Nitric oxide synthase and guanylate cyclase levels in canine basilar artery after subarachnoid hemorrhage

HIDETOSHI KASUYA, M.D., BRYCE K. A. WEIR, M.D., MASAKI NAKANE, PH.D., JENNIFER S. POLLOCK, PH.D., LYDIA JOHNS, LINDA S. MARTON, PH.D., AND KARI STEFANSSON, M.D.

Section of Neurosurgery and Department of Neurology, University of Chicago, Chicago; and Vascular Biology Group, Abbott Laboratories, Abbott Park, Illinois

Endothelium-dependent vasodilation may be impaired during cerebral vasospasm following subarachnoid hemorrhage. Under normal circumstances nitric oxide (NO) released by endothelial cells induces relaxation of smooth muscle by activating the soluble form of guanylate cyclase within muscle cells. In this study the levels of both endothelial NO synthase, the enzyme that produces NO, and soluble guanylate cyclase were determined in canine basilar arteries in a double-hemorrhage model using Western blot immunoassays. Thirty dogs were assigned to three groups: Group D0, control; Group D2, dogs sacrificed 2 days after cisternal injection of blood; and Group D7, dogs given double cisternal injections of blood and sacrificed 7 days after the first injection. Constriction of the basilar artery was confirmed by arterial angiography. Portions of the affected arteries or the corresponding region in control animals were solubilized for sodium dodecylsulfate–polyacrylamide gel electrophoresis and Western blotting. A specific monoclonal antibody against endothelial NO synthase was used. The extract from basilar arteries showed two bands on the blots: 135 kD, characteristic of endothelial NO synthase, and 120 kD, which may be a degradation product of the enzyme. The densitometer values of the bands were presented as percentages of D0 control values. Although the total signal in the D7 group was less than that of the D0 control group (D2, 97% ± 22%; D7, 78% ± 40%), it was not statistically significant. The proportion of the 135-kD form decreased between Groups D0 and D7, but the difference was not significant. A single major band corresponding to the α-subunit of soluble guanylate cyclase was seen at 70 kD in the basilar artery extracts. The signals of D2 and D7 samples were 69% ± 40% and 25% ± 18%, respectively. There was a significant difference between D7 and D0 (p < 0.001). The reduced expression of soluble guanylate cyclase may be related to the impairment of endothelium-dependent vasodilation in vasospasm.

Key Words • subarachnoid hemorrhage • soluble guanylate cyclase • endothelial nitric oxide synthase • vasospasm • endothelium-dependent relaxation • dog
Nitric oxide synthase and guanylate cyclase levels after SAH

the arterial wall was examined using a Western blot immunoassay method in the canine SAH model, and we found significantly reduced expression of soluble guanylate cyclase following SAH.

Materials and Methods

Cerebral Vasospasm Model

Thirty mongrel dogs each weighing between 17 and 28 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg), intubated, and ventilated mechanically to maintain an end-tidal pCO2 of 38 to 42 mm Hg. Anesthesia was maintained by halothane (0.5% to 1.5%) and nitrous oxide (60%) in oxygen. Body temperature was maintained with a heating pad. Arterial blood pressure and pulse rate were monitored continuously via the femoral artery. Using fluoroscopic guidance, a transfemoral catheter was placed into the left vertebral artery and advanced to the C-5 spinal level. Cerebral angiography was performed by hand injections of 5 to 8 ml isofothalamate meglumine. After a control angiogram was obtained, the animal was placed prone. In 20 of the 30 dogs the cisterna magna was punctured percutaneously with a No. 21 needle, and 0.3 ml/kg cerebrospinal fluid was removed by spontaneous egress. Subsequently, 0.5 ml/kg of the blood removed from the arterial catheter was injected into the cisterna magna over a 60-second period. The head was left positioned downward on the table for 15 minutes. The catheter was then removed and the animals were allowed to recover. The dogs were randomly assigned to one of three treatment groups, with 10 animals in each group. Group D0 consisted of normal control animals, sacrificed on Day 0 without blood injection. Group D2 animals were sacrificed on Day 2 following the first cisternal injection of blood. Angiography was repeated on Day 2. Group D7 animals were sacrificed on Day 7 following cisternal injections of blood on Days 0 and 2. Angiography was repeated on Day 7. The experimental protocol was evaluated and approved by the Institutional Animal Care and Use Committee of the University of Chicago. Care of the animals and surgical procedures were performed according to the standards of the University of Chicago Manual on Laboratory Animals.

Vessel Caliber Assessment

With a calibrated optical micrometer, the diameters of basilar arteries were measured on the angiographic films at three separate, equally spaced locations along the artery by an experienced person who was blinded to the treatment groups. The average diameter value at each of three locations was used.

Sacrifice and Removal of Artery

The animals were sacrificed by injection of 100 mg/kg pentobarbital. Following sacrifice, exsanguination and perfusion with 1000 ml normal saline was performed. Each animal’s calvaria was removed and the brain taken out. The basilar artery was removed carefully, stripped of periarterial clot using a microscope, immediately frozen on dry ice, and stored at −70°C.

Cultured Endothelial Cells and Smooth-Muscle Cells

Bovine pulmonary artery endothelial cells* were cultured to confluence in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml), at 37°C in an atmosphere of 5% CO2/95% air. Culture medium was changed twice weekly. Confluent cells were subcultured by 0.05% trypsin/0.02% ethylenediamine tetraacetic acid. Vascular smooth-muscle cells were isolated from dog thoracic aorta and cultured as described previously.28

* Bovine pulmonary artery endothelial cells, type 209-CCL, were obtained from American Type Culture Collection, Rockville, Maryland.

Expression of Endothelial NO Synthase Following SAH

The basilar arteries were minced and homogenized in 20 μl/mg tissue of solubilizing mix (1% sodium dodecylsulfate (SDS), 40% urea, and 1% 2-mercaptoethanol). Cultured cells, in 100-mm flasks, were lysed and pooled in 500 μl of solubilizing mix. The samples were sonicated, boiled for 2 minutes, and then stored at −80°C until use. Protein content was measured by a modified Lowry procedure.46 Solubilized proteins (60 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis. For immunoblot analysis, gels were preincubated in transfer buffer (48 mmol Tris/39 mmol glycine/0.7 mmol SDS/10% methanol; pH 9.2) for 15 minutes at room temperature, and the proteins were electrothermally transferred to presoaked nitrocellulose filters for 45 minutes at 100 mA using a dry-blot apparatus.† The filters were air dried for 1 hour and incubated with 6% nonfat dry milk/Tris-buffered saline (TBS) at 4°C overnight to block nonspecific binding. The filters were then incubated with antiendothelial NO synthase monoclonal antibody (1:3000)‡ or anti–α subunit of soluble guanylate cyclase monoclonal antibody (1:3000)§ in 1% nonfat dry milk/TBS at room temperature for 2 hours, followed by a wash with TBS. The filters were incubated with horseradish peroxidase–anti-immunoglobulin antibody (1:3000) in 1% nonfat dry milk/TBS at room temperature for 2 hours followed by a wash with TBS. The filters were then incubated with an enhanced chemiluminescent detection solution;¶ and exposed to x-ray film. The films were individually quantified using Bromma Ultroscan XL.§

Statistical Analysis

Comparisons within groups were made by paired t-test; comparisons between groups were made by analysis of variance followed by Scheffé’s test for pairwise comparisons. All tests of significance were for p less than 0.05.

Results

Cerebral Vasospasm Model

The change in vessel caliber of basilar arteries following cisternal injection of blood is shown in Fig. 1. A significant reduction in vessel caliber was seen on angiography in the D2 and D7 groups. In the D7 group, arterial spasm was significantly more intense than in the D2 group (p < 0.0001).

Electrophoresis and Immunoblotting

Figure 2 shows immunoblot analysis using a monoclonal antibody specific to endothelial NO synthase.23 In cultured bovine arterial endothelial cells, a single band of 135 kD, corresponding to endothelial NO synthase, was detected. The extract from canine basilar arteries contained this 135-kD band as well as a 120-kD band, a possible degradation product of endothelial NO synthase. Because brain and macrophage synthases have molecular weights of 160 kDa and 125 kD, respectively, and the antibody we used does not cross-react with brain and macrophage NO synthases,29 this 120-kD band is unlikely to be either of these. No such degradation product was noted in the cultured cells, which could be solubilized for electrophoresis much more quickly than the cerebral arteries. The total densitometer values of 135 kD and 120 kD

* Bovine pulmonary artery endothelial cells, type 209-CCL, were obtained from American Type Culture Collection, Rockville, Maryland.

† Dry-blot electrophoretic apparatus obtained from Bio Rad, Richmond, California.

‡ Enhanced chemiluminescent detection solution obtained from Amersham Corp., Arlington Heights, Illinois.

§ Bromma Ultroscan XL manufactured by Pharmacia LKB Biotech, Piscataway, New Jersey.
were presented as percentages of the D0 control value measured in the same experiment. Although the signal in the D7 group was less than that of the control group, it was not statistically significant (Fig. 3). The proportions of the 135-kD form in Groups D0, D2, and D7 were 49% ± 15%, 44% ± 14%, and 38% ± 14%, respectively. In both the D2 and D7 groups there was less staining of the 135-kD polypeptide than at the previous time point, but the differences were not statistically significant.

Expression of Soluble Guanylate Cyclase Following SAH

Figure 4 shows immunoblot analysis using a monoclonal antibody specific to the α subunit of soluble guanylate cyclase. The extract from the canine basilar arteries showed a clear single major band at 70 kD corresponding to the α subunit of soluble guanylate cyclase. In cultured endothelial cells and smooth-muscle cells, no specific band was detected. The densitometer values were calculated as percentages of D0 group’s control value measured in the same experiment (Fig. 5). The signal of the D2 group was 69% of the control value, but this reduction was not statistically significant. The signal strength in the D7 group was reduced to 25% of the control value. There was a significant difference between the D7 value and the D0 control value (p < 0.001).

Discussion

The canine basilar artery model of SAH in combination with an immunoblotting technique was used to examine the levels of endothelial NO synthase and soluble guanylate cyclase in vasospastic arteries. As expected, the caliber of the vessels in the animals sacrificed on Days 2 and 7 was less than in the control animals sacrificed on Day 0, with arterial spasm significantly greater in D7 than D2 animals. On the immunoblots there was some indication of a progressive reduction in the total amount of endothelial NO synthase and degradation to a smaller-size peptide at Day 2 and Day 7, but these changes were not statistically significant. More important, however, was the observation of a loss of soluble guanylate cyclase from the arteries, the amount detected in the D7 group being only 25% of the control value from Group D0. This change could impair the relaxation response of arterial smooth-muscle cells.

Thus far, three isoforms of NO synthase have been described and purified. The type I and type III isoforms are constitutive and are responsible for the release of NO from neurons and from blood vessels, respectively, whereas type II, found mainly in macrophages, is inducible with endotoxin and/or cytokines and as a part of the immunological reaction. Brain NO synthase (type I) was the first to be isolated and purified to a denatured molecular mass of 160 kD, and its complementary deoxyribonucleic acid has been cloned and sequenced. Brain NO synthase has been immunohistochemically localized in cerebral arteries...
using polyclonal antibodies, and this enzyme has been found in both endothelium and dense innervation of adventitia. Recently, endothelial NO synthase (type III) has been purified to a denatured molecular mass of 135 kD. A full-length endothelial NO synthase clone has been isolated. Because the loss of endothelium-dependent relaxation has been reported following SAH, we focused on endothelial NO synthase. The monoclonal antibody against endothelial NO synthase used in this study does not cross-react with brain NO synthase.

The extract from canine basilar arteries contained 135-kD and 120-kD polypeptides. The latter band is most likely a degradation product of endothelial NO synthase. The time required to dissect the arteries may have permitted some degradation of the endothelial NO synthase protein. The proportion of the 135-kD form at Day 2 and Day 7 was less than at the previous time point. If the distribution of immunostaining between the 135-kD and 120-kD polypeptides at Day 0 represents the combined results of degradation in situ and during dissection under normal conditions, the relative increases in the 120-kD band compared to the 135-kD band at Day 2 and Day 7 indicates that there may be a progressive degradation of endothelial NO synthase in situ. However, the differences were not statistically significant.

The total densitometer values of 135-kD and 120-kD polypeptides at Day 2 and Day 7, presented as percentages of Day 0 control values, show a decrease in total enzyme but the differences are not statistically significant. The luminal release of EDRF as determined under bioassay conditions is not decreased during cerebral vasospasm. More recently, it was reported that oxyhemoglobin increased the NO synthase activity and restored the rate of NO formation. The inhibition of the production or release mechanism of NO in endothelium may not have significance in the cause of impairment of endothelium-dependent relaxation following SAH.

Soluble guanylate cyclase has a molecular mass of 150 kD and consists of two subunits with reported molecular masses of 70 to 82 kD. The messenger ribonucleic acid encoding both α and β subunits has been cloned recently from bovine and rat lung. The α and β subunits from rat lung differ in primary structures and antigenicity. A single major band of 70 kD was obtained in the extract from canine basilar arteries with the anti–rat α subunit monoclonal antibody, which was reported to recognize soluble guanylate cyclase from other species. This compares to 73 kD for the bovine, 78 kD for the human, and 82 kD for the rat α subunits. The densitometer values of the 70-kD protein were compared for each group in the current experiment, and a significant difference of expression between the control and vasospastic arteries was shown. It has been reported that the resting levels of cGMP in the arterial wall and those evoked by NO were diminished in SAH. Inactivation of NO by oxyhemoglobin...
globin in the arterial wall, a decreased supply of the substrate guanosine triphosphate, and/or reduced release of NO have been considered as the causes of impairment of endothelium-dependent relaxation after SAH. The reduced expression of soluble guanylate cyclase protein should also be considered.

Soluble guanylate cyclase is considered to be a constitutively expressed protein. The regulatory mechanism of this enzyme is not clear. Oxyhemoglobin inhibits the activation of soluble guanylate cyclase and impairs the production of cellular cGMP. Oxyhemoglobin captures EDRF/NO and competes with the heme moiety of soluble guanylate cyclase. The reduced level of soluble guanylate cyclase may be a consequence of the presence of oxyhemoglobin in the arterial wall. Another possibility is the change in characteristics of smooth-muscle cells in the arterial wall. Subarachnoid hemorrhage causes a breakdown of the blood–arterial wall barrier in the cerebral arteries, which may allow penetration of plasma factors into the vessel wall. The smooth-muscle cells exposed to a variety of plasma factors may modulate phenotypically to a synthetic state from a contractile state and may lose the usual properties of smooth-muscle cells. This may cause the impaired expression of soluble guanylate cyclase protein. Smooth-muscle cells that we cultured from canine aorta are considered to be modulated to the synthetic phenotype, and we were unable to detect soluble guanylate cyclase in the cells by immunoblotting.

Another possibility is that the guanylate cyclase protein is more degraded in spastic arteries. The monoclonal antibody we used may not recognize degradation products. Using similar immunoblotting techniques, Minami, et al. recently demonstrated the degradation of contractile and cytoskeletal proteins and their attachment proteins in spastic basilar arteries. Their group also showed that the activity of μ-calpain was continuously enhanced in spastic arteries and suggested that all proteins responsible for contraction might be similarly degraded. The degradation of soluble guanylate cyclase in smooth-muscle cells in the arterial wall could be catalyzed by calpain activated by elevated intracellular Ca++ following SAH.

In conclusion, the reduced expression of soluble guanylate cyclase in basilar arteries in the canine double-hemorrhage model suggests that the impairment of endothelium-dependent vasodilation may involve the altered activity of soluble guanylate cyclase and the inability of vascular muscle to produce cGMP.

Acknowledgments

The authors thank Dr. David Michael White and Mr. Ben Lim of the Department of Neurology and Ms. Audrey Lee, Mr. Michael Mays, Ms. Mary Williams, and Ms. Kristi Hollingsworth of the Animal Resources Center for their technical assistance.

References


Fig. 5. Graph showing levels of soluble guanylate cyclase in a canine subarachnoid hemorrhage model. Values are shown as mean ± standard deviation (10 dogs/group). DO = control group; *= p < 0.001; NS = not significant.
Nitric oxide synthase and guanylate cyclase levels after SAH


Manuscript received February 16, 1994. Accepted in final form June 12, 1994. This work was supported by National Institutes of Health Grant R01NS25946-04 to Dr. Weir.

Address for Dr. Kasuya: Department of Neurosurgery, Tokyo Women’s Medical College, Tokyo, Japan.
Address reprint requests to: Bryce K. A. Weir, M.D., Section of Neurosurgery, University of Chicago, 5841 South Maryland Avenue, Chicago, Illinois 60637.