Systemic administration of an inhibitor of endothelin-converting enzyme for attenuation of cerebral vasospasm following experimental subarachnoid hemorrhage

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The potent vasoconstrictor peptide, endothelin-1 (ET-1), has been implicated in the pathophysiology of cerebral vasospasm that occurs after subarachnoid hemorrhage (SAH). This peptide is synthesized as a large prepropeptide that requires a series of modifying steps for its activation. The last of these steps involves the proteolytic conversion of a relatively inactive propeptide, Big ET-1, to its active, 21–amino acid peptide form. The enzyme responsible for converting Big ET-1 to ET-1 is a metalloprotease called endothelin-converting enzyme (ECE). In the present study the authors examined the effects of a newly developed inhibitor of ECE on responses to ET peptides in the normal basilar artery and on pathophysiological constriction in the spastic basilar artery after SAH.

In the first series of experiments the authors examined normal basilar arteries in the rabbit, which were exposed transclinically and measured on-line using videomicroscopy. Intravenous administration or topical application of an active inhibitor of ECE, CGS 26303, blocked vasoconstrictor responses to topically applied Big ET-1 but not to ET-1. In contrast, topical application of a structurally related compound that does not inhibit ECE, CGS 24592, was ineffective in blocking vasocostriction that was elicited by a topical application of Big ET-1. These findings indicate that CGS 26303 when administered systemically is capable of blocking the conversion of Big ET-1 to ET-1 in the basilar artery without affecting the ability of the vessel to respond to ET-1. In the second series of experiments the authors examined the effects of the ECE inhibitor on cerebral vasospasm after experimental SAH. Intraperitoneal administration of CGS 26303 via osmotic minipumps significantly attenuated the delayed spastic response of the basilar artery to an intracisternal injection of autologous blood.

This study provides the first evidence that systemic administration of an inhibitor of ECE is capable of preventing cerebral vasospasm after SAH. The results reinforce a growing body of evidence that ETs play a critical role in the development of spastic constriction after SAH. Moreover, the findings indicate that blocking the conversion of Big ET-1 to its active ET-1 form using CGS 26303 may represent a feasible strategy for ameliorating cerebral vasospasm.

KEY WORDS • cerebral vasospasm • endothelin • endothelin-converting enzyme • subarachnoid hemorrhage

S ubstantial evidence has accrued that implicates endothelin (ET)-induced constriction in the vascular pathophysiology that emerges after subarachnoid hemorrhage (SAH). Endothelins are bicyclic 21–amino acid peptides with potent vasoactivity \(^{41,42}\) (see Rubanyi and Polokoff\(^{29}\) for a review). Several studies have shown that ETs are elevated in the cerebrospinal fluid (CSF) of patients after they suffer SAH.\(^{6,11,20,25,32-34}\) Although some controversy exists in the literature regarding the relationship of ET levels to cerebral vasospasm, a recent and comprehensive study by Seifert, et al.,\(^{29}\) has helped clarify the time course of ET concentrations after SAH. These investigators demonstrated that the increase in levels of ET-1 and Big ET-1 correlated well with cerebral vasospasm in patients with SAH.\(^{29}\)

The precise mechanisms by which the levels of ETs become increased after SAH are not known. One plausible scenario is that the release of hemoglobin and/or other substances from the subarachnoid blood clot stimulates the production and release of ETs from endothelial cells. Hemoglobin is released from lysed blood cells in the subarachnoid clot (for a review see Macdonald and Weir\(^{11}\)) and gains access to both endothelial and smooth-muscle cells.\(^{9}\) Moreover, the production and release of ET have been shown to be increased in endothelial cultures treated with hemoglobin.\(^{11,23}\) This series of events, and perhaps other routes of activation, could lead to the elevation of ETs after SAH.

Endothelins are among the most potent endogenous vasoactive agents yet identified. Endothelin-1, the most extensively studied of the ET family, acts as a powerful vasoconstrictor in most vascular beds. The initial product of the gene responsible for producing ET-1 is a large prepropeptide form of ET composed of approximately 212 amino acids.\(^{13,14}\) Generation of the active form of ET-1 requires multiple conversion steps that ultimately result
in a 21–amino acid polypeptide with nanomolar affinity for specific membrane receptors. The final conversion step in this process involves the proteolytic modification of Big ET-1 (approximately 40 amino acids in length) by a phosphoramidon-sensitive metalloprotease.23,26,31,40 This metalloprotease, ET-converting enzyme (ECE), is positioned at a crucial point in the production of ET because it converts relatively inactive Big ET-1 to its physiologically active form; ET-1 then becomes available to specific extracellular receptors, the activation of which can alter cerebrovascular tone.8,18,19

Because of the important roles that the ETs appear to play in cerebral vasospasm and a variety of other disease states, considerable effort has been directed toward developing agents that block the actions of ETs. Most of this effort has involved the development of antagonists for ET receptors. Over the last 5 years, a variety of peptidic and nonpeptidic antagonists have been developed with a range of specificity for ET₄, and ET₃ receptor subtypes (for recent reviews see Warner and colleagues36 and Wilson and Hargreaves38). In several recent studies the utility of ET receptor antagonists for preventing or reversing cerebral vasospasm in animal models of SAH has been tested. The majority of these studies have demonstrated a beneficial effect of ET receptor antagonists on cerebral vasospasm,2,3,9,12,15,16,24,27,37,44 although at least one study did not result in improved outcome.4 The encouraging results from most of the animal studies using ET receptor antagonists have stimulated early-phase clinical trials with several of the most promising compounds.

Another general strategy for limiting the deleterious effects of ETs is to inhibit the production of these molecules. One such strategy is to block the conversion of Big ET-1 to ET-1. The metalloprotease inhibitor, phosphoramidon, is capable of blocking ECE and thus inhibits the conversion of Big ET-1 to ET-1. Mixed results have been obtained in studies examining the effects of phosphoramidon on cerebral vasospasm. Matsumura and colleagues22 have shown that topical application of phosphoramidon ameliorates cerebral vasospasm in a canine model of SAH. Shigeno, et al.,30 presented evidence that phosphoramidon attenuates vasospasm by approximately 40% in a similar model; however, an insufficient number of animals was evaluated in this study to permit statistical analyses.

Finally, Cosentino and coworkers,4 who also used a canine model of SAH, did not observe reduced spasm after intracisternal application of phosphoramidon. The mixed findings of investigations using phosphoramidon could be the result of the pharmacokinetics of the compound or the particular treatment paradigms used in the individual studies. The actual effectiveness of ECE inhibition as a therapeutic strategy for limiting vasospasm, therefore, remains to be defined. A critical link in evaluating this issue will be the development of effective inhibitors of ECE that are capable of altering ET-mediated constrictions when administered systemically. Compounds of this type could then be tested for their utility as therapeutic agents for cerebral vasospasm.

In the present study we examined a recently identified inhibitor of ECE, CGS 26303, which was discovered as a result of a directed search of the chemical library of Ciba-Geigy Pharmaceuticals.3 This compound is a nonpeptidic inhibitor of ECE that has been shown to block the pro-duction of ET-1 after exogenous administration of Big ET-1 and reduce arterial pressure in spontaneously hypertensive rats during long-term administration.5 The goals of the present study were twofold. First, the ECE inhibitor was administered topically or systemically to test its effects on ETs that had been topically applied to normal cerebral arteries. This was undertaken to evaluate both the efficacy of the compound and its bioavailability after systemic administration. Second, the inhibitor was administered over a long period to animals subjected to experimental SAH to test its ability to limit the development of cerebral vasospasm. The results of this study indicate that systemic administration of an inhibitor of ECE can prevent the development of cerebral vasospasm after SAH.

Materials and Methods

Acute Transclival Experiments

The first series of experiments used normal (non-SAH) rabbits to test the effects of an intravenously administered ECE inhibitor. Twenty-seven New Zealand white rabbits weighing 3.3 to 3.5 kg were anesthetized intramuscularly with ketamine (40 mg/kg) and xylazine (6 mg/kg). The animals were then intubated, immobilized with pancuronium bromide (0.1 mg/kg), and ventilated with 100% O₂ using a rodent ventilator. A femoral artery and vein and a brachial vein were cannulated with polyethylene tubing. The femoral arterial line was used for monitoring heart rate and blood pressure, which were obtained every 5 minutes. Blood gas measurements were obtained at least every 60 minutes. The femoral venous line was used for fluid replacement as necessary. The brachial venous line was used for intravenous administration of drugs. Ventilation was adjusted to maintain a PaCO₂ of 35 to 45 mm HG.

A transclival approach was used to expose the basilar artery of normal (non-SAH) rabbits. After a midline incision had been made from the mandible to the jugular fossa, the hyoid bone was removed. The sternomastoid, sternohyoid muscles, and trachea were dissected and retracted laterally. The capitus muscles overlying the clivus bone were reflected using a self-retaining retractor. The clivus bone was removed using a high-speed drill and the dura and the arachnoid membranes were opened. Artificial CSF (aCSF) was superfused continuously (1 ml/minute) over the basilar artery such that the artery was never exposed to the air. A suction line on the opposite side of the artery was used to remove the aCSF and to keep the fluid at a constant level above the artery. Opening of the dura and the arachnoid membranes was done cautiously so as not to touch the artery or to cut any small branches of arteries or veins on the brainstem. If arterial constriction or bleeding of the basilar artery was observed during surgical preparation, the affected animal was removed from the study. After full exposure of the basilar artery, a stereomicroscope with an attached computer-controlled display (CCD) camera was used to visualize the basilar artery on a video monitor. Every 5 minutes, the investigator positioned a video caliper at the midpoint of the artery and read the diameter of the basilar artery on a digital display. In these experiments, the investigator was not blinded to the treatment condition.

The drugs used in this study were the ECE inhibitor, (S)-2-biphenyl-4-yl-1-[(1H-tetrazol-5-yl)-ethylamino-methyl phosphonic acid (CGS 26303), and a structurally related compound with no ECE-inhibiting activity, (S)-N-[2-(phosphonomethylamino)-3-(4-biphenyl)-propionyl]-1-aminopropionic acid (CGS 24592). The chemical structures of these compounds have been published by De Lombaert, et al.1 The CGS 26303 has previously been shown to inhibit both ECE and neutral endopeptidase (NEP) in both in vivo and in vitro preparations.1 In contrast, CGS 24592 is a potent inhibitor of NEP but not ECE.5 It is therefore possible to evaluate the actions of CGS 26303 that are mediated by ECE inhibition by comparing its effects with those of the specific NEP inhibitor CGS 24592. In the present studies, the CGS 26303 and CGS 24592 compounds were dissolved in 0.1 N sodium hydroxide, then added


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to aCSF (final pH 7.4) for topical application. The aCSF was composed of: 124 mmol/L NaCl, 3.3 mmol/L KCl, 1.25 mmol/L KH₂PO₄, 2.4 mmol/L MgSO₄, 2 mmol/L CaCl₂, 25.7 mmol/L NaHCO₃, and 10 mmol/L glucose. The aCSF was kept at 37°C and the PCO₂ pressure in the solution was maintained between 45 and 50 mm Hg by bubbling with a mixture of air and CO₂. An overall mean value for the resting diameter of the basilar arteries was calculated by averaging the combined values for Groups 1 through 4 (see below). All values are expressed as the mean ± the standard error of the mean.

The basic paradigm in these studies was to examine the vascular responses to topically applied ET peptides in the presence of either a topically or an intravenously administered ECE inhibitor. The rabbits were divided into six groups. The first group (four animals) was topically administered 20 nmol/L ET-1 for 90 minutes to determine the magnitude of the ET response in the rabbit basilar artery. The second group (five animals) was topically administered 100 nmol/L Big ET-1 for 50 minutes followed by 20 nmol/L ET-1 for 20 minutes. The third group (five animals) received topical applications of 100 nmol/L Big ET-1 for 50 minutes followed by 100 nmol/L CGS 26302 and 20 nmol/L ET-1 for 50 minutes. The fourth group (five animals) received topical applications of 100 nmol/L CGS 26303 for 30 minutes followed by 100 nmol/L CGS 26303 and 100 nmol/L Big ET-1 for 50 minutes. The fifth group (three animals) was treated topically with 100 nmol/L CGS 24592 for 30 minutes followed by 100 nmol/L CGS 24592 and 100 nmol/L Big ET-1 for 50 minutes. This treatment was followed by a topical application of 20 nmol/L ET-1 alone for 20 minutes. The sixth group (five animals) received an intravenous injection of 30 mg/kg CGS 26303 followed 15 minutes later by a topical application of 100 nmol/L Big ET-1 for 50 minutes. After these treatments, a final 20-minute topical application of 20 nmol/L ET-1 alone was tested. A 100-μmol concentration of both CGS 26303 and CGS 24592 was selected for the studies in which topical drug application was used because this concentration is greater than the 90% inhibiting concentration of CGS 26303 but less than the median inhibiting concentration of CGS 24592 for inhibiting ECE in vitro.⁴

Experimental Subarachnoid Hemorrhage

In the second series of experiments we examined the effects of systemically administered CGS 26303 on the development of cerebral vasospasm after SAH. In these experiments, 10 New Zealand White rabbits weighing 3.3 to 3.5 kg were subjected to experimental SAH after being treated with either ECE inhibitor or vehicle. The night before the experiment food and water were withheld from the rabbits, and the following morning they were anesthetized and ventilated as described above. Osmotic minipumps with a 2-ml capacity and a flow rate of 10 μl/hour were filled with either 60 mg CGS 26303 in 2 ml of 0.25 M sodium bicarbonate buffer or with 2 ml of 0.25 M sodium bicarbonate buffer alone. Four minipumps were placed intraperitoneally in each animal under sterile conditions. Five rabbits were treated with CGS 26303 and five were treated with vehicle (0.25 M sodium bicarbonate buffer). This treatment protocol was chosen because it sustained a concentration of approximately 1 μmol/L CGS 26303 in the blood (A. Trapani, personal communication, 1995). Twenty-four hours later, the animals were reanesthetized and ventilated. Five milliliters of arterial blood was obtained from the ear artery and injected into the cisterna magna. The rabbits were subjected to ventricular recumbency for at least 15 minutes after the injection of blood to allow ventral clot formation. Two days after intracisternal injection of blood, the animals were reanesthetized and, by means of transthoracic cannulation of the left ventricle, were perfused fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) under a pressure of 120 cm H₂O. The brainstem was removed and the basilar artery was carefully dissected free and placed in the same fixative solution. The tissue was embedded in an epoxy resin, cross sections were cut at a thickness of 0.5 μm, and stained with toluidine blue. The slides containing the vascular cross sections were coded and examined in a blind manner using light microscopy. At least three cross sections were examined for each vessel. The general appearance of the vessels, such as the presence or absence of corrugation of the internal elastic lamina, was noted. In addition, computerized videomicroscopic measurements of the cross-sectional area of at least three sections were performed using commercially available image analysis software. This software permits an automatic measurement of the luminal area without the need for the investigator to trace the vessel. The values obtained from the cross sections of a given vessel were averaged to provide a mean area value for that vessel. The values for individual vessels were then averaged and compared.

Sources of Supplies and Equipment

The rabbits were ventilated by means of a model 683 rodent ventilator, available from Harvard Apparatus, South Natick, MA. The CCD camera used to display the basilar arteries was purchased from World Video, Inc., Boyertown, PA; the video caliper (model 601 HM) used to measure the diameters of the arteries was purchased from Stahl Research Laboratories, Inc., Port Chester, NY.

In the second series of experiments, osmotic minipumps (model ALZET 2ML1), available from Alza Co., Palo Alto, CA, were used to administer treatment drugs or vehicle. Computerized videomicroscopic measurements of arterial cross sections were made using Image 1 software, produced by Universal Imaging, West Chester, PA.

Both CGS 26303 and CGS 24592 were provided by Ciba-Geigy Pharmaceuticals, Inc., Summit, NJ. Big ET-1 was purchased from Peptides International, Louisville, KY, and ET-1 from Chemical Co., St. Louis, MO.

Results

Acute Transclival Experiments

Basilar arteries in normal (non-SAH) rabbits were exposed transclivally and measured using videomicroscopy. The average resting diameter for all arteries examined at the outset of the experiment was 763 ± 20 μm. Topical application of ET-1 (Fig. 1A) or Big ET-1 (Fig. 1B) elicited a substantial constriction of the arteries. The addition of ET-1 (20 nM) to vessels that were preconstricted with Big ET-1 did not elicit a further constriction (Fig. 1B). Topical pretreatment with CGS 26303 did not inhibit the vasoconstrictor response to ET-1 (Fig. 1C). In contrast, the constrictor response to Big ET-1 was inhibited by pretreatment with CGS 26303 (Fig. 1D). This inhibitory effect appeared to be due to the blockade of ECE because the structurally similar compound, CGS 24592 (which does not inhibit ECE), did not block the constrictor response to Big ET-1 (Fig. 1E). This finding also indicates that an inhibitory effect of CGS 26303 on NEP is not responsible for its ability to block vasoconstriction induced by Big ET-1. Intravenously administered CGS 26303 also inhibited the constrictor response to topically applied Big ET-1 (Fig. 1F). Physiological parameters, including mean arterial blood pressure, blood pH, PCO₂, PO₂, and heart rate, did not differ significantly among the groups.

It is noteworthy that topical or intravenous administration of CGS 26303 alone elicited a small vasoconstriction in most experiments. In addition, treatment with ET-1 in the presence of CGS 26303 elicited a transient and small vasodilatation. These findings are consistent with the presence of a small vasodilatory component of the response to ETS in addition to the large vasoconstrictor component.

Experimental Subarachnoid Hemorrhage

In the second series of experiments we examined the effects of intraperitoneal treatment with the ECE inhibitor or vehicle on cerebral vasospasm resulting from experi-
mental SAH. In each animal with SAH, a thick subarachnoid clot was observed over the basal surface of the brainstem. The basilar arteries in the vehicle-treated group showed moderate or severe vasoconstriction when examined under the dissecting microscope during removal of the vessels. In contrast, constriction was not obvious in the group treated with CGS 26303. The cross-sectional area of the basilar arteries in animals receiving continuous intraperitoneal infusions of vehicle was 0.170 mm$^2$ on the 2nd day post-SAH. Corrugation of the internal elastic lamina was typically observed in the vehicle-treated group. The cross-sectional area of vessels from animals treated with CGS 26303 was 0.348 mm$^2$; this value was substantially and significantly larger than that observed in the vehicle-treated animals (0.398 mm$^2$) in a previous study from this laboratory.

**Discussion**

A central role for ETs in the development of cerebral vasospasm after SAH has been suggested by numerous studies. The potential role of ETs in the spastic constriction of cerebral vessels, as well as other disease states, has triggered considerable interest in therapeutic strategies that modulate ET function. Several of these strategies have shown promise in laboratory studies of cerebral vasospasm. These include: 1) blocking the synthesis of ETs; 2) inhibiting the conversion of ET precursors to their active form; 3) buffering extracellular ET levels with specific antibodies; and 4) antagonizing ET receptors.

Although there remains some controversy as to the efficacy of certain of these approaches, the majority of the available evidence supports the concept that targeting specific components of ET function may be of therapeutic value in the treatment of cerebral vasospasm.

A critical impediment to the development of an appropriate therapeutic agent for treating cerebral vasospasm is the identification of compounds with adequate bioavailability. Most laboratory studies that have targeted ET function after experimental SAH have relied on intracerebral or topical application of drugs; this approach allows direct access of the compounds to the affected arteries and obviates most problems of bioavailability. This mode of delivery is of clear value when surgical intervention is undertaken. However, an optimal agent should also be efficacious when administered systemically. Only a few studies have been performed in which researchers have examined the effects of systemically administered compounds targeting ET function. Moreover, these studies have met with only mixed success. The ET$_A$-selective ET...
receptor antagonist, BQ-123, which is generally effective in limiting cerebral vasospasm when administered intracisternally, appears to be ineffective when administered intravenously. In contrast, bosentan, an orally active ET receptor antagonist that exhibits a slight selectivity for the ET$_A$ type ET receptor, has shown promising results after systemic administration. Intravenous administration of bosentan attenuates cerebral vasospasm in rabbit and canine models of SAH. Although some of the results from experimental studies using bosentan have been negative, encouraging findings from the initial laboratory studies have already stimulated clinical trials using this compound. Substantial efforts within the pharmaceutical industry to develop novel antagonists of ET receptors are continuing; these efforts are likely to provide a valuable source of ET-directed therapies in the future.

An important goal of ongoing research remains the discovery of ET-blocking agents that are effective when administered systemically. In addition to the development of ET receptor antagonists, the identification and refinement of inhibitors of ECE is an important objective of this research. Endothelin-converting enzyme is a membrane-associated metalloprotease that converts the relatively ineffective Big ET-1 into its physiologically active form. Some evidence suggests that phosphoramidon, an inhibitor of ECE, is capable of limiting cerebral vasospasm when applied intracisternally. However, studies using phosphoramidon have provided mixed results and the pharmacokinetics of this compound have not been shown to be appropriate for systemic use in the treatment of vasospasm. In contrast, intravenous administration of CGS 26303, a recently described ECE inhibitor, has been shown to reduce arterial pressure in spontaneously hypertensive rats and to block the conversion of Big ET-1 to ET-1. However, it is noteworthy that systemic administration of CGS 26303 did not affect the mean arterial pressure in normotensive rats (A. Trapani, personal communication, 1995) or in normal rabbits (present study). These findings indicate that systemic administration of the ECE inhibitor does not alter mean arterial pressure and suggest that ET does not play a major role in the tonic regulation of systemic blood pressure under normal conditions.

The present study demonstrates that topical administration of CGS 26303 attenuates the vasoconstrictor response to Big ET-1 in the transclivaly exposed basilar artery of the rabbit. This finding indicates that CGS 26303 is capable of inhibiting the conversion of Big ET-1 to ET-1 in cerebral arteries. The present study also demonstrates that intravenous administration of CGS 26303 attenuates the constrictor response to topically applied Big ET-1, but not topically applied ET-1. These findings indicate that sufficient levels of CGS 26303 can be achieved in the basilar artery to block the conversion of Big ET-1, even after systemic injection, but that this compound does not interfere directly with contractile mechanisms.

One important issue regarding the actions of CGS 26303 concerns the specificity of the compound and its mechanism of action. In addition to inhibiting ECE, CGS 26303 is capable of inhibiting NEP. The possibility therefore exists that the effects of CGS 26303 could be due to its ability to block NEP rather than ECE. To evaluate this possibility, the effects of a structurally similar compound, CGS 24592, were tested. A potent and selective inhibitor of NEP that does not inhibit ECE, CGS 24592, did not inhibit vasoconstriction elicited by Big ET-1 and did not alter baseline tone in the arteries in the present study. These findings indicate that the effects of CGS 26303 are not the result of inhibiting NEP.

The findings from the transclival experiments in this study provided a rational foundation for investigating the effects of systemically administered CGS 26303 on cerebral vasospasm. If ET is indeed a crucial participant in cerebral vasospasm and if CGS 26303 can inhibit the conversion of Big ET-1 to ET-1, it is plausible that treatment with this ECE inhibitor would be of benefit in limiting cerebral vasospasm. The results of the present study support this assertion; continuous intraperitoneal infusion of CGS 26303 substantially and significantly limits the magnitude of cerebral vasospasm after SAH. To our knowledge, this finding represents the first evidence that a systemically administered inhibitor of ECE is capable of attenuating cerebral vasospasm. This finding also reinforces the critical role of ECE in generating active ET under pathological, as well as physiological, circumstances.

It is clear that a considerable amount of experimental work remains to be done to characterize the actions of CGS 26303 (or related compounds) during cerebral vasospasm. An important goal of future studies will be to identify the effects of CGS 26303 when administered at various stages after SAH. Such studies will provide valuable information regarding the potential utility of this compound for preventing the development of vasospasm after SAH occurs and for reversing an established vasospasm. In addition, the optimal dose range of this compound for limiting vasospasm needs to be established in conjunction with parallel analyses of the levels of the compound in the targeted vessel. Nonetheless, the results described herein portray CGS 26303, and thus the strategy of targeting ECE, as a potentially viable therapeutic approach for treating cerebral vasospasm after SAH. It is also conceivable that this compound will be of value for the treatment of other forms of cerebral injury, such as ischemic neuronal injury, in which ETs are postulated to play a role.

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