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Prevention and Reversal of Vasospasm and Ultrastructural Changes in Basilar Artery by Continuous Infusion of CGS 35066 Following Subarachnoid Hemorrhage

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Endothelin-1, a potent vasoconstrictive peptide, has been implicated in the pathogenesis of cerebral vasospasm following subarachnoid hemorrhage (SAH). The goal of this study was to evaluate the effect of continuous intravenous infusion of a highly selective endothelin-converting enzyme-1 inhibitor, CGS 35066, on the prevention and reversal of cerebral vasospasm following SAH. New Zealand white rabbits were subjected to SAH by injecting autologous arterial blood into the cisterna magna. Infusion of CGS 35066 at dosages of 1, 3, or 10 mg/kg/day was initiated either 1 hr and 24 hrs later in the prevention and reversal protocols, respectively. Animals were sacrificed by perfusion-fixation 48 hrs after SAH induction. The cross-sectional areas of basilar arteries were measured using computer-assisted videomicroscopy. Ultrastructural changes in basilar arteries were determined using electron microscopy. CGS 35066 significantly prevented and reversed the arterial narrowing after SAH in all three groups. The mean cross-sectional areas of arteries from animals in both the prevention and reversal protocol groups that received 10 mg/kg/day of CGS 35066 did not differ significantly from those of the healthy controls. Histological studies of the basilar artery in the 10 mg/kg/day treatment group did not show pathomorphological changes, such as corrugation of the endothelium seen at 2 days after SAH induction or vacuole formation in the endothelial cells noted in the vehicle-treated SAH group. These findings suggest that CGS 35066 is a promising therapeutic agent for the prevention and reversal of cerebral vasospasm after SAH. It also prevents the pathological changes in vascular walls due to SAH. Exp Biol Med 231:1069–1074, 2006

Key words: cerebral vasospasm; endothelin-converting enzyme; subarachnoid hemorrhage; CGS 35066; electron microscopy

Cerebral vasospasm is the leading cause of mortality and morbidity among patients with aneurysmal subarachnoid hemorrhage (SAH) (1). However, the mechanism and adequate treatment of vasospasm are still elusive. In recent years, increasing evidence has implicated endothelin-1 (ET-1) in the pathophysiology of cerebral vasospasm (2). It is envisaged that suppression of the production of this vasoconstrictor by inhibitors of endothelin-converting enzyme-1 (ECE-1), an enzyme involved in the final step of posttranslational processing of ET-1, should have beneficial effects for the treatment of SAH-induced cerebral vasospasm (3).

We have previously investigated the effect of an ECE inhibitor, CGS 26303 ((*S*)-2-biphenyl-4yl-1-(1*H*-tetrazol-5-yl)-ethylaminomethyl phosphonic acid), on SAH-induced cerebral vasospasm (4, 5). Continuous ip infusion of CGS 26303 via osmotic minipumps beginning 24 hrs before onset of SAH significantly attenuated the delayed spastic response of the basilar artery following experimentally induced SAH (5). In addition, iv bolus injections of CGS 26303 beginning either before or after the establishment of arterial narrowing were protective. However, in a vasospasm reversal paradigm in which treatment began after vascular narrowing had been initiated, CGS 26303 did not provide significant protection unless a relatively high dosage was used (30 mg/kg two times a day).

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CGS 26303 is a dual inhibitor of ECE-1 and neutral endopeptidase 24.11 (NEP), with IC_{50} values of 410 n*M* and 1 n*M* for ECE-1 and NEP inhibition, respectively (6). Subsequent optimization of the structure of CGS 26303 led to the discovery of CGS 35066, a novel and selective ECE-1 inhibitor that inhibits human ECE-1 with an IC_{50} of 22 n*M* and rat kidney NEP with an IC_{50} of 2.3 μ*M* (7). To further demonstrate the usefulness of an ECE-1 inhibitor for the treatment of SAH-induced cerebral vasospasm, we evaluated the effects of continuous iv infusion of CGS 35066 in an experimental model of SAH. Ultrastructural changes of the basilar artery 7 days after SAH induction were also examined.

Materials and Methods

Animal Preparation and General Procedures. All procedures were approved by the Kaohsiung Medical University Animal Research Committee. A total of 96 immunized and conditioned male New Zealand white rabbits weighing 3.4–3.8 kg were anesthetized by im injection of a mixture of 55 mg/kg KetaVed (Phoenix Scientific, St. Joseph, MO) and 9 mg/kg xylazine (Phoenix Scientific) and intubated endotracheally. Experimental SAH was induced as detailed in the following section. This animal model has been widely used in our previous studies and studies from other groups (8–10). CGS 35066 or vehicle was administered 1 hr or 24 hrs after SAH induction, depending on the purpose of the study. All animals were sacrificed by perfusion-fixation 48 hrs after SAH, except for 24 animals, which were used in electron microscopy (EM) examination.

Induction of Experimental SAH. Rabbits were anesthetized, and 3 ml of autologous arterial blood was injected over 3 mins into the cisterna magna by means of a 23-gauge butterfly needle. The animals were then positioned in ventral recumbency for at least 15 mins to allow ventral clot formation. Rabbits were monitored postoperatively for respiratory distress and ventilated as needed. They were then extubated and were returned to the vivarium after they were fully awake.

Prevention Study. In the prevention study, 35 animals were divided into the following six groups: (i) control (that is, no SAH; $n = 6$), (ii) SAH only ($n = 6$), (iii) SAH plus vehicle ($n = 6$), (iv) SAH plus low-dose CGS 35066 (1 mg/kg/day; $n = 5$), (v) SAH plus medium-dose CGS 35066 (3 mg/kg/day; $n = 5$), and (vi) SAH plus high-dose CGS 35066 (10 mg/kg/day; $n = 7$). The left ear vein was cannulated for iv delivery of CGS 35066 or vehicle. Infusion pumps with a 20-ml capacity and a flow rate of 0.5 ml/hr were filled with 6, 18, or 60 mg of CGS 35066 in 20 ml of 0.25 *M* sodium bicarbonate buffer or vehicle. Continuous iv administration of drug or vehicle was given to animals 1 hr after induction of SAH and was discontinued 35 hrs later. All animals were sacrificed by perfusion-fixation 48 hrs after SAH induction. No treatment was given during the 12-hr period before the sacrifice.

Reversal Study. In the reversal study, 37 animals were divided into the same six groups described in the prevention study. Six rabbits were used in each group except the SAH only group, which had 7 animals. Infusion of CGS 35066 or vehicle was initiated 24 hrs after the induction of SAH and was continued for 12 hrs. The animals were sacrificed 48 hrs after SAH induction. No treatment was given during the 12-hr period before the sacrifice.

Perfusion-Fixation. A total of 48 hrs after induction of SAH, animals were reanesthetized, intubated, and ventilated. The central ear artery was cannulated to monitor blood pressure and determine the blood gas levels. Perfusion-fixation was performed in the following manner. The thorax was opened, a cannula was placed in the left ventricle, the descending thoracic aorta was clamped, and the right atrium was opened. Perfusion was begun with 300 ml of Hank's balanced Salt Solution (HBSS; catalog number H-1387; Sigma, St. Louis, MO), pH 7.4, at 37°C, followed by 200 ml of a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in HBSS at a pressure of 120 cm H_2O. Following perfusion-fixation, the brain was removed and immersed in the same fixative overnight at 4°C.

Tissue Embedding. Arterial segments were removed from the middle third of each basilar artery and washed several times in 0.1 *M* phosphate buffer (pH 7.4). The specimens were postfixed with osmium tetroxide, rinsed, dehydrated, and embedded in Epon 812. Cross-sections of basilar arteries were cut at a thickness of 0.5 μm with an ultramicrotome, mounted on glass slides, and stained with toluidine blue for morphometric analysis.

Tissue Morphometry and Statistical Analysis. Morphometric measurements were performed by an investigator blinded to the treatment group to which the arteries belonged. At least five randomly selected arterial cross-sections from each animal were evaluated qualitatively for the extent of corrugation of the internal elastic lamina, and the cross-sectional area of each section was measured using a computer-assisted image analysis system (Image 1; Universal Imaging Corp., West Chester, PA). The areas of the five cross-sections from a given artery were averaged to provide a single value for each animal. Group data are expressed as mean ± SEM. Group comparisons were performed using a one-way analysis of variance with a Tukey post-hoc test. Differences were considered to be significant at the $P < 0.05$ level.

Ultrastructural Examination. A total of 24 animals were divided equally into four groups: (i) control (that is, no SAH), (ii) 2-day post-SAH, (iii) 7-day post-SAH, and (iv) 7-day post-SAH plus treatment with high-dose CGS 35066 (10 mg/kg/day) as described in the prevention study. Two days or 7 days after SAH, animals were reanesthetized, intubated, and ventilated. Perfusion-fixation was performed in the same manner as detailed before. The brains were

removed and immersed in the same fixative for 2 hrs and then placed in 0.1 *M* phosphate buffer overnight. Basilar artery was then dissected out from the brain. The specimens were washed in 0.1 *M* phosphate buffer and fixed in 0.1% osmium tetroxide in 0.1 *M* phosphate buffer for 30 mins. Afterward, they were rinsed in distilled water, stained in 0.1% aqueous uranyl acetate for 30 mins, dehydrated, and embedded in araldite. Ultrathin sections were cut and mounted on 200-mesh grids to be stained with aqueous uranyl followed by lead citrate and were examined by EM.

Results

General Observations. All animals subjected to SAH were found to have a thick blood clot over the basilar artery. No significant differences were observed among the groups with respect to blood pH, blood partial carbon dioxide pressure, blood partial oxygen pressure, mean arterial blood pressure, or body weight. Morphologically, the basilar arteries in the SAH only and SAH plus vehicle groups exhibited substantial corrugation of the internal elastic lamina (Fig. 1B). Corrugation of the internal elastic lamina was less prominent in animals treated with CGS 35066. The vessels from the healthy control and high dose CGS 35066 groups had similar internal elastic lamina (Fig. 1A and C).

Cross-Sectional Luminal Area Measurements. *Prevention Study.* The cross-sectional area of basilar arteries in the healthy rabbits was 0.343 ± 0.017 mm^2 ($n = 6$). In the SAH only and SAH plus vehicle groups, the cross-sectional areas were reduced by 59% and 57%, respectively, compared with the control group. The magnitude of cerebral vasospasm was significantly and dose-dependently attenuated in animals treated with CGS 35066 (Fig. 2). The cross-sectional areas were reduced by 26% and 25% in the groups receiving CGS 35066 at the doses of 1 and 3 mg/kg/day, respectively. In the high-dose treatment group, the cross-sectional area increased by 3%. The protective effect of CGS 35066 achieved statistical significance in all three treatment groups ($P < 0.01$, compared with the SAH only or SAH plus vehicle group). In addition, the cross-sectional area of arteries from animals in the 10 mg/kg/day treatment group did not differ significantly from those from healthy controls.

Reversal Study. Treatment with CGS 35066 also significantly attenuated the magnitude of cerebral vasospasm when infusion was initiated 24 hrs after SAH induction. Under these conditions, the cross-sectional area of basilar arteries in the healthy controls was 0.335 ± 0.021 mm^2 ($n = 6$). Similar to the prevention study, the cross-sectional areas in the SAH only and the SAH plus vehicle groups were reduced by 58% and 54%, respectively, compared with the control group (Fig. 3). In the reversal paradigm, the cross-sectional areas were reduced by 26%, 14%, and 4% in the groups receiving 1, 3, and 10 mg/kg/day of CGS 35066, respectively. The reversal effect of CGS 35066 in all three treatment groups was statistically significantly different from the effects in the SAH only or SAH plus vehicle group ($P < 0.05$). Furthermore, the cross-sectional areas in all three treatment groups did not differ significantly from that of the healthy control group ($P =$

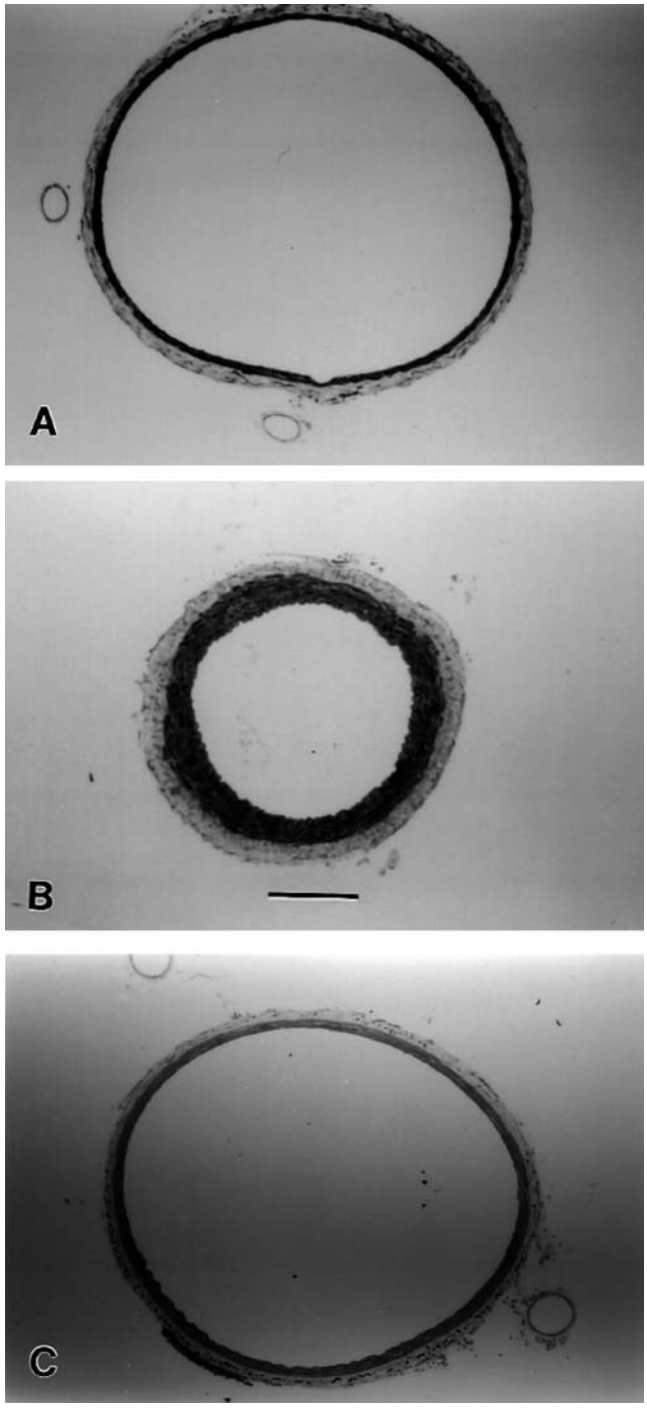

Figure 1. Micrographs of representative cross-sections of basilar arteries from healthy control rabbits (A), rabbits with SAH that did not receive treatment (B), and rabbits with SAH that were treated with CGS 35066 (10 mg/kg/day) in a reversal study (C). Calibration bar, 100 μm.

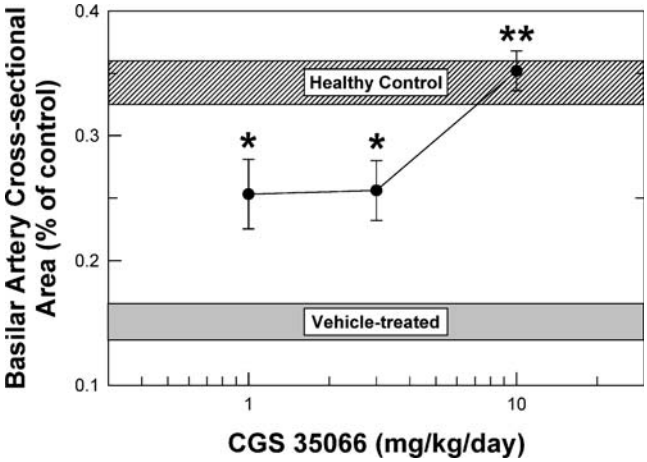

Figure 2. Prevention of cerebral vasospasm by CGS 35066. Treatment with this compound at the indicated dosage began 1 hr after the induction of SAH and was discontinued 35 hrs later. The basilar artery cross-sectional areas for the vehicle-treated and healthy rabbits are shown by horizontal bars. All values represent mean ± SEM. *$P < 0.01$, compared with vehicle-treated SAH animals. **$P < 0.01$, compared with vehicle-treated SAH animals, but values were not significantly different from those of the healthy controls.

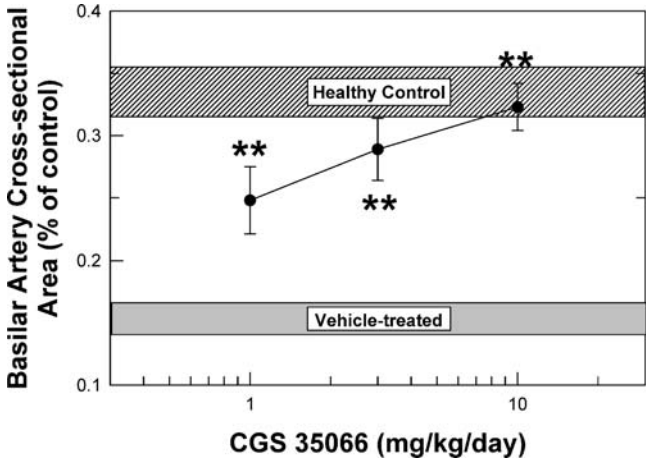

Figure 3. Reversal of cerebral vasospasm by CGS 35066. Treatment with this compound at the indicated dose began 24 hrs after the induction of SAH and was discontinued 12 hrs later. The basilar artery cross-sectional areas for the vehicle-treated and healthy rabbits are shown by horizontal bars. All values represent mean ± SEM. **$P < 0.05$, compared with vehicle-treated SAH animals, but values were not significantly different from those of the healthy controls.

0.074, 0.667, and 0.998, respectively, for the 1, 3, and 10 mg/kg/day treatment groups).

Electron Microscopic Examination of Basilar Artery. In the 2-day post-SAH group, basilar arteries had pathomorphological changes, such as infolding and corrugation of the endothelium (Fig. 4B). Seven days after SAH, vacuolization, disorientation, and desquamation of endothelial cells, as well as thickening and discontinuities of the elastic lamina, were observed (Fig. 4C). However, these pathological changes were not found in the high-dose CGS 35066 treatment group (Fig. 4D) or in healthy controls (Fig. 4A).

Discussion

Although cerebral vasospasm associated with aneurysmal SAH has been recognized for more than 50 years, it is still a major complication in patients with SAH. Because adequate treatment for vasospasm is not available, the mechanisms contributing to this form of arterial dysfunction remain a topic of intensive study. Recently, lines of evidence have implicated ET-1 in the spastic response to SAH (11, 12). ET-1 is a member of a family of structurally related peptides that cause potent and long-lasting vasoconstrictive effects in nearly all vascular beds examined. The final step of biosynthesis of ET-1 is the conversion of the relatively inactive precursor peptide big ET-1 to the mature peptide by ECE-1. Thus, inhibitors of ECE-1 should suppress the production of ET-1 and are expected to have beneficial effects on the treatment of cerebral vasospasm following SAH. It should be noted that four isoforms of ECE-1, designated as ECE-1a, ECE-1b, ECE-1c, and ECE-1d, have been identified to date, and they differ only in their *N*-terminal amino acid sequences as a result of alternative splicing of the same gene. However, it is not clear which of the isoforms are involved in SAH-induced cerebral vasospasm.

Although CGS 26303 has been shown previously to have beneficial effects for the treatment of cerebral vasospasm after SAH, it is nevertheless a potent inhibitor of NEP, with modest ECE-1 inhibitory activity. To demonstrate that these effects of CGS 26303 are due to ECE-1 but not NEP inhibition, a more potent and selective ECE-1 inhibitor is needed. Optimization of the structure of CGS 26303 has led to the discovery of CGS 35066 (9, 13). Compared with CGS 26303, CGS 35066 is approximately 48,000-fold more selective towards ECE-1 inhibition versus NEP inhibition. In addition, this selective ECE-1 inhibitor produced the best *in vivo* activity among the aminophosphonate series of compounds (7). Therefore, CGS 35066 could be a novel compound for assessing the pathogenic role of ET-1 overproduction in various disease states. In the present study, significant vascular narrowing is established in the rabbit model of SAH-induced cerebral vasospasm 24 hrs after SAH induction. At a dose as low as 1 mg/kg/day, CGS 35066 not only prevented but also reversed vasospasm in this animal model, even when treatment was delayed for up to 24 hrs after induction of SAH. Interestingly, the mean cross-sectional areas of basilar arteries in all three treatment groups in the reversal study were statistically significantly different from that of the healthy rabbits. These results further support the hypothesis that endogenous endothelins play a major role in SAH-induced cerebral vasospasm.

A number of animal models of SAH have demonstrated

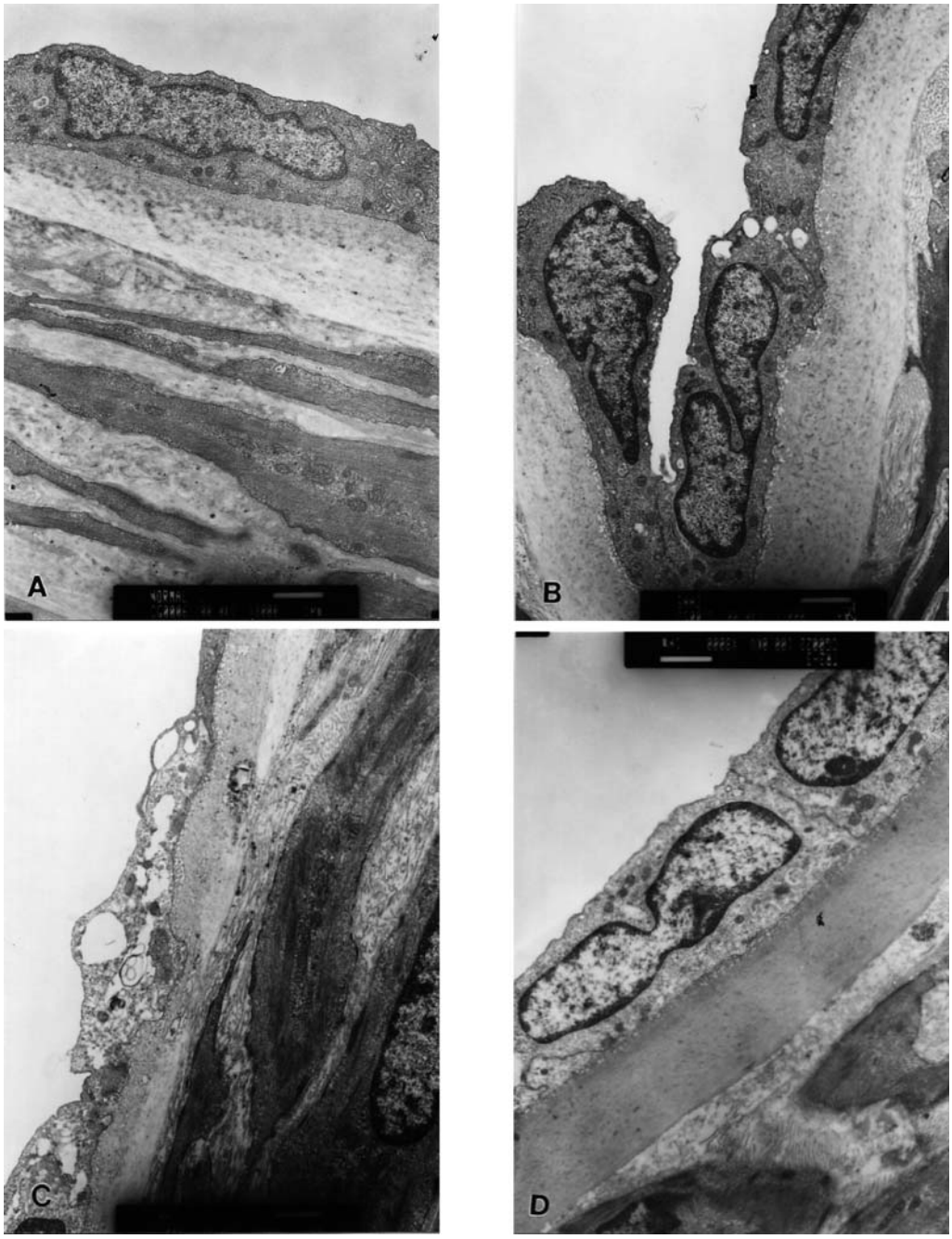

Figure 4. Ultrastructure of rabbit basilar arteries observed under transmission electron microscopy. Basilar arteries from healthy control animals showed smooth, intact, and regular endothelial cells without corrugation of elastic lamina or smooth-muscle cells (A). Two days after SAH induction, infolding and corrugation of basilar arteries were observed. Disorientation, desquamation, and vacuolization of endothelial cells were noted. Prominent corrugation was also observed in elastic lamina and smooth muscle (B). Seven days after SAH, damage to endothelial cells was observed (C). In animals with SAH, treatment with high-dose CGS 35066 prevented damage to endothelial cells and released elastic lamina and smooth muscle contracture (D).

ultrastructural changes in the vascular wall after SAH (14). These changes in cerebral artery morphology are characterized by infolding and corrugation of the endothelium, disorientation and desquamation of endothelial cells, vascuolation, and ingrowth of fibrous tissue between the endothelial and muscular layers. Similar findings in basilar arteries 2 and 7 days after SAH induction were also observed in our EM study. Continuous iv infusion of CGS 35066 at 10 mg/kg/day preserved the integrity of endothelium of the vascular wall. These findings suggest that endothelial changes may be attributed to the pathogenesis of SAH-induced vasospasm and that a highly selective ECE-1 inhibitor could attenuate cerebral vasospasm after SAH by preventing endothelial injury. Nevertheless, it should be

noted that the animal model used in the present study does not develop a sustained and chronic vasospasm. Thus, the usefulness of CGS 35066 remains to be confirmed in a clinical setting.

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