Inhibition of Poly(ADP-Ribose) Polymerase Attenuates Cerebral Vasospasm After Subarachnoid Hemorrhage in Rabbits

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Background and Purpose—Poly(ADP-ribose) polymerase (PARP) is important in modulating inflammation, which has been implicated in cerebral vasospasm after subarachnoid hemorrhage (SAH). We investigated the role of PARP in vasospasm using 3-aminobenzamide (3-AB), a PARP inhibitor, in a rabbit model.

Methods—Twenty-four New Zealand White rabbits were divided into 4 groups: (1) no treatment (control group, n=6); (2) blood injection without pretreatment (SAH-only group, n=6); (3) blood injection with pretreatment by vehicle (SAH + vehicle group, n=6); and (4) blood injection with pretreatment by 3-AB (SAH + 3-AB group, n=6). We used the single-hemorrhage model of SAH, injecting autologous arterial blood into the cisterna magna. Angiography was performed before (baseline) and after (day 2) SAH, and the diameter of the basilar artery (BA) was measured. Animals were euthanatized after the second angiogram. After perfusion and fixation, the brains were cut into sections for hematoxylin and eosin and immunohistochemical staining for poly(ADP-ribosyl)ation.

Results—In the control group, there were no differences in the BA lumen caliber between baseline and day 2 (96.8±10.4%). Cerebral vasospasm in the SAH + 3-AB group (88.2±6.2%) was remarkably attenuated in comparison with that in the SAH-only group (64.9±8.0%) and the SAH + vehicle group (65.6±10.8%). The BA in the SAH + 3-AB group showed less corrugation of the tunica elastica interna than that in the SAH-only and SAH + vehicle groups. Staining for poly(ADP-ribosylation) was markedly inhibited in smooth muscle and adventitial cells of the BA in the SAH + 3-AB group compared with other groups.

Conclusions—Inhibiting ADP-ribosylation attenuates cerebral vasospasm after SAH in rabbits, and PARP activation may play an important role in the development of cerebral vasospasm. (Stroke. 2001;32:225-231.)

Key Words: nitric oxide synthase • subarachnoid hemorrhage • vasospasm • rabbits

Free radicals, such as peroxynitrite and hydroxyl radical, trigger the breakage of single strands of DNA and subsequently activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP). When PARP is activated, nicotinamide adenine dinucleotide is dissipated, and its regeneration requires adenosine triphosphate. Therefore, PARP activation leads to cell death through energy depletion. Inhibiting PARP has reduced ischemic and traumatic brain injury in experimental animal models. Szabó et al have demonstrated that PARP may also modulate the course of inflammation by regulating the expression of adhesion molecules, neutrophil infiltration, nuclear factor-κB (NF-κB), and inducible nitric oxide synthase (iNOS), all of which are responsible for inflammation. Recent experimental data show that PARP activation is associated with the pathogenesis of a variety of inflammatory diseases, such as arthritis and meningitis.

Cerebral vasospasm is an important cause of morbidity and mortality after subarachnoid hemorrhage (SAH). Although the pathogenesis of vasospasm is not fully understood, an inflammatory response has been suggested to be involved. Oxyhemoglobin in hemolysate within a subarachnoid clot is considered one of the candidates responsible for delayed vasospasm because it releases free radicals, such as superoxide anions and hydroxyl radicals, during autooxidation to methemoglobin. In addition, the induction of iNOS has been observed in the cerebral vessel wall and the mononuclear and polymorphonuclear cells infiltrating the subarachnoid space after SAH in rats. Thus, the nitric oxide metabolite peroxynitrite may be associated with vasospasm. These free radicals may cause inflammation by

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injuring the vessel wall. These findings may also indicate the possible activation of PARP during cerebral vasospasm. Polin et al. showed that adhesion molecules were elevated in the cerebrospinal fluid of patients after SAH. We previously demonstrated that inhibiting the NF-κB transcription factor, which is known to be essential in iNOS induction and closely related to inflammation, attenuated cerebral vasospasm. On the basis of these findings, we assumed that an inflammatory response after SAH plays a significant role in the pathogenesis of cerebral vasospasm. In this study we investigated the role of PARP in cerebral vasospasm using 3-aminobenzamide (3-AB), a specific inhibitor of PARP, in a rabbit SAH model.

Materials and Methods

Experimental Design

Twenty-four male New Zealand White rabbits weighing 1.9 to 2.6 kg were assigned randomly to 4 groups. We picked 1 animal from each group randomly, and the experiments were also performed randomly on a set of 4 animals each time. Thus, the experiments in different groups were dispersed over time. All procedures were performed by 6 investigators working in tandem. Group 1 served as the control group and did not undergo SAH or receive treatment (control group, n=6). All other animals were subjected to SAH as described below.

Group 2 underwent SAH without pretreatment (SAH-only group, n=6). In group 3, a vehicle (phosphate-buffered saline [PBS]) was injected into the subarachnoid space before the induction of SAH (SAH+vehicle group, n=6). Group 4 was treated with 3-AB before the induction of SAH (SAH+3-AB group, n=6). 3-AB was dissolved in PBS at a dose of 10 mg/kg. We used 10 mg/kg of 3-AB because this dosage has shown significant effect on cerebral ischemia. We did not detect cerebrospinal fluid levels. All procedures used in this study were performed according to the public health standards at Okayama University Medical School.

Induction of Experimental SAH

The rabbit single-hemorrhage model of SAH was used. All animals subjected to SAH were anesthetized by the administration of ketamine (50 mg/kg IM) and pentobarbital (20 mg/kg IV). The atlanto-occipital membrane was exposed through an occipitocervical midline incision, and a 24-gauge butterfly needle was inserted. After the withdrawal of 1 mL of cerebrospinal fluid, the vehicle (PBS) or drug (3-AB+PBS) was given. One minute later, autologous arterial blood (1 mg/kg) from the auricular artery was carefully injected into the cisterna magna. Thereafter, the animals were placed head down for 30 minutes so that the basilar artery (BA) was suffused with the autologous blood.

Cerebral Angiography

In all animals, left verteobasilar angiography was performed 5 days before (baseline) and 2 days after (day 2) the induction of SAH to measure the diameter of the BA. The animals were anesthetized as described above under spontaneous respiration. The angiography catheter was inserted into the right femoral artery and positioned at the origin of the left vertebral artery. We injected 1 mL of contrast medium through the catheter and performed the angiography using a digital subtraction angiography system (Advantx/AFM, GE Company) at the same magnification for each animal. During these procedures, arterial blood pressure was recorded on a monitor (Omnice RT3100, NEC Corp) connected to the catheter, and blood gases and body temperature were measured and strictly maintained at standard levels. For the second angiogram, the left femoral artery was used.

Perfusion and Fixation

All animals were euthanized after the second angiogram. To do this, we performed a thoracotomy, closed the descending aorta, inserted a catheter into the left ventricle, and perforated the right atrium. After perfusion and fixation with 4% paraformaldehyde, each animal’s brain was removed and stored overnight in paraformaldehyde at 4°C.

Histological Examination

For histological examination, the brains were embedded in paraffin and cut into 4-μm sections. They were then stained with hematoxylin and eosin and observed under a light microscope.

Immunohistochemistry

To clarify poly(ADP-ribosyl)ation through PARP activation, we performed immunohistochemical staining with the Vectastain Elite ABC kit (Vector Laboratories). The paraffin-embedded sections were deparaffinized, and the production of endogenous peroxidase was halted by placing the specimens in 0.6% H2O2 in methanol for 10 minutes. The sections were blocked with normal horse serum for 20 minutes and then were incubated with a primary monoclonal antibody at 1:200 dilution for 1 hour at 37°C. The primary monoclonal antibody used for this study was the mouse anti-poly(ADP-ribose) monoclonal antibody (4335-MC-100, Trevigen), which recognizes poly(ADP-ribose)polymerized proteins. The slides were washed in PBS. They were then incubated with biotinylated anti-mouse IgG secondary antibody at 1:200 dilution for 30 minutes and with ABC reagent solution for 30 minutes. The sections were stained with diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Negative controls were performed without the primary antibody.

Statistical Analysis

We measured the diameter of the BA at 3 points: 0.2 mm above the union of the bilateral vertebral arteries, just below the anterior inferior cerebellar arteries, and 0.2 mm below the top of the BA. Three blinded investigators measured the arterial diameter, and the mean of the 3 points was calculated as the diameter of the BA. We calculated a percentage of the BA diameter on day 2 relative to the baseline for each rabbit, and the results were expressed as the mean±SD. Angiographic measurement was statistically evaluated by ANOVA with the Bonferroni/Dunn post hoc test; these statistical differences were considered significant when P<0.05. There was no mortality. All of the operated animals were analyzed.

Results

Cerebral Angiography

In the control group (96.8±10.4%), there were no differences in the caliber of the BA lumen between baseline and day 2. Two days after the injection of blood, vertebrobasilar angiograms showed severe narrowing of the BAs in comparison with that seen on the angiograms done before the injection of blood in both the SAH-only group (64.9±8.0%) and the SAH+vehicle group (65.6±10.8%). In contrast, arterial narrowing in the SAH+3-AB group (88.2±6.2%) after the injection of blood was significantly less than in the SAH-only and SAH+vehicle groups (Figures 1 and 2).

Histological Analysis

The BAs exhibited subendothelial thickening and severe corrugation of the tunica elastica interna in the SAH-only (Figure 3A and 3B) and SAH+vehicle groups (Figure 3C). In contrast, the tunica elastica interna of the SAH+3-AB vessels had a smooth appearance, and morphological changes in the vascular structure did not occur (Figure 3D).
Immunohistochemistry of Poly(ADP-Ribose)

Poly(ADP-ribose)-positive cells could be identified clearly in the endothelial cells, smooth muscle cells, and adventitial cells of the SAH-only group (data not shown) and the SAH+vehicle group (Figure 4A and 4B). Immunoreactivity was shown mainly in the nuclei. Poly(ADP-ribosyl)ation after SAH was inhibited by 3-AB, especially in smooth muscle cells and adventitial cells. In the SAH+3-AB group, approximately one third of the smooth muscle cells and adventitial cells were positive for poly(ADP-ribose), although almost all of them were positive in the SAH-only and SAH+vehicle groups. In addition, the staining intensity of positive cells was decreased in the SAH+3-AB group (Figure 4C and 4D). The negative controls did not show poly(ADP-ribose) immunoreactivity.

Discussion

PARP is activated in endothelial cells and smooth muscle cells through the breaking of single-strand DNA by free radicals such as hydroxyl radical and peroxynitrite. This activity is attenuated by a pharmacological inhibitor, 3-AB. Free radicals are also produced during SAH and are believed to be important in the pathogenesis of cerebral vasospasm. The auto-oxidation of oxyhemoglobin within a subarachnoid clot to methemoglobin releases free radicals, such as the superoxide anion or hydroxyl radical, via the Haber-Weiss and Fenton reactions. The generation of these free radicals causes lipid peroxidation and damages vessel walls, especially endothelial cells, thereby leading to vasospasm. In our study we identified the activation of PARP during vasospasm (Figure 4). Poly(ADP-ribose) immunoreactivity on day 2 after SAH was increased in endothelial cells, smooth muscle cells, and adventitial cells in the SAH-only and vehicle-treated animals. These findings support the hypothesis that PARP is activated by free radicals released after SAH. In addition, rabbits treated with 3-AB showed decreased poly(ADP-ribosyl)ation in smooth muscle cells and adventitial cells. Inhibiting PARP with 3-AB reduced the degree of vasospasm; thus, the activation of PARP plays a role in inducing cerebral vasospasm after SAH.

The insult of SAH elevates cytokines in cerebrospinal fluid. Either these cytokines or oxidative stress, both of which are related to an inflammatory response, mediates iNOS expression and NF-κB activation in smooth muscle cells and macrophages. Although the relationship between iNOS and vasospasm remains controversial, some authors report the expression of iNOS in the cerebral vessel wall and the mononuclear cells and polymorphonuclear cells.
infiltrating into the subarachnoid space during vasospasm.19–21 Widenka et al.21 demonstrated iNOS induction in endothelial cells, smooth muscle cells, and, above all, in adventitial cells of the rat BA after SAH. The overproduction of nitric oxide by iNOS may produce peroxynitrite, a free radical, which leads to tissue damage and lipid peroxidation.40 Moreover, we previously demonstrated that preventing the development of NF-κB, a transcription factor whose activation is necessary for iNOS expression,35,36,41 inhibited cerebral vasospasm.22 Peroxynitrite may also further activate PARP.

PARP is involved in regulating the NF-κB signaling pathway leading to the induction of iNOS, and the pharmacological and genetic inhibition of PARP impairs the expression of iNOS by decreasing NF-κB activation.42–45 These findings are consistent with ours, which show that inhibiting PARP with 3-AB reduced the degree of vasospasm in smooth muscle cells and adventitial cells.

Cytokines produced during SAH stimulate the expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin on the endothelial surface.46 These adhesion molecules may contribute to vasospasm because they promote leukocytic adhesion to and migration across the vascular endothelium, thereby initiating tissue injury. They are found to be elevated in cerebrospinal fluid of patients with SAH because of endothelial damage and breaking of the blood-brain barrier.17 In a rat model of SAH, the expression of ICAM-1 was observed on the endothelial surface and in the medial layer of the vessel wall.16 Bavbek et al.47 demonstrated that administering monoclonal antibodies against ICAM-1 or CD18 attenuated vasospasm in the BA after SAH in rabbits. Thai et al.48 documented similar findings in a rat femoral artery model of vasospasm with ibuprofen, an inhibitor of both ICAM-1 and VCAM-1 expression. The expression of ICAM-1 in myocardial ischemia and reperfusion injury is inhibited by the genetic disruption of PARP, and in vitro studies have demonstrated that the pharmacological inhibition of PARP with 3-AB reduces ICAM-1 expression.49 ICAM-1 expression by inflammatory cytokines has been also shown to be under the control of NF-κB.50–52 Since PARP is required to activate NF-κB, it is reasonable to assume that the activation of PARP participates in regulating ICAM-1 expression. On the basis of these findings, we assume that downregulating adhesion molecules by inhibiting PARP attenuates vasospasm. Our experiment did not inhibit PARP activation in the endothelial layer but only in the medial and adventitial layers. The route used to administer the drug, ie, the adventitial side, may be the reason that PARP expression could not be inhibited in the endothelial layer. Further study is required to clarify this issue.

A high concentration of 3-AB acts as a scavenger of the hydroxyl radical,9,13,53 and free radical scavengers are of benefit in the treatment of vasospasm. In experimental and clinical studies, free radical scavengers and lipid peroxidation inhibitors have been shown to suppress the contraction of the cerebral artery after SAH.54–57

In this study the pharmacological inhibition of PARP with 3-AB attenuated vasospasm. These findings provide the first evidence of the role of PARP in the pathogenesis of cerebral vasospasm. We anticipate that inhibiting PARP can offer a new therapy for patients suffering vasospasm, but further studies are required to elucidate the mechanism of vasospasm that involves PARP activation.

**Figure 3.** Representative histological structural changes in the arterial wall of the BA. A, Control group; B, SAH-only group; C, SAH+vehicle group; D, SAH+3-AB group (hematoxylin and eosin staining, magnification ×100). The BA wall in the SAH+3-AB group showed less narrowing and corrugation of the tunica elastica interna than in the SAH-only and the SAH+vehicle groups.
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Figure 4. Photomicrographs show representative findings of immunohistochemical staining for poly(ADP-ribose): low (>100, A) and higher (>400, B) magnification of the SAH+ vehicle group; low (>100, C) and higher (>400, D) magnification of the SAH+3-AB group. The brown products showed poly(ADP-ribose) immunoreactivity. In animals that received vehicle, the poly(ADP-ribose) immunoreactivity was the same as in the SAH-only animals (A, B). Treatment with 3-AB markedly decreased the immunoreactivity (C, D).


Satoh et al performed a baseline cerebral angiogram and then gave a single injection of vehicle or 3-aminobenzamide followed by blood into the cisterna magna of rabbits. Forty-eight hours later, the animals had another angiogram and were then euthanized. Immunoreactivity to poly(ADP-ribosyl)ated protein was assessed in cross-sections of the basilar artery. 3-Aminobenzamide reduced the degree of arteriographic narrowing and the amount of immunoreactivity to poly(ADP-ribosyl)ated protein in the adventitia and media of the basilar artery of treated rats. The authors conclude, therefore, that activation of poly(ADP-ribose) polymerase is important in the development of vasospasm and that it can be a new therapy for patients with vasospasm.

These results are interesting and seem to provide more support for the idea that an inflammatory process possibly mediated by free radicals contributes to vasospasm. Let us address the limitations of this experiment first before recommending another drug for treatment of vasospasm that might be destined to fail like so many others. No appropriate randomization schedule was used, and interpretation of the results was not blinded. There is no result presented that proves that the effects observed were due to inhibition of poly(ADP-ribose) polymerase. Reduction of poly(ADP-ribosyl)ated protein is associated with the reduction in vasospasm but only association is shown. Any treatment that reduces vasospasm in this model might reduce markers of arterial injury, such as poly(ADP-ribosyl)ated protein, if it is a marker of injury. Finally, at 2 days after SAH in rabbits, there is moderate vasospasm that is not resistant to vasodilators,2 which might correspond to the early, mildest form of vasospasm in man; whether inhibiting processes that account for this will reduce severe, prolonged vasospasm is open to question.

The theory that free radicals and inflammation contribute to vasospasm is simplistic. Inflammation and generation of free radicals are complex processes that potentially have beneficial and detrimental effects. Nonspecific inhibition of either process may not be beneficial. Indeed, tirilazad, a potent inhibitor of iron-dependent lipid peroxidation and probably other free radical reactions, was not of unequivocal benefit to patients with subarachnoid hemorrhage and may have been detrimental to patients with ischemic stroke.1 Similarly, the role of inflammation in vasospasm is unclear. Vasospasm was not recognized as a clinical problem in patients with infectious meningitis, and when they do develop infarcts, the topography is not that of the vasospasm which occurs after SAH. Finally, one would like to have some rational basis for the therapy. While 3-aminobenzamide may inhibit inflammation, why would it reduce vasospasm 48 hours after SAH, at a time when the arterial narrowing in this model is due mainly to active smooth muscle contraction and is reversible with papaverine? It is the authors’ responsibility now to present detailed data to confirm or refute the hypothesis that activation of poly(ADP-ribose) polymerase mediates vasospasm 48 hours after SAH in rabbits.

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