor of ATP-sensitive K⁺ (KATP) channels, on vascular reactivity after inhibition of NO using L-NA. TEA and glibenclamide were added 20 minutes before testing the relaxant effect of oxymetazoline. In the presence of TEA or glibenclamide, the relaxation mediated by oxymetazoline was not modified (Fig 4).

Relaxations induced by pinacidil, an ATP-sensitive K⁺ channel agonist, represented 19±4%, 68±5%, and 92±6% of relaxation at 1, 3, and 10 μmol/L, respectively. In the presence of glibenclamide, relaxations were decreased to 0±0%, 0±0%, and 54±4% of relaxation at 1, 3, and 10 μmol/L, respectively (n=3 to 4, P<.05 versus in the absence of glibenclamide).

**Discussion**

The results of this study suggest first that ET is involved in the tonic response induced by NO synthase inhibition; second, part of the contractile response induced by serotonin is endothelium-dependent and sensitive to BQ123; and third, the data suggest that activation of α-adrenergic receptors generated an endothelium-dependent relaxation that was selectively inhibited by exogenous ET and insensitive to Kca and KATP channel inhibition.

Acetylcholine mediated endothelium-dependent relaxation of a similar amplitude as reported before and was sensitive to NO synthase inhibition. It is important to note that inhibition of NO production increased basal tone. In experimental conditions in which isometric myographs are used, arterial...
segments do not develop myogenic tone by opposition to what is observed in isobaric conditions. This can be demonstrated by the addition of sodium nitroprusside or papaverine, which do not relax isometrically mounted vessels. Consequently, it is likely that any increase in tone observed in the presence of L-NA is induced by endothelium-derived constricting factors, since L-NA had no effect in denuded arteries. This hypothesis is further supported by the inhibitory effect of BQ123, suggesting that ET is actively involved in the constriction induced by L-NA. It would also confirm and give a functional correlate to previous studies showing that NO actively inhibits the release of stimulated endothelium-derived ET. However, it is possible that NO masks the constricting influence of ET in our experimental conditions without directly affecting ET production. In canine cerebral arteries, cyclooxygenase derivatives have been shown to be involved in both basal and agonist-stimulated tone in similar experimental conditions. Since all experiments were performed in the presence of indomethacin, by blocking the production of vasoactive cyclooxygenase products, we could not confirm that these previous findings are applicable to humans.

Thus, this first set of data suggests that basal tone of human pial arteries is actively regulated by the endothelium. Although we previously reported that both constricting and dilating endothelial factors were involved in the regulation of the overall vascular tone of the rat tail artery, this is the first time that ET is shown to be physiologically involved in the regulation of the human cerebrovascular tone.

In the presence of an intact endothelium, the contraction mediated by serotonin was reduced by BQ123, suggesting an endothelium-dependent component of the serotoninergic response that may involve ET. The concentration of BQ123 used in this study has been shown to antagonize angiotensin II–induced endothelium–dependent contraction of the isolated rat tail artery. As mentioned in the introduction, most stimuli regulate ET release at the level of gene transcription. However, our finding that serotonin has an immediate effect on ET release is not without precedent. The importance of endothelium-derived ET in the net contractile response to serotonin is actively counteracted by NO. Inhibition of NO synthase significantly potentiated the contractile response to serotonin is actively counteracted by NO. Inhibition of NO synthase significantly potentiated the contractile response induced by stimulating smooth muscle cell serotoninergic receptors. The release of EDHF has been shown to be involved in the regulation of the human cerebral vascular tone. Alkayed and coworkers showed that miconazole, a cytochrome P450 inhibitor, decreased rat cerebral blood flow, reinforcing the idea that NO is not the sole regulator of the vascular tone. It is therefore likely that in our experimental conditions, an endothelium–derived relaxing factor, possibly EDHF, counteracts smooth muscle cell contraction. We will not be able to confirm this hypothesis until selective inhibitors of EDHF become available.

It has been reported that oxymetazoline induced endothelium–dependent relaxation of rabbit cerebral arteries through a selective activation of \( \alpha_2 \)–adrenergic receptors and via inhibition of ET release. Contrary to findings in rabbits, inhibition of NO production significantly attenuated the relaxant properties of oxymetazoline (Fig 1). Activation of endothelial \( \alpha_2 \)–adrenergic receptors induced NO release, triggering relaxation of large conductance arteries. In rat cerebral arteries, NO has been shown to have a permissive role on the relaxation induced by \( \alpha_2 \)–adrenergic receptor agonists. However, as in our experimental conditions, NO appears not to be the only factor contributing to the \( \alpha_2 \)–adrenergic receptor–mediated relaxation as previously reported by others.

Since the preconstricting tone is highly dependent on ET release, we hypothesized that oxymetazoline may cause relaxation of serotonin–preconstricted human pial arteries by decreasing ET production, counterbalancing the stimulatory effect of serotonin. It has been reported that oxymetazoline decreased ET production from cultured human pial artery ECs and isolated segments of rabbit middle cerebral artery. Since BQ123 abolished the contractile response to serotonin (Fig 1, Table 1), we were unable to construct relaxant concentration–response curves to oxymetazoline in these conditions. Thus, we postulated that if our hypothesis was valid, the addition of exogenous ET would not only potentiate the preconstricting tone but would also selectively antagonize the relaxation mediated by activation of \( \alpha_2 \)–adrenergic receptors. Indeed, by adding ET we believed we would artificially inhibit the relaxant pathway, ie, the decrease in endothelial ET release by \( \alpha_2 \)–adrenergic receptor occupation. As shown by Fig 3, the relaxation mediated by oxymetazoline was fully antagonized by ET, whereas substance P still induced a potent relaxation. Substance P has been shown to cause relaxation of human cerebral arteries by stimulating the release of both NO and EDHF; this would suggest that EDHF is not the mediator of the \( \alpha_2 \)–adrenergic receptor–dependent pathway. Rather, a functional inhibition of ET release is likely, as previously reported in rabbit cerebral arteries and cultured human pial artery ECs.

This hypothesis is reinforced by the absence of effect of two well characterized inhibitors of potassium channels (Fig 4). TEA is an inhibitor of \( K_{\text{ir}} \) channels; a TEA-sensitive pathway has been shown to be a key regulator of the mesenteric and brain circulations. In some vascular preparations, receptor-mediated endothelium–dependent smooth muscle cell relaxation could...
be blocked by inhibitors of K<sub>Ca</sub> channels but not K<sub>ATP</sub> channels in the absence of NO production. 

The absence of effect of GLI is therefore not surprising.

In conclusion, our results reveal for the first time that ET may have a relevant physiological function in the human cerebral circulation in vitro. They show that NO actively counteracts ET production and/or action. They also suggest that receptor-activated ET regulation may play an important role in the overall control of the local cerebrovascular tone. It is noteworthy that in humans, serotonin has been proposed to be involved in the pathogenesis of migraine, and it has been suggested that treatment with ET receptor antagonists may be an efficient therapy for some forms of migraine. Although we acknowledge the possible limitations of this study due to the limited numbers of experiments performed in some protocols, altogether these studies suggest that there may be a link between ET, serotonin, and certain cerebrovascular disorders in humans.

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References

Editorial Comment
Endothelial Regulation of Human Pial Arteries

An additional interesting aspect of the present study relates to potential interaction of endothelin with nitric oxide. Previous studies suggest that in addition to being a powerful vasodilator, nitric oxide also inhibits gene expression and/or synthesis of endothelin. In the present study, pharmacological inhibition of nitric oxide synthase appeared to unmask a constrictor effect of endogenous endothelin. An implication of this finding is that endothelin may exert a greater influence on vascular tone under pathophysiological conditions which are associated with impairment of the nitric oxide signaling pathway.

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