Nonpeptide Endothelin Antagonist
Cerebrovascular Characterization and Effects on Delayed Cerebral Vasospasm

Robert N. Willette, PhD; Hong Zhang, MD; Marcus P. Mitchell, BS; Charles F. Sauermelch, MS; Eliot H. Ohlstein, PhD; Anthony C. Sulpizio, BS

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 (Kd = 4.6 mmol/L and apparent Kd = 2.7 mmol/L, respectively). The effects of (±)-SB 209670 were mediated by inhibition of ETA receptors since the ETs selective agonist sarafotoxin 6c did not contract these posterior cerebral vessels. (±)-SB 209670 also produced a concentration-dependent inhibition (IC50 = 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.1,2 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 ETB2 receptors.3

The cerebral circulation is extremely sensitive to the abluminal/vasoconstrictor actions of ET-1.4-6 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.7,9 Evidence also suggests that the mitogenic effects of ET-1 may play a role in vascular remodeling (e.g., subintimal proliferation) associated with delayed cerebral vasospasm.10-12

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.15,16 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.15,16 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.17,18 The increase in ET level may be secondary to the liberation of oxyhemoglobin, which has been shown to increase ET production.10,11 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.19,20

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.21 This group obtained similar results when the rats were pretreated, intravenously, with a nonpeptide ET antagonist, RO 46-2005.22 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 [(+)-(1RS, 2RS, 3RS)-3-(2-carboxy-methoxy-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 spine column and the occipital portion of the skull, the underlying muscle was retracted, and the atlanto-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 (composition listed below). Under a dissection microscope, ring segments (2 to 3 mm wide) were prepared from each vessel with the endothelium intact and carefully placed on a tungsten wire (0.005-in diameter) hooks. The segments were suspended between an isometric transducer (Grass FT.03) and a stationary glass rod in 50-mL organ baths containing aerated Krebs-Henseleit buffer maintained at 38°C. An optimum resting tension of 750 mg was applied to each segment and equilibrated for 45 minutes before testing. Changes in tension applied to or generated by the tissues were recorded continually (SensorMedics R611). The composition of the Krebs-Henseleit buffer was (mmol/L) NaCl 119, KCl 4.7, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25, and glucose 11.

The reactivity of each vascular segment was determined by increasing the concentration of KCl (by addition to the bath) in the bath buffer to 60 mmol/L to induce contraction and by adding arginine vasopressin (100 mmol/L) to produce an endothelium-dependent relaxation. After the tissue was washed completely, the appropriate concentration of (±)-SB 209670 was added to the bath and equilibrated for 30 minutes before a cumulative concentration-response curve to ET-1 was constructed. In control experiments, tissues were pretreated with the appropriate volume of the (±)-SB 209670 vehicle (water) before ET-1 treatment. The contractile response elicited by ET-1 was expressed as a percentage of the maximum ET response obtained in that tissue. Only one concentration of (±)-SB 209670 and one ET-1 concentration-response relation were explored in each vascular segment.

The nature of the antagonism and the dissociation constant (Kd) for (±)-SB 209670 at the ET receptor was determined in the basilar and anterior spinal arteries by the technique of Arunlakshana and Schild. Briefly, dose ratios (ie, the concentration of ET required to produce a half-maximal response [EC50] in the presence of (±)-SB 209670 divided by the EC50 obtained in untreated tissues) were calculated for several concentrations of (±)-SB 209670. The log (dose ratio−1) was plotted against the −log (±)-SB 209670 on the abscissa. The slope of the regression will not differ significantly from −1 if the antagonism is competitive and the x intercept is equal to the pA2 (−log Kd). When the slope of the Schild regression was significantly different from −1, the antagonism was assumed to be noncompetitive, and an estimate of potency was obtained by calculating the Kd as (±)-SB 209670 apparent ratio−1 for each concentration (molar) of (±)-SB 209670 in individual tissues. The mean±SEM of the resulting values was designated as an "apparent" Kd.

**Vascular Smooth Muscle Mitogenesis**

Vascular smooth muscle cells were isolated from thoracic aortas of male Sprague-Dawley rats as described previously. The purity of the vascular smooth muscle cells was estimated to be greater than 90% by cell morphology and the immunoperoxidase of myosin. Smooth muscle cell viability was greater than 98% as determined by trypan blue exclusion.

Vascular smooth muscle cells were grown to confluence (3 days) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in 24-well plates. Cells were made quiescent (G0) 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 GFC/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 aseptic 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 modified 5F 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
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. The percent reduction in the cross-sectional area was determined, and the results were compared in vehicle and treated animals with Student's unpaired t test.

Angiographic images of the basilar and anterior spinal arteries were captured every 100 milliseconds and stored digitally for off-line analysis and animation (NIH IMAGE 1.43). The diameters of the spastic segments in vehicle and treated animals were measured during systole by means of an edging routine, and the cross-sectional area was calculated. The cross-sectional areas of the spastic segments on day 7 were compared with the same segments in the baseline angiograms. The percent reduction in the cross-sectional area was determined, and the results were compared in vehicle and treated animals with Student's unpaired t test.

**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.

**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 ET₄ 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 Kᵦ 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-
tile response to ET-1 in the spinal artery, the shifts in the ET-1 concentration-response curve were not linear. Consequently, the slope of the Schild regression was -0.72, which was found to be significantly different than -1. Since a nonlinear relation existed between the concentration of (±)-SB 209670 and the magnitude of the shift in the ET-1 concentration-response curve, it was impossible to calculate a pA\textsubscript{2} from the data. The apparent K\textsubscript{a} of (±)-SB 209670 (2.7±0.6 nmol/L) was estimated in the spinal artery by calculating the mean±SEM of the K\textsubscript{BS} determined for individual tissues at each concentration of (±)-SB 209670.

**Vascular Smooth Muscle Mitogenesis**

The addition of oxyhemoglobin to cultured vascular smooth muscle cells produced a concentration-dependent increase in [\textsuperscript{3}H]thymidine incorporation. The EC\textsubscript{50} for oxyhemoglobin was approximately 0.5 μmol/L, and the maximal mitogenic response (fivefold increase) was observed at 10 μmol/L (data not shown). The coinoculation of (±)-SB 209670 (0.1 to 1000 nmol/L), added to the vascular smooth muscle cell culture with oxyhemoglobin, produced a concentration-dependent inhibition of [\textsuperscript{3}H]thymidine incorporation stimulated by 1 μmol/L oxyhemoglobin (Fig 3). The inhibitory concentration (IC\textsubscript{50}) for (±)-SB 209670 was approximately 1 nmol/L, and the maximal inhibition was observed at approximately 10 nmol/L. (±)-SB 209670 had no significant effect on the basal [\textsuperscript{3}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 μg. 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\textsubscript{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\textsubscript{D}=0.2 nmol/L) and ETB (K\textsubscript{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\textsubscript{D}=0.41 nmol/L) and the isolated rabbit pulmonary artery (K\textsubscript{D}=55 nmol/L), respectively.\textsuperscript{25} This compound is highly selective for ET receptors and has no significant interactions (≤10 μmol/L) with a variety of impor-
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 μmol/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 mediating these structural and functional arterial changes through an ET-dependent mechanism.

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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