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Gene Transfer of Cu/ZnSOD to Cerebral Vessels Prevents Subarachnoid Hemorrhage-induced Cerebral Vasospasm

Mi Ran Yun, Dong Eun Kim, Hye Jin Heo, Ji Young Park, Ji Young Lee, Sun Sik Bae, and Chi Dae Kim

Department of Pharmacology, College of Medicine and Medical Research Center for Ischemic Tissue Regeneration, Pusan National University, Busan 602–739, Korea

The preventive effects of gene transfer of human copper/zinc superoxide dismutase (Cu/ZnSOD) on the development of cerebral vasospasm after subarachnoid hemorrhage (SAH) were examined using a rat model of SAH. An experimental SAH was produced by injecting autologous arterial blood twice into the cisterna magna, and the changes in the diameter of the middle cerebral artery (MCA) were measured. Rats subjected to SAH exhibited a decreased diameter with an increased wall thickness of MCA that were significantly ameliorated by pretreatment with diphenyleneiodonium (DPI, 10 μM), an inhibitor of NAD(P)H oxidase. Furthermore, application of recombinant adenovirus (100 μl of 1×10^{10} pfu/ml, intracisternally), which encodes human Cu/ZnSOD, 3 days before SAH prevented the development of SAH-induced vasospasm. Our findings demonstrate that SAH-induced cerebral vasospasm is closely related with NAD(P)H oxidase-derived reactive oxygen species, and these alterations can be prevented by the recombinant adenovirus-mediated transfer of human Cu/ZnSOD gene to the cerebral vasculature.

Key Words: Cu/ZnSOD, SAH, NAD(P)H oxidase, Cerebral vasospasm

INTRODUCTION

Cerebral vasospasm after subarachnoid hemorrhage (SAH) is characterized as a delayed but prolonged contraction of the major cerebral arteries and seriously affects the prognosis of patients, but the pathophysiological mechanisms of SAH-induced cerebral vasospasm still remains unclear.

A variety of mechanisms have been proposed to explain these phenomena and, in particular, oxidant stress has been implicated in the pathogenesis of cerebral vasospasm (Sano et al, 1980; Sasaki et al, 1981; MacDonald & Weir, 1991). A plethora of reports support the significance of reactive oxygen species (ROS) in causing vasospasm through activation of protein kinase C and/or inactivation of nitric oxide (Gryglewski et al, 1986; Chakraborti and Michael, 1993).

Convincing evidences have been presented to show that production of ROS occurs in non-phagocytic cells, including endothelial cells (Matsubara & Ziff, 1986), vascular smooth muscle cells (Griendling et al, 1994) and aortic adventitial fibroblasts (Pagano et al, 1998), and superoxide anion has a variety of biological functions that accompany the induction of gene expression (Sen & Packer, 1996), cellular proliferation (Rao & Berk, 1992), apoptosis (Li et al, 1997; Maron et al, 1999), and hypertrophy (Zafari et al, 1998). However, the exact roles of vascular production of ROS in the development of cerebral vasospasm in SAH remain unknown.

Thus, we employed diphenyleneiodonium (DPI), an NAD(P)H oxidase inhibitor, to clarify the involvement of NAD(P)H oxidase-derived ROS anion in the development of SAH-induced cerebral vasospasm. Furthermore, the potential effect of gene transfer by recombinant adenovirus encoding human Cu/ZnSOD was also determined.

METHODS

Experimental SAH and drug injection

The experimental protocols used in this study were approved by the Pusan National University Animal Research Committee. The animals used in this experiment were Sprague-Dawley male rats, weighing 300~400 g. The rats were anesthetized by an intraperitoneal injection of thiopental sodium (50 mg/kg), placed in a supine position and allowed to breathe spontaneously. The left femoral artery was exposed and cannulated to obtain fresh autologous arterial blood. The animals were then fixed on stereotaxic frame. Aided by a surgical microscope, the atlantooccipital membrane was tapped carefully into the cisterna magna with a 27-gauge butterfly needle. After aspirating 0.3 ml of cerebrospinal fluid (CSF), an equal amount of autologous blood from the femoral artery was injected for a period of 3 minutes or longer. The drugs were coadmini-

Corresponding to: Chi Dae Kim, Department of Pharmacology, College of Medicine, Pusan National University, 10 Ami-dong 1-ga, Seo-gu, Busan 602-739, Korea. (Tel) 82-51-240-7730 (Fax) 82-51-244-1036, (E-mail) chidkim@pusan.ac.kr

ABBREVIATIONS: Cu/ZnSOD, copper/zinc superoxide dismutase; SAH, subarachnoid hemorrhage; MCA, middle cerebral artery; DPI, diphenyleneiodonium; ROS, reactive oxygen species.

stered with blood at first injection (DPI, 25 μl of 100 μM, from Sigma Chemical Co. or AdCMVSOD, 100 μl of 1×10^{10} pfu/ml). The rats were then placed in a head-down prone position at a 30° angle for 30 min to hold the blood in the basal cisterns. For the second injection, the same procedure was repeated after 48 hours. Sham-operated rats were injected with 0.9% sterile NaCl solution instead of blood as a control.

Morphological examination with light microscope

After anesthesia, the hearts were cannulated and perfused with phosphate buffer solution (60 ml) followed by phosphate buffer solution containing 2% glutaraldehyde (60 ml), which maintaining at 7.8 ml/min with peristaltic pump (Cole-Parmer Inc., Chicago, IL, USA). Brain was then fixed with 10% neutral formalin for 24 hours, and embedded in paraffin, sliced, and stained with hematoxylin and eosin for light microscopy. The middle cerebral artery (MCA) was divided into three portions. The wall thickness and cross-sectional diameter of the vessel were measured using an image analysis system (Image-Pro Plus, Media Cybernetics, Maryland, USA). To correct for vessel deformation and off-transverse sectioning, the diameter was determined by measuring the circumference of vessel lumen and calculating the diameter as a generalized circle, based on the measured circumference of vessel.

Cerebral angiography

Digital angiography was performed 7 days after experimental SAH. The anesthetized rat was placed in supine position. The femoral artery was exposed and catheterized. Then the catheter was advanced to right carotid artery, and angiography was performed after injection of nonionic contrast medium through the catheter.

Preparation of adenoviral vector

Replication-deficient recombinant adenoviral vector (serotype 5, produced in HEK293 cells) driven by the cytomegalovirus immediate early promoter was used to transfer the gene to the cerebral vasculature. Adenoviral vectors containing human SOD gene (AdCMVSOD) and E.coli β-galatoxidase gene (AdCMVLacZ) were obtained from Dr. John Engelhardt (Gene Therapy Core Center, University of Iowa, IA, USA). The DNA constructs of replication-deficient adenovirus comprised almost a full-length copy of adenoviral genome, in which SOD1 and LacZ expression cassettes were incorporated at the site of E1 region deletion. For each vector, high titer adenoviral vectors were prepared by double cesium gradient purification, and viral titer (pfu/ml) was determined by the standard methods. Virus preparation was suspended in phosphatebuffered saline (PBS) containing 3% sucrose, and stored at −70°C until use.

To transfer the genes to the cerebral vasculature *in vivo*, rats were anesthetized with thiopental sodium (50 mg/kg, i.p.), and 27-gauge needle was aseptically inserted into the cistern magna as previously described. An equal volume (100 μl) of CSF was removed before injection of viral suspension (100 μl of 1×10^{10} pfu/ml) to avoid an increase of intracranial pressure.

X-Gal staining

Isolated brains were placed in plastic vials and fixed for 10 minutes in a fixative (PBS containing 2% paraformaldehyde and 0.025% glutaraldehyde) at room temperature. The samples were rinsed well in PBS and then immersed in X-Gal stain solution [PBS containing 20 mmol/L $K_4Fe(CN)_6 \cdot 3H_2O$, 20 mmol/L $K_3Fe(CN)_6$, 2 mmol/L $MgCl_2$, and 1 g/L X-Gal stain] for 2 hours at room temperature. The samples were evaluated to determine whether β-galactosidase had been produced in the blood vessels and brain tissue (blue staining). For histochemical analysis, the fixed brain was processed for paraffin embedding, and microtome sections (5 μm) were cut from the block, placed on slide glasses, and counterstained with hematoxylin-eosine.

Immunohistochemial analysis for Cu/ZnSOD

For immunohistochemical analysis for Cu/ZnSOD, serial 5-μM-thick frozen sections of MCA were adhered to poly-L-lysine-coated slides, allowed to dry in room air, and fixed in acetone. After treatment with H_2O_2 (0.6%) and bovine serum albumin (2%), the preparations were incubated with the goat anti-rat Cu/ZnSOD polyclonal antibody (Santa Cruz Biotechnology). After the primary incubation, the slides were incubated with the biotinylated anti-goat secondary antibody (Santa Cruz Biotechnology). The slides were then incubated with avidine and biotinylated horseradish peroxidase complex (Vector Laboratories, Inc. Burlingame, CA) for 1 hour. The slides were washed in PBS, incubated with diaminobenzidine (Vector Laboratories, Inc.), and then washed with water. Vessel sections were counterstained with hematoxyline (purple) and examined for positive staining for Cu/ZnSOD (gray-black) by light microscopy. Immunoreactivity for Cu/ZnSOD was quantified by Image-Pro plus Imaging soft ware (Media Cybernetics, Silver Spring, MD), and the stain density was expressed as a pixels per μm^2 for tissue.

Statistical analysis

Data were presented as meansSEM or percentage of control. Statistical comparisons between groups were performed with two-tailed Student's *t*-test for unpaired data. A value of $p<0.05$ was considered statistically significant.

RESULTS

Temporal changes in diameter of middle cerebral artery

The baseline values of middle cerebral artery (MCA) diameter and wall thickness were 78.911 ± 2.56 μm and 16.77 ± 1.54 μm, respectively. Fig. 1 demonstrates the sequential changes in diameter and wall thickness of MCA after SAH. The diameter of MCA started to decrease on day 1, peaking on day 7, and the wall thickness increased. On day 14, they recovered almost to the values of the control group. Angiographic cerebral arteries between groups on day 7 were compared, and illustrated in Fig. 2 shows that the small cerebral arteries of SAH group were indistinct or disappeared, compared with those of the control group.

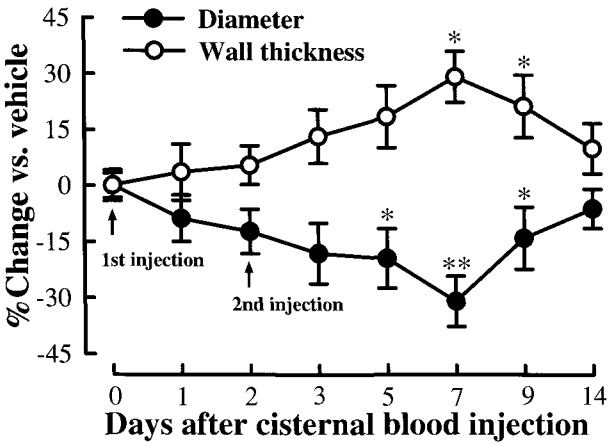

Fig. 1. Temporal changes in diameter and wall thickness of rat middle cerebral artery after intracisternal injection of autologous blood. Values are expressed as means±SEM from 6 animals. *p< 0.05; **p<0.01 vs. baseline value on day 0.

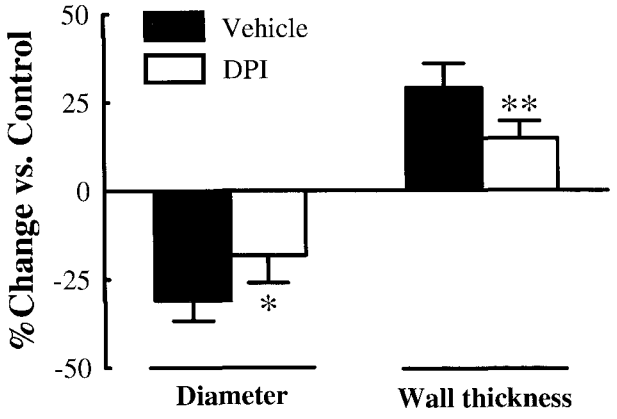

Fig. 3. Effect of diphenyleneiodonium (DPI, 25 μl of 100 μM) on the SAH-induced changes in the wall thickness and diameter of middle cerebral artery. Values were obtained from samples on day 5 after the second injection of blood into cisterna magna, and expressed as means±SEM from 6 animals. *p<0.05; **p<0.01.

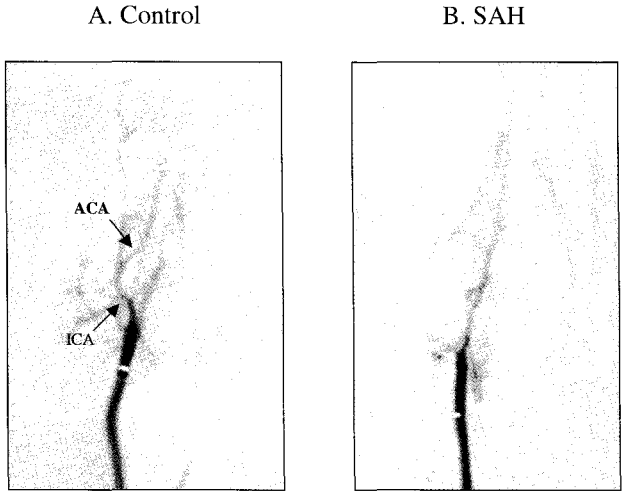

Fig. 2. Representative cerebral angiogram of rat on day 7 after intracisternal injection of autologous blood (double hemorrhage model). Many of small cerebral arteries in SAH are not distinct.

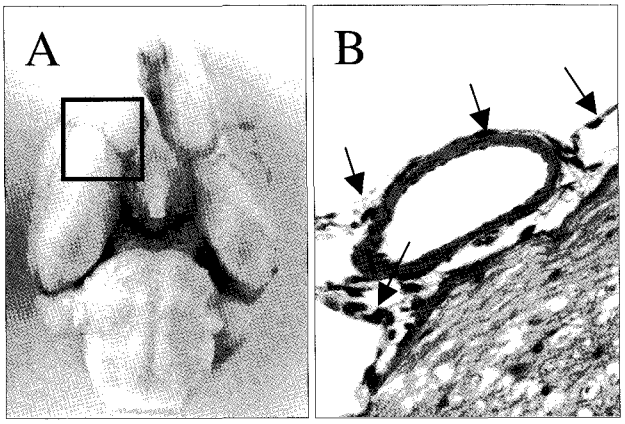

Fig. 4. (A) shows the expression of β-galatoxidase in cerebral vasculature 3 days after intracisternal injection of AdCMVLacZ. (B) represents the microscopic photograph of box in (A) The transgene expression emerged on adventitia mainly (arrows).

Effect of DPI on SAH-induced cerebral vasospasm

Light microscopic examination of the MCA in the vehicle-treated group revealed quantitatively substantial corrugation of the internal elastic lamina, whereas arterial corrugation was less prominent in animals treated with DPI (data not shown). Fig. 3 shows the result of comparison of cross-sectional diameter and wall thickness of MCA between vehicle- and DPI-treated groups on day 5 after the second injection of autologous blood into cisterna magna. The vehicle-treated group exhibited a decrease of vascular diameter with an increase of diameter. These variables were significantly attenuated by treatment with DPI (25 μl of 100 μM).

AdCMVLacZ-mediated transgene expression on the cerebral vasculature

Staining with X-gal for -galactosidase was performed on the brain to confirm the transgene expression after intracisternal administration of AdCMVLacZ. Positive staining merged mainly along the vasculature (Fig. 4A). Microscopic examination revealed that positive staining for β-galactosidase was observed mostly in adventitial cells of MCA and leptomeningeal cells overlying MCA (Fig. 4B).

Transgene expression was observed on day 1 and maximally on days 3 after intracisternal administration of AdCMVLacZ (100 μl of 1×10^{10} pfu/ml). β-Galactosidase activities in the cerebral vasculature from rats treated with 1×10^{9}, 3×10^{10}, 1×10^{10} and 3×10^{10} pfu/ml were 20±10, 250±60, 423±138, and 370±114 mU/mg protein, respectively (n=4). These results suggest that injection of

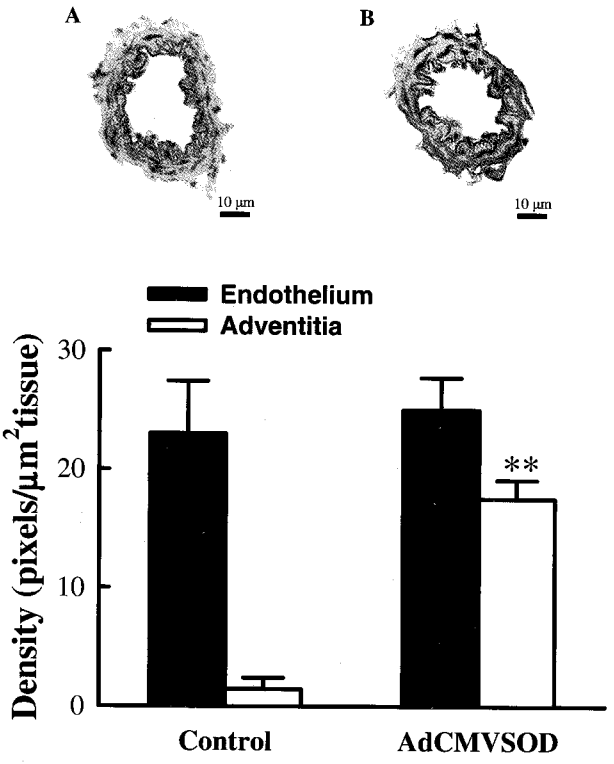

Fig. 5. Comparison of densities of immunohistochemical staining for Cu/Zn SOD in middle cerebral artery. (A) Positive staining (gray-black) for Cu/Zn SOD was observed in the endothelium, but not in the media or adventitia in vessels from the control group. (B) Positive staining for Cu/Zn SOD was observed in both endothelium and adventitia in vessels from AdCMVSOD-treated group. The densities of staining in middle cerebral artery section were expressed as pixels per μm^2 of tissue. Values are means ± SEM from 5 experiments. **$p<0.01$ vs. control.

AdCMVLacZ 1×10^{10} pfu/ml was optimal for adenoviral vector.

AdCMVSOD-mediated transgene expression on the cerebral vasculature

In the MCA from vehicle-treated group, positive staining for Cu/ZnSOD was observed in the endothelium, but not in adventitia. Positive staining for Cu/ZnSOD was observed in both the endothelium and adventitia of the vessels from AdCMVSOD-treated group. The amount of Cu/ZnSOD expression in the endothelium was not different from that of vehicle-treated group (Fig. 5).

Effect of AdCMVSOD-mediated gene transfer

In the MCA from rats treated with AdCMVSOD (100 μl of 1×10^{10} pfu/ml), the reduction of diameter (13.05 ± 5.96%) and augmentation of wall thickness (10.04 ± 7.24%) accompanied by corrugation of the internal elastic lamina were significantly attenuated, compared with vehicle- and LacZ-treated group (diameter: 31.05 ± 6.74%, and wall thickness: 29.03 ± 6.68% in the vehicle-treated group) (Fig. 6).

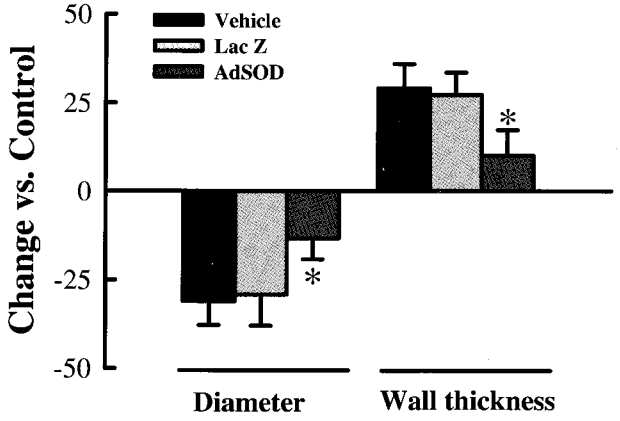

Fig. 6. Effect of AdCMVSOD and AdCMVLacZ on the SAH-induced changes for in diameter and wall thickness of middle cerebral artery. The cerebral vasospasm of AdCMVSOD-treated group was significantly ameliorated. However there was no significant difference between AdCMVLacZ-treated and vehicle-treated group (SAH only). Values were obtained 5 days after the second injection of blood, and were expressed as means ± SEM from 6 animals. *$p<0.05$; **$p<0.01$ vs. control.

DISCUSSION

In the present experiment with the two-hemorrhage model of SAH rats, the time course of the development of vasospasm was similar to vasospasm after SAH in clinical situation. Minimal narrowing occurred in 1 to 2 days and has progressively increased with 5 days after the second injection of autologous blood into cisterna magna. Such delayed cerebral vasospasm in SAH rats was significantly attenuated by pretreatment of the rats with DPI, an NAD(P)H oxidase inhibitor. Together with our previous study, (Kim et al, 2002), the above results suggest close involvement of NAD(P)H-derived ROS in the pathogenesis of cerebral vasospasm in SAH.

Although cerebral vasospasm in SAH patients is a major cause of morbidity and mortality, the precise mechanisms for the delayed cerebral vasospasm remain unclear. In the previous reports, production of superoxide in the vascular wall has been shown to inactivate nitric oxide, which leads to impairment of endothelium-dependent vasodilation (Mugge et al, 1991; Rubbo et al, 1995), oxidization of low density lipoprotein (Aviram et al, 1996), and increase of the adhesion molecule expression in endothelial cells which resulting in monocyte infiltration (Marui et al, 1993) and activating matrix metalloproteinases, leading to vascular remodeling (Rajagopalan et al, 1996). With regard to SAH, ROS has been suggested to be involved in cerebral vasospasm in conjunction with protein kinase C-dependent augmentation of contraction, and suppression of vasodilation (Wang et al, 1998). Furthermore, the Cu/ZnSOD activity is significantly reduced in brain after induction of SAH in rat (Marzatico et al, 1993; Gaetani et al, 1997). Steele et al. (1991) and Shishido et al. (1994) have reported that cerebral vasospasm after SAH was attenuated by local administration of SOD, suggesting the pivotal role of oxygen radicals in the development of cerebral vasospasm.

Among various sources for ROS such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, nitric oxide (NO) synthase, and mitochondrial electron transport, NAD(P)H

oxidase has been considered to be the major source of ROS in the vasculature (Zalba et al, 2000) and been implicated in numerous cellular processes and vascular diseases (Griendling et al, 2000). Recently, Wang et al. (1998) demonstrated NADH/NADPH oxidase localized in the adventitia as the primary site for superoxide production. Furthermore, our recent studies (Choi et al, 2001; Kim et al, 2002) clearly showed that periarterial blood application to the vasculature significantly augmented the NADH/NADPH-dependent production of superoxide. Our present experimental results that DPI prevented delayed cerebral vasospasm together with the previous reports suggest that NAD(P)H oxidase-derived superoxide is involved in the experimental vasospasm induced by SAH.

Although SOD has been reported to be effective in preventing development of cerebral vasospasm, native SOD in rat plasma has a short half-life with less than 6 minutes (Odlind et al, 1988). Furthermore, the entry of exogenous SOD into the brain is normally very limited by the blood brain barrier (BBB), although the amount of SOD that enters the brain after moderate brain damage is increased several fold due to altered vascular permeability (Yoshida et al, 1992). Therefore, it is not easy to maintain therapeutic concentration of SOD in the targeted tissues after the bolus injection.

Adenoviral vectors have widely been used to efficiently transfer and express recombinant genes in different vasculatures both in *ex vivo* and *in vivo* experiments, thereby raising the possibility of their use to treat vascular disorders (Schneider & French, 1993; Nabel & Nabel, 1994). Recently, it has been demonstrated that intracisternal administration of adenoviral vector encoding -galactosidase effectively transfers recombinant genes to the cerebral vasculatures of rat, mice and rabbit (Ooboshi et al, 1995; Christenson et al, 1998; Toyoda et al, 2000). In the present study, the expression of Cu/ZnSOD was demonstrated in the adventitial cells of pail artery one day after intracisternal administration of recombinant adenovirus encoding human Cu/ZnSOD and maximized after 3 days.

Considering the fact that an NAD(P)H oxidase in the adventitial cells is a major source of superoxide in the vascular cells (Griendling et al, 2000), periadventitial transfer of Cu/ZnSOD gene is expected to prevent oxygen radical-mediated alterations of vascular function. Our results showed that recombinant adenovirus-mediated transfer to the cerebral vasculature of Cu/ZnSOD cDNA prevented alterations of cerebral vasculatures after SAH, resulting suggest of alterations in cerebral vasculature by SAH were most likely due to the production of an NAD(P)H oxidase-derived ROS. Therefore, recombinant adenovirus-mediated transfer of Cu/ZnSOD cDNA to cerebral vasculature might have therapeutic potential to minimize the oxidative injury caused by ROS until the endogenous free radical scavenger system recovers. This approach will hopefully contribute dramatically to reducing the mortality and to improving outcome following traumatic brain injury.

In summary, SAH-induced NAD(P)H oxidase-derived ROS appears to be, at least, one mechanism underlying the SAH-induced cerebral vasospasm, and such phenomena can be prevented by recombinant adenovirus-mediated transfer of Cu/ZnSOD gene to the cerebral vasculature.

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