Nonpeptide Endothelin Antagonist
Cerebrovascular Characterization and Effects on Delayed Cerebral Vasospasm

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Background and Purpose  (+)–SB 209670, a potent nonpeptide endothelin (ET) receptor antagonist, was used to investigate the potential role of ET in cerebral vasospasm associated with subarachnoid hemorrhage.

Methods  The effects of (+)–SB 209670 were evaluated in isolated segments of canine posterior cerebral arteries in vitro, vascular smooth muscle cells in culture, and in the canine two-hemorrhage model of delayed cerebral vasospasm in vivo.

Results  In the canine basilar and anterior spinal arteries, (+)–SB 209670 caused a dose-related inhibition of contractile responses mediated by ET (K<sub>A</sub>=4.5 mmol/L and apparent K<sub>0</sub>=2.7 mmol/L, respectively). The effects of (+)–SB 209670 were mediated by inhibition of ETA receptors since the ETα selective agonist sarafotoxin 6c did not contract these posterior cerebral vessels. (+)–SB 209670 also produced a concentration-dependent inhibition (IC<sub>50</sub>=1 nmol/L) of the mitogenic response induced by ET-1 in vascular smooth muscle cell culture. In the canine model of delayed cerebral vasospasm, animals received intracisternal vehicle (saline) or (+)–SB 209670 (360±10 µg/d) via osmotic minipump for 7 days. On day 7, the cross-sectional areas in the (+)–SB 209670 group were significantly greater than those in the vehicle group in both the basilar artery (68% versus 27%) and anterior spinal artery (78% versus 38%). No differences in blood pressure or heart rate were noted in the two groups, and the vasospasm in the vehicle group did not differ from that of historic controls in this model.

Conclusions  The results suggest that ET plays a significant role in the development of delayed cerebral vasospasm via an interaction with ETA receptors. Furthermore, ETA receptor antagonists may represent a novel therapeutic approach to the treatment of subarachnoid hemorrhage. (Stroke. 1994;25:2450–2456.)

Key Words  • cerebral vasospasm • endothelin • subarachnoid hemorrhage • dogs

Endothelin (ET)-1 is a potent and long-acting vasoactive peptide isolated originally from endothelial cell culture. The contractile effects of ET-1 are mediated by the activation of a specific family of receptors on smooth muscle. The ET receptors responsible for vasoconstriction have been subdivided tentatively into ETA and ETβ2 receptors.

The cerebral circulation is extremely sensitive to the abluminal/vasoconstrictor actions of ET-1. In various animal models, reports of cerebral hyperperfusion, prolonged periods of cerebral vasospasm, and cerebral ischemia produced by exogenous ET have led to the suggestion that this peptide may be an important mediator of cerebrovascular and neuronal sequelae associated with subarachnoid hemorrhage (SAH) and/or ischemic stroke. Evidence also suggests that the mitogenic effects of ET-1 may play a role in vascular remodeling (eg, subintimal proliferation) associated with delayed cerebral vasospasm.

A number of clinical studies aimed at investigating the correlation of ET levels in the cerebrospinal fluid (CSF) and plasma with the symptomatology associated with delayed cerebral vasospasm in SAH support a potential role of ET. In particular, a distinct parallel peak in the CSF levels of ET-1 coincided with clinically documented signs of cerebral vasospasm in a small group of patients. No increases in ET-1 CSF levels were noted in two SAH patients who did not develop cerebral vasospasm. Some evidence suggests that ET associated with SAH originates not only from the endothelium but also derives from neurons and glia.

The increase in ET level may be secondary to the liberation of oxyhemoglobin, which has been shown to increase ET production. However, not all of the clinical studies are in agreement. For example, some studies of SAH patients with cerebral vasospasm have failed to detect any significant increases in CSF and plasma levels of ET-1 or big-ET.

Recent laboratory studies demonstrating a beneficial action of ET antagonists in models of SAH support a role for ET in the pathogenesis of cerebral vasospasm. The intracisternal administration of BQ-123, an ETA receptor antagonist, abolished the acute reduction in cerebral blood flow in an acute rat model of SAH. This group obtained similar results when the rats were pretreated, intravenously, with a nonpeptide ET antagonist, RO 46-2005. More relevant perhaps, another ETA receptor antagonist, FR139317, was moderately...
effective in the canine chronic two-hemorrhage model of delayed cerebral vasospasm. These results are controversial, and several investigators have not detected a significant effect of ET inhibition in models of cerebral vasospasm.

The purpose of the present study was to further evaluate the potential role of ET in the pathogenesis of delayed cerebral vasospasm. Specifically, the effects of (±)-SB 209670 [(±)-(RS, RS, 3RS, 2S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid, disodium salt, hydrate], a selective nonpeptide ETA and ET receptor antagonist, on ET-1-induced contraction and oxyhemoglobin-induced mitogenesis were characterized in isolated canine cerebral vessels and vascular smooth muscle cell culture, respectively. In addition, the effects of (±)-SB 209670 were evaluated in the two-hemorrhage canine SAH model of delayed cerebral vasospasm.

Materials and Methods

Isolated Canine Cerebral Vessels

Adult mongrel dogs were killed via intravenous pentobarbital overdose. An incision was made lengthwise through the skin covering the upper cervical spinal column and the occipital portion of the skull, the underlying muscle was retracted, and the atloido-occipital membrane was dissected away. The medulla oblongata and the rostral spinal cord were removed, and the basilar artery and the anterior spinal artery (dorsal spinal artery) were dissected into a Krebs-Henseleit buffer containing 10% fetal calf serum in 24-well plates. Cells were made quiescent (Go) by substituting serum-containing medium with DMEM containing insulin (5 μg/mL), transferrin (5 μg/mL), and sodium selenite (5 ng/mL) for 48 hours. Cells were replenished with fresh medium once during and after the 48-hour quiescent period. Oxyhemoglobin and/or (±)-SB 209670 was added to the cells for an additional 24-hour incubation. DNA synthesis was assessed by measuring the radioactivity incorporation of [3H]thymidine into the trichloroacetic acid (TCA)-insoluble fractions as follows. First, 1 μCi of [3H]thymidine was added to each well for an additional 4-hour incubation. The cells were then washed three times with 1 mL DMEM and treated with 0.25 mL of 0.2N NaOH for 30 minutes followed by the addition of 1 mL TCA (15%) for at least 2 hours. The total sample was transferred from each well and filtered under vacuum with GF/C Whatman glass microfiber filters (Whatman Inc). Filters were washed three times with 2 mL TCA (5%), and radioactivity was counted in a liquid scintillation counter. Oxyhemoglobin-induced stimulation of DNA synthesis was calculated by expressing the percent increase in [3H]thymidine incorporation in the presence of oxyhemoglobin over basal values obtained in the absence of oxyhemoglobin. Treatments were compared with an ANOVA followed by a Bonferroni t test.

Canine Two-Hemorrhage Model of Cerebral Vasospasm

The 20 male mongrel dogs used for these experiments weighed 10 to 12 kg and were housed in a thermally controlled (22°C), 12-hour light-cycled (6 AM to 6 PM) laboratory animal facility with free access to food and water. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (US Department of Health, Education, and Welfare [Department of Health and Human Services] publication NIH 85-23) and were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals, plc.

The procedure used for the two-hemorrhage model of cerebral vasospasm was similar to that described by Chyatte et al. This procedure produces a reproducible delayed cerebral vasospasm. Briefly, anesthesia was induced in all dogs by administering thiamylal sodium (8 mg/kg IV) on day 1. The animals were intubated immediately and placed supine on a heated fluoroscopy table. Anesthesia was then maintained by mechanical ventilation with isoflurane (2.5% in oxygen), and the femoral artery was exposed by means of an aortic technique. A hemostasis device (Cordis) was placed in the left femoral artery, which allowed continuous monitoring of arterial blood pressure, sampling for arterial blood gas analysis, and passage of the vertebral catheter. The end-tidal CO₂ was also monitored continuously. A transfemoral modifiedSF Lehman catheter (Bard) was guided fluoroscopically into the left vertebral artery and advanced to the vertebral column. The animal was then placed prone. Automated arterial blood pressure...
gases were determined periodically, and ventilation parameters were adjusted, if necessary, to maintain normocapnia within a limited range (Pco2=35 to 40 mm Hg) before obtaining the baseline (day 1) vertebrobasilar angiogram. All angiograms were recorded at identical magnification and injection parameters with Omnipaque 300 (Winthrop Pharmaceuticals) delivered rapidly (5 mL/s) with a pneumatic syringe (Cook Inc).

Immediately after the day 1 angiogram a 6-cm midline incision was made to expose the occipital bone (Fig 1). A burr hole (3.5 mm in diameter) was made 3 mm lateral to the midline, and an 18.5-gauge catheter (Delmed) was advanced caudal 1.5 cm into the subarachnoid space (Fig 1). CSF flowed freely through the catheter, and 5 mL was removed. The burr hole was closed around the catheter with bone wax, and a miniature screw was placed into the adjacent bone for securing the catheter with dental cement. The catheter was filled with vehicle or drug solution (1.2 mg/mL), and 0.1 mL was injected intracisternally. The catheter was then connected to a preconditioned osmotic minipump (Alzet), filled with vehicle (saline) or drug, and placed into the adjacent subcutaneous tissue.

The animal was then placed in a head-down position (30°), the atlanto-occipital membrane was punctured aseptically with a 22-gauge spinal needle, and 4 mL of autologous venous blood was injected slowly (2 minutes). The animal remained in the head-down position for 30 minutes. During this time the dorsal surgical wounds were closed, and the animal was prepared for recovery. The procedure for the intracisternal administration of autologous blood was repeated on day 3 of the study. On day 7, each animal was again prepared for a transfemoral vertebrobasilar angiogram. All other materials were obtained from common commercial sources.

### Drugs and Solutions

The novel nonpeptide ET antagonist (±)-SB 209670 was synthesized at SmithKline Beecham Laboratories. In vivo experiments, (±)-SB 209670 solutions (1.2 mg/mL) were prepared fresh in sterile saline and filtered (Millex-GV) before filling the sterile catheter and osmotic minipump (Alzet, model 2ML1). All other materials were obtained from common commercial sources.

### Results

#### Effects on Isolated Cerebral Vessels In Vitro

ET-1 produced a concentration-related contractile response in canine basilar and spinal arteries. The potency of ET-1 was similar in both vessels, with an EC50 of 3.9 nmol/L in the basilar artery and 2.0 nmol/L in the spinal artery. The maximum contractile response to ET-1 was 2.2±0.4 g and 1.8±0.3 g in the basilar and spinal arteries, respectively. Sarafotoxin 6c, a selective ETB receptor agonist, did not contract either blood vessel at concentrations up to 1 μmol/L (data not shown), indicating that the contractile response to ET-1 in the basilar and spinal arteries is most likely mediated by the ETA subtype of ET receptors. (±)-SB 209670 inhibited the contractile response to ET-1 in both vessels (Fig 2A and 2B). In the basilar artery the antagonism was competitive, resulting in parallel and linear shifts in the ET-1 concentration response and having no effect on the maximum contractile response. The slope of the Schild regression for (±)-SB 209670 in the basilar artery was −1, and the intercept on the x axis (pA2) was 8.34, which yields a Ki of 4.6 nmol/L (Fig 2A). The antagonism of ET-1 produced by (±)-SB 209670 in the spinal artery was not entirely consistent with a purely competitive interaction (Fig 2B). Although (±)-SB 209670 had no effect on the maximum contrac-
A. Day 1 | B. Day 7

C. Day 1 | D. Day 7 + SB209670

Fig 3. Bar graph shows inhibition of oxyhemoglobin (OxyHb) (1 \( \mu \)mol/L)-mediated stimulation of [\( \text{H} \)]thymidine incorporation in rat aortic smooth muscle cell culture by (±)-SB 209670. All values are mean ± SEM from three observations, each performed in triplicate. Controls were treated with (±)-SB 209670 vehicle (saline).

Vascular Smooth Muscle Mitogenesis

The addition of oxyhemoglobin to cultured vascular smooth muscle cells produced a concentration-dependent increase in [\( \text{H} \)]thymidine incorporation. The EC_{50} for oxyhemoglobin was approximately 0.5 \( \mu \)mol/L, and the maximal mitogenic response (fivefold increase) was observed at 10 \( \mu \)mol/L (data not shown). The coincubation of (±)-SB 209670 (0.1 to 1000 \( \mu \)mol/L), added to the vascular smooth muscle cell culture with oxyhemoglobin, produced a concentration-dependent inhibition of [\( \text{H} \)]thymidine incorporation stimulated by 1 \( \mu \)mol/L oxyhemoglobin (Fig 3). The inhibitory concentration (IC_{50}) for (±)-SB 209670 was approximately 1 \( \mu \)mol/L, and the maximal inhibition was observed at approximately 10 \( \mu \)mol/L. (±)-SB 209670 had no significant effect on the basal [\( \text{H} \)]thymidine incorporation (6790±1690 cpm per well).

Effects on the Development of Cerebral Vasospasm In Vivo

In the vehicle group receiving intracisternal saline, significant cerebral vasospasm developed in both the basilar and anterior arterial arteries in the canine two-hemorrhage model of cerebral vasospasm (Fig 4A and 4B). The degree of vasospasm in the intracisternal vehicle group was reproducible and did not differ from historic vehicle controls in previous studies, which received saline intravenously on day 7 (Fig 5). In contrast, the continual intracisternal administration of (±)-SB 209670 inhibited the development of delayed cerebral vasospasm in both arteries in the canine two-hemorrhage model (Fig 4C and 4D). Reductions in the cross-sectional area of the basilar and anterior arterial arteries were significantly greater in the vehicle group when compared with the (±)-SB 209670-treated group (Fig 5). Based on the residual volume in the osmotic minipump, the average daily dose of (±)-SB 209670 was 360.4±10.4 \( \mu \)g. The blood pressure did not differ significantly in the (±)-SB 209670 and vehicle groups on day 7 (data not shown). In addition, the arterial PCO_{2} did not differ significantly in the (±)-SB 209670 and vehicle groups on day 7 (38.6±0.4 mm Hg versus 37.8±0.4 mm Hg, respectively).

Discussion

It has been suggested that potent ET antagonists may represent a therapeutic approach to the treatment of cerebral vasospasm.\textsuperscript{21-23} Recently a novel nonpeptide ET antagonist, (±)-SB 209670, has been described. (±)-SB 209670 produces potent competitive inhibition of ET-1 binding to cloned human ETA (\( K_{d} = 0.2 \) nmol/L) and ETB (\( K_{d} = 18 \) nmol/L) receptors.\textsuperscript{25} (±)-SB 209670 also produces competitive antagonism of ETA- and ETB-mediated contraction in the isolated rat aorta (\( K_{d} = 0.41 \) nmol/L) and the isolated rabbit pulmonary artery (\( K_{d} = 55 \) nmol/L), respectively.\textsuperscript{25} This compound is highly selective for ET receptors and has no significant interactions (≤10 \( \mu \)mol/L) with a variety of impor-
ant vasoactive agents, eg, norepinephrine, calcitonin gene-related peptide, thrombin, and angiotensin II. In the present study contraction of canine posterior cerebral arteries produced by ET-1 was inhibited potently by (+/-)-SB 209670 in vitro. The contractile effects of ET-1 in the canine basilar and anterior spinal arteries appeared to be mediated via activation of ETA receptors since sarafotoxin 6c, a selective ETB receptor agonist, failed to contract these arteries. Thus, (+/-)-SB 209670 acts as a competitive antagonist of ETA receptors in the canine basilar artery ($K_a=4.6$ nmol/L). Similar results were obtained in the anterior spinal artery; however, the slope of the Schild regression showed a slight, albeit significant, deviation from unity.

In view of the competitive nature of the antagonism of ET-1 by (+/-)-SB 209670 in the basilar and other vascular preparations, the slight deviation of the Schild regression from unity was unexpected and is most likely the product of a limited sample size. The apparent $K_a$ of 2.7 nmol/L for (+/-)-SB 209670 in the spinal artery was essentially the same as the $K_a$ in the basilar artery, suggesting an interaction with the same ET receptor (ETA) in both arteries.

In the canine two-hemorrhage model of SAH, (+/-)-SB 209670 inhibited significantly the development of delayed basilar and anterior spinal artery spasms. To demonstrate efficacy in this model, (+/-)-SB 209670 had to be delivered continually into the cisterna magna via osmotic minipump for the duration of the study. In the absence of pharmacokinetic data this dosing regimen may achieve a concentration of 10 $\mu$mol/L in the CSF assuming steady-state distribution restricted to the CSF.

In preliminary studies (not presented) (+/-)-SB 209670 had no effect on the delayed cerebral vasospasm in this model when delivered intracisternally or intravenously on day 7 only. Others have obtained similar results in the same animal model when using an ET-1 monoclonal antibody. The ET antibody failed to alter the delayed cerebral vasospasm when applied topically on day 7. The failures observed after acute treatments are not entirely unexpected given the delayed treatment and the near-irreversible binding of ET-1 to ETA receptors.

The canine two-hemorrhage model elicits highly reproducible delayed cerebral vasospasm; both the basilar and anterior spinal artery spasms observed in the vehicle group were very similar to those in historic vehicle controls from our laboratory. In this model, structural (eg, endothelial damage and subintimal proliferation) and functional (eg, vasoconstriction and impaired dilation) changes observed in spastic cerebral arteries are believed to be similar to the human condition.

Recent evidence suggests that oxyhemoglobin liberated from the subarachnoid clot may play an important role in mediating these structural and functional arterial changes through an ET-dependent mechanism. In this regard, oxyhemoglobin has been shown to increase the production and release of ET-1 from endothelial and vascular smooth muscle cells in culture. In the present study the potent ET antagonist (+/-)-SB 209670 inhibited the proliferative effects of oxyhemoglobin in vascular smooth muscle cell culture. (+/-)-SB 209670 also inhibited the mitogenic effects of ET-1 but did not inhibit the mitogenic response produced by thrombin, angiotensin II, and platelet-derived growth factor (data not shown). These results suggest that the mitogenic actions of oxyhemoglobin may be mediated by ET, most likely via interactions with ETA receptors. Thus, ET may play an important role in both the functional and structural changes observed in spastic cerebral arteries associated with SAH.

Several previous studies have evaluated various ET antagonists in animal models of cerebral vasospasm. In an acute model of SAH in the rat, the intravenous administration of a peptide ET antagonist, BQ-123, and a nonpeptide ET antagonist, RO 46-2005, inhibited the reduction in cerebellar blood flow at 30, 60, and 90 minutes after the intracisternal administration of autologous blood. Perhaps more relevant is the evidence that ET receptor antagonists are also effective in the canine two-hemorrhage model of delayed cerebral vasospasm. In these studies the basilar artery spasm was attenuated by the intracisternal administration of...
FR139317 on day 7. Similar results were obtained with the ETA receptor antagonist BQ-485 after systemic administration. Taken together, these studies support the present results, which suggest that ET plays a major role in the development of delayed cerebral vasospasm.

In summary, ET-induced contraction of the canine basilar and anterior spinal arteries is mediated by ETA receptors. These effects are inhibited by (+)-SB-209670, a potent nonpeptide ET receptor antagonist. (+)-SB-209670 also inhibits oxyhemoglobin-mediated proliferation of vascular smooth muscle cells and attenuates the development of delayed cerebral vasospasm. However, further studies are needed to determine the effects of ET antagonists on the functional and structural vascular changes associated with delayed cerebral vasospasm. In conclusion, ETA receptor antagonists may represent novel therapeutic agents for the treatment of SAH.

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