Endothelin B Receptor Antagonists Attenuate Subarachnoid Hemorrhage–Induced Cerebral Vasospasm

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Background and Purpose—While it has been widely reported that the vasospasm following subarachnoid hemorrhage (SAH) is prevented/reversed by endothelin (ET) receptor antagonists selective for the ET$_{\alpha}$ receptor and by nonselective ET receptor antagonists, ie, antagonists of both the ET$_{\alpha}$ and ET$_{\beta}$ receptors, there are no reports on the possible attenuation of the spasm by selective ET$_{\beta}$ receptor antagonists. The purpose of this study was to investigate whether (1) ET$_{\beta}$ receptor antagonists prevent and reverse SAH-induced spasm and (2) attenuation of the spasm results from blockade of smooth muscle ET$_{\beta}$ (ET$_{\beta2}$) receptor–mediated constriction and/or endothelial ET$_{\beta}$ (ET$_{\beta1}$) receptor–mediated ET-1–induced ET-1 release.

Methods—SAH-induced spasm of the rabbit basilar artery was induced with the use of a double hemorrhage model. In vivo effects of agents on the spasm were determined by angiography after their intracisternal infusion (10 $\mu$L/h) by mini osmotic pump. In situ effects of agents on the spasm were determined by direct measurement of vessel diameter after their suffusion in a cranial window.

Results—SAH constricted the basilar artery by 30%. Intracisternal infusion with 10 $\mu$mol/L BQ788, an ET$_{\beta1/\beta2}$ receptor antagonist, reduced the spasm to 10%. To investigate whether BQ788 prevented the spasm by blockade of ET$_{\beta1}$ receptor–mediated ET-1–induced ET-1 release, as opposed to ET$_{\beta2}$ receptor–mediated constriction, we tested whether ET$_{\beta1}$ receptor blockade also prevented the spasm. Indeed, intracisternal infusion with 10 $\mu$mol/L RES-701-1, a selective ET$_{\beta1}$ receptor antagonist, reduced the spasm to 10%. Similarly, in situ superfusion with 1 $\mu$mol/L BQ788 reversed the spasm by 40%, and 1 $\mu$mol/L RES-701-1 reversed the spasm by 50%. However, both BQ788 and RES-701-1 enhanced by 40% to 50% the 3 nmol/L ET-1–induced constriction elicited in spastic vessels previously relaxed with 0.1 mmol/L phosphoramidon, an ET-converting enzyme inhibitor.

Conclusions—These results demonstrate that ET$_{\beta}$ receptor antagonists prevent and reverse SAH-induced cerebral vasospasm in an animal model. The likely mechanism underlying the attenuation of the spasm is blockade of ET$_{\beta1}$ receptor–mediated ET-1–induced ET-1 release of newly synthesized ET-1. These studies provide rationale for the therapeutic use of ET$_{\beta1}$ receptor antagonists to relieve the vasospasm following SAH, as well as other pathophysiological conditions involving possible ET-1–induced ET-1 release. (Stroke. 1998;29:1924-1929.)

Key Words: basilar artery  ■  cerebral ischemia, transient  ■  endothelins  ■  vasodilation

While it has been widely reported that the vasospasm following subarachnoid hemorrhage (SAH) is prevented/reversed by endothelin (ET) receptor antagonists selective for the ET$_{\alpha}$ receptor and by nonselective ET receptor antagonists, ie, antagonists of both the ET$_{\alpha}$ and ET$_{\beta}$ receptors, there are no reports on the possible attenuation of the spasm by selective ET$_{\beta}$ receptor antagonists. This lack of inquiry presumably reflects the considerations that ET-1 constriction of the cerebral vasculature is largely ET$_{\alpha}$ receptor mediated$^{12-19}$ and that ET$_{\beta}$ receptor blockade might actually enhance the spasm by prevention of ET$_{\beta}$ receptor–mediated nitric oxide release.$^{11,20}$

However, there is also some evidence to suggest that ET$_{\beta}$ receptor blockade may decrease the spasm. First, the ET-1–dependent spasm may be mediated not only by ET$_{\alpha}$ receptor activation but also by ET$_{\beta}$ receptor activation. This suggestion is supported by our previous demonstration in situ that spasm of the rabbit basilar artery was only partially reversed by a selective ET$_{\alpha}$ receptor antagonist, and subsequent addition of an ET$_{\alpha/\beta}$ receptor antagonist was required to induce complete relaxation.$^{21}$ Also consistent with the involvement of smooth muscle ET$_{\beta}$ receptor activation in the spasm is the possible induction of functional ET$_{\beta}$ receptors following SAH, as demonstrated by increased ET$_{\beta}$ receptor binding and mRNA levels.$^{7,22}$

Second, we recently suggested that endothelial ET$_{\beta}$ receptor activation maintains the spasm by the further release of ET-1.$^{23}$ This suggestion was based on the demonstration that intracistern-
nal infusion of ET-1, and then cessation of the infusion, still induced ET-1-dependent spasm of the rabbit basilar artery.\(^{23}\) Lending support to the possibility that endothelial ET\(_A\) receptor activation causes further ET-1 release\(^{23}\) are reports of ET\(_A\) receptor–mediated ET-1/ET-3–induced ET-1 release/preproET-1 mRNA expression in cultured endothelial and mesangial cells.\(^{24-27}\) Therefore, the purpose of this study was to investigate whether (1) ET\(_A\) receptor antagonists prevent and reverse SAH-induced spasm and (2) attenuation of the spasm results from blockade of smooth muscle ET\(_A\) (ET\(_{A_2}\)) receptor–mediated constriction and/or endothelial ET\(_A\) (ET\(_{A_1}\)) receptor–mediated ET-1–induced ET-1 release. Some of these results have appeared in abstract form.\(^{28}\)

**Materials and Methods**

**Anesthesia**

Procedures were approved by the Institutional Animal Care and Use Committee. A total of 69 New Zealand White male rabbits (weight, 3 to 4 kg) were used. Anesthesia was induced with ketamine HCl (30 mg/kg IM) and xylazine (6 mg/kg IM) and was maintained with sodium pentobarbital (25 mg/kg) administered through a catheter inserted into the subclavian artery (in vivo studies) or femoral vein (in situ studies).

**Angiography**

Vertebrobasilar angiograms were obtained on day 0 (pre-SAH) and on day 7 as follows. The left or right subclavian artery was catheterized, and the tip of a 5F catheter was directed toward the ipsilateral vertebral artery to obtain a selective injection of the vertebrobasilar system. Contrast medium (Angiostat 282) was injected (5 mL/s for 5 seconds), and images (4° left anterior oblique angle) of the vertebrobasilar system were obtained at 2 per second for 14 seconds with a rapid sequential angiographic technique. Digital subtraction analysis was performed with the small focal spot at 60 kV and 0.8 mA. Basilar artery diameter was measured by image analysis (ImagePro). Images were collected by placement of angiograms on a light box fixed with a video camera. Three measurements were made at levels just below the basilar-posterior cerebral artery junction, just above the basilar-vertebral artery junction, and midway between these locations, and these 9 values were averaged. Constriction was expressed as a percentage of the basilar artery diameter on day 7 relative to day 0.

**Mini Osmotic Pump**

Immediately after the day 0 angiogram, a mini osmotic pump (10 μL/h; Alza) containing either 10 μmol/L BQ788, an ET\(_B\) receptor antagonist,\(^{29}\) RES-701-1, an ET\(_A\) receptor antagonist,\(^{30-33}\) or vehicle was implanted in the neck, with the catheter placed into the cisterna magna.

**Subarachnoid Hemorrhage**

On days 1 and 3 after angiography, arterial blood was withdrawn from the central ear artery and injected (0.75 mL/kg) into the cisterna magna over 3 minutes through an additional catheter. For the in situ studies (no angiography or mini osmotic pump implantation), on days 0 and 2, blood was injected through a 21-gauge butterfly needle into the cisterna magna. Similar magnitudes of spasm were induced by blood injected through a catheter or needle inserted into the cisterna magna, as measured 6 days after the initial injection (see Results).

**In Situ Studies**

Rabbits were intubated and mechanically ventilated with room air supplemented with O\(_2\). The respiratory rate and tidal volume were adjusted to maintain expiratory P\(_{CO_2}\) between 35 and 37 mm Hg. Heart rate and systemic pressure were measured with the use of a femoral artery catheter. Arterial P\(_O_2\) and P\(_{CO_2}\) were monitored and maintained within normal levels by adjusting the respiratory rate and/or tidal volume. Core body temperature was monitored rectally and maintained at 37°C with a heating pad.

To prepare the basilar artery cranial window, rabbits were placed in a head holder in the supine position, the clivus was exposed by blunt dissection between the carotid sheath and trachea, and the trachea and esophagus were retracted laterally. Compression of the carotid arteries and the descending vagus nerves was avoided. The muscle covering the basioccipital bone was removed by electrocautery. A rectangular osteotomy (4 to 5 mm wide) was then made at the base of the skull between the tympanic bullae with the use of a microdrill and microrongeur under an operating microscope. After a perfect hemostasis was achieved, the dura was opened and excised with microscissors, and the basilar artery was exposed. The blood clot was gently removed with microforceps. The surgical field was illuminated with a 100-W halogen lamp, which was fitted with a heat filter to avoid warming the cranial window, and was visualized through a trinocular microscope. Head temperature was monitored with a needle inserted in the residual longus colli muscle and was maintained at 37°C to 38°C.

The cranial window was suffused (1 mL/min) with artificial cerebrospinal fluid (mmol/L: NaCl 121.8, KCl 3.2, CaCl\(_2\) 2.5, MgCl\(_2\) 1.26, NaHCO\(_3\) 25.0, g-glucose 3.7, urea 6.0), maintained at 37°C, and gassed with 7% O\(_2\)/6% CO\(_2\)/87% N\(_2\). Vessel diameter, blood pressure, heart rate, and arterial P\(_O_2\) and P\(_{CO_2}\) stabilized within 45 minutes after suffusion with artificial cerebrospinal fluid, and agents were then suffused over the craniotomy. Basilar artery diameter, measured by image analysis with a video camera mounted on the phototube of the microscope, was recorded at the time of the plateau response to each agent.

Each value of vessel diameter was the mean of 13 consecutive measurements taken at approximately 10-second intervals. The magnitude of constriction was expressed as a percentage of basal diameter, measured in micrometers. The basal diameter used to calculate the magnitude of SAH-induced spasm was the mean of 156 measurements from present and historic non-SAH vessels and was 831 μm (SEM=7). The magnitude of relaxation was expressed as a percentage of the constriction, the latter measured as the difference in micrometers between basal and agonist-induced tone.

**Statistical Methods**

Statistical significance between 2 means and multiple means was determined with Student’s unpaired t test and ANOVA, respectively. Significance was accepted at the 0.05 level of probability. Values are expressed as mean±SEM; n represents the number of animals.

**Materials**

Reagent sources were as follows: American Peptide Company for ET-1 and RES-701-1; Henry Schein for ketamine and xylazine; and Peptides International, Inc for BQ610, BQ788, and phosphoramidon. RES-701-1 was also obtained from Kyowa Hakko Kogyo Company, Ltd (gift), and PD145065 was obtained from Parke-Davis (gift).

**Results**

**In Vivo Studies**

SAH decreased basilar artery diameter by 30% (Figure 1). To test whether ET\(_A\) receptor blockade prevented the spasm, the selective ET\(_A\) receptor antagonist BQ788\(^{39}\) was infused intracranially in rabbits subjected to SAH. Indeed, BQ788 infusion (10 μmol/L; 10 μL/h) decreased the magnitude of spasm to 10% (Figure 1). BQ788 infusion in the absence of SAH did not alter basal diameter (Figure 1). Vehicle infusion also did not alter basal diameter or the magnitude of SAH-induced spasm (data not shown).

To test whether the ability of BQ788 to prevent the spasm resulted from inhibition of ET-1–induced (1) constriction, due to blockade of smooth muscle ET\(_A\) (ET\(_{A_2}\)) receptors, or (2) ET-1 release, due to blockade of endothelial ET\(_A\) (ET\(_{A_1}\))
receptors, we utilized the ET\textsubscript{B} selective antagonist RES-701-1.\textsuperscript{30–33} Intracisternal infusion of RES-701-1 (10 $\mu$mol/L; 10 $\mu$L/h) in rabbits subjected to SAH also decreased the spasm to 10% (Figure 1). RES-701-1 infusion in the absence of SAH did not alter basal diameter (Figure 1). Systolic and diastolic pressures of rabbits subjected to SAH were not altered by BQ788 and RES-701-1 in the absence of SAH and significantly less than SAH.

In Situ Studies

We then investigated whether ET\textsubscript{B} receptor antagonists also reversed the spasm due to SAH. Thus, we tested whether BQ788 and RES-701-1 relaxed the spasm in situ. SAH induced 28% constriction (Figure 2), which was a magnitude of constriction similar to the spasm observed in vivo (Figure 1). BQ788 and RES-701-1 (1 $\mu$mol/L) in situ relaxed the constriction by 39% and 52%, respectively, and these magnitudes of relaxation were not significantly different (Figure 2).

To further investigate whether the ability of the ET\textsubscript{B} receptor antagonists to prevent as well as reverse the spasm resulted from inhibition of ET-1–induced constriction, due to blockade of smooth muscle ET\textsubscript{B2} (ET\textsubscript{B} receptors), or ET-1 release, due to blockade of endothelial ET\textsubscript{B1} (ET\textsubscript{B} receptors), we tested whether BQ788 and RES-701-1 also relaxed the constriction due to exogenous ET-1. That is, if the attenuation of the spasm by the ET\textsubscript{B} receptor antagonists was the result of blockade of ET\textsubscript{B2}-receptor–mediated constriction, then the ET\textsubscript{B} receptor antagonists would also be predicted to relax the constriction elicited by exogenous ET-1.

Importantly, this test needed to be performed in vessels from rabbits subjected to SAH, since there is some evidence to suggest that SAH causes the induction of ETB receptors.\textsuperscript{7,22} Thus, it was first necessary to eliminate the spasm associated with SAH, as well as any endogenous ET-1 release, before ET-1 challenge. Thus, we considered that the ET-1 released in spastic vessels was newly synthesized and tested whether spastic vessels were relaxed by phosphoramidon, an ET-converting enzyme inhibitor.\textsuperscript{34} Phosphoramidon (0.1 mmol/L) relaxed the spasm by 74%. The magnitude of phosphoramidon-induced relaxation was not significantly different from the magnitude of relaxation to the ET\textsubscript{B} receptor antagonist PD145065 (1 $\mu$mol/L; 81%; Figure 2).\textsuperscript{35} Phosphoramidon-treated spastic vessels were not further relaxed by 1 $\mu$mol/L BQ788, RES-701-1, and PD145065 (data not shown). Phosphoramidon (100 $\mu$mol/L)
Mechanism of Spasm Attenuation

The present results further suggest that the mechanism of ETβ receptor antagonist attenuation of the spasm is largely through blockade of ETα receptor–mediated ET-1–induced ET-1 release, rather than blockade of ETβ receptor–mediated ET-1–induced constriction, as supported by the following (see working model of Figure 4). First, the ETβ receptor antagonists differentially affected the constriction associated with SAH, which is due to ET-1 release,21 and the constriction due to exogenous ET-1. Specifically, BQ788 and RES-701-1 relaxed the constriction associated with SAH and enhanced the constriction due to exogenous ET-1 (Figures 2 and 3, respectively). Thus, if an ET-1 release mechanism independent of ETβ receptor activation were not responsible for the SAH-induced spasm, then the ETβ receptor antagonists should have enhanced the constriction resulting from both SAH and exogenous ET-1. It should also be considered that an additional ET-1 release mechanism independent of ETβ receptor activation may be present, since the magnitude of reversal of the spasm by the ETβ receptor antagonists was less than that due to phosphoramidon and PD145065 (Figure 2).

A second observation in support of the suggestion that the ETα receptor antagonist attenuation of the spasm is largely through blockade of ETα receptor–mediated ET-1–induced ET-1 release is that RES-701-1, a selective ETβ receptor antagonist and thus an antagonist of endothelial ETβ receptors,30–33 effectively attenuated the spasm (Figures 1 and 2). RES-701-1 appears selective for the ETβ receptor since, in the basilar artery in situ, (1) the ET-1 constriction that remained after exposure to the selective ETα receptor antagonist BQ610 was enhanced by RES-701-1 but was relaxed by BQ788 and (2) BQ788 relaxed the ET-1 constriction elicited in the presence of BQ610 and RES-701-1 (M. Zuccarello, MD, et al, unpublished data, 1998). However, the presence of ETβ as well as ETα receptors in the basilar artery awaits more direct confirmation.

With respect to the selectivity of RES-701-1 as an ETβ receptor antagonist, such a selectivity was not observed by others.36,37 The inability to demonstrate ETβ selectivity for RES-701-1 may have been due to differences in the tertiary structure of the natural compared with the synthetic product.38 In this regard, the present studies were performed with both natural and synthetic RES-701-1 (see Materials and Methods), and similar results were obtained. The apparent ETβ receptor selectivity of the currently used synthetic RES-701-1, compared with the synthetic RES-701-1 used by other investigators, may relate to differences in synthetic procedures (S. Lee, PhD, personal communication, 1997; although see Reference 36).

Third and finally, it should be noted that the ability of ET-1 to induce further ET-1 release through activation of ETβ receptors is consistent with several observations in the literature: (1) ET-1 activation of ETβ receptors induced ET-1 release24–27; (2) intravenous bolus infusion of ET-1 in rats induced a delayed pressor response that was inhibited by the ETα receptor antagonist BQ123 and by phosphoramidon42; and (3) intravenous bolus infusion of the selective ETβ

Spasm Attenuation

The present results represent the first demonstration that selective ETβ receptor antagonists attenuate SAH-induced cerebral vasospasm. Approximately two thirds of the spasm was prevented by intracisternal treatment with BQ788 and RES-701-1, i.e., the spasm of 30% was reduced to 10% (Figure 1). A similar magnitude of protection was observed in this model after oral administration of the selective ETβ receptor antagonist PD155080 and the ETαβ receptor antagonist bosantan.10

Consistent with the in vivo prevention of the spasm by the ETβ receptor antagonists, these antagonists also reversed the spasm in situ (Figure 2). Furthermore, the magnitude of reversal of the spasm in situ was similar to that observed in vivo (Figures 1 and 2), after the apparent magnitude of relaxation of the spasm due to phosphoramidon and PD145065 in situ was taken into account (74% and 81%, respectively; Figure 2).

Discussion

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ET-1 constricted phosphoramidon-treated spastic vessels by 26% (Figure 3). In contrast to the relaxation of the spasm by the ETβ receptor antagonists (Figure 2), 1 μmol/L BQ788 and RES-701-1 enhanced the ET-1 constriction elicited in phosphoramidon-treated spastic vessels (51% and 47%, respectively; Figure 3). BQ788 and RES-701-1 (1 μmol/L) also relaxed the ET-1 constriction elicited in non-SAH vessels unexposed to phosphoramidon (M. Zuccarello, MD, et al, unpublished data, 1998).

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receptor agonist IRL1620 in guinea pigs induced initial and secondary phases of bronchospasm that were blocked by BQ788, while only the secondary phase was inhibited by BQ123.39

The present results also suggest that newly synthesized ET-1 is released as a result of ET_B receptor activation, since phosphoramidon greatly, if not completely, relaxed the vasospasm (Figure 1). It should be noted that these results represent the first demonstration of the reversal of chronic vasospasm through inhibition of ET-1 synthesis and are consistent with previous demonstrations that intraperitoneal infusion or intracisternal injection of ET-converting enzyme inhibitor initiated before SAH prevented the development of spasm in the rabbit and dog basilar artery40–41 and the recent demonstration of the reversal of acute spasm in the rabbit basilar artery.42

In summary, these results demonstrate that ET_B receptor antagonists prevent and reverse SAH-induced cerebral vasospasm in an animal model. The likely mechanism underlying the attenuation of the spasm is blockade of ET_B receptor–mediated ET-1–induced ET-1 release of newly synthesized ET-1. Clearly, the direct measurement of ET-1 release would further test this proposed mechanism. These studies provide rationale for the therapeutic use of ET_B receptor antagonists to relieve the vasospasm following SAH, as well as other pathophysiological conditions involving possible ET-1–induced ET-1 release.

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References

Editorial Comment

Like so many other substances implicated in the induction of vasospasm after aneurysmal SAH, the role of ET becomes more and more complicated. The authors describe the roles of three different ET-1 receptors, one of which generates both a positive self-enhancing feedback loop (the ET<sub>B1</sub> receptor-mediated ET-1–induced ET-1 release) and a negative modulating effect through the release of nitric oxide.

The experiments were cleverly designed and well executed.

Although vasospasm in the rabbit basilar artery narrows the lumen by only 30%, which is much less than the clinically manifest vasospasm in aneurysmal SAH and therefore of uncertain significance, there is an interesting aspect to the results described in the accompanying article: Phosphoramidate, a blocker of ET-1 synthesis, reversed for a good part the chronic vasospasm in this model. This is, to my knowledge, the first time that any substance has been shown to reverse (not prevent) chronic (not acute) vasospasm in the conducting arteries, and it makes one wonder what parts are played by smooth muscle contraction, vessel wall inflammation and thickening, and collagen lattice contraction in this particular model compared with the human situation.

Despite this encouraging finding, I remain skeptical about the prospect of identifying a single substance that works as a “silver bullet” in the prevention and treatment of aneurysmal vasospasm.

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