ALTERATIONS IN CEREBRAL BLOOD FLOW (CBF) after traumatic brain injury may be one factor that affects the pathophysiology and neurological outcomes associated with brain trauma. Reduction of CBF after brain injury is well documented in rats (17, 33). Fluid percussion-induced brain injury (FPI), as a model of human concussive trauma, has been reported (1, 3) to cause considerable alterations in neurohumoral control of the cerebral circulation, including impairment of K⁺ channel function with reduced vasorelaxation. Several studies have revealed that oxygen radical-mediated cerebrovascular abnormalities occur in the first few hours after brain trauma (8, 16), which is associated with a reduction of CBF (31, 33). We (14) recently demonstrated an altered autoregulatory vasodilation in response to acute hypotension in association with reduced vasodilation in response to calcitonin gene-related peptide (CGRP) and levcromakalim after FPI. In our previous experiment, we (14) demonstrated a mechanistic result that FPI-induced activation of tyrosine kinase links the inhibition of K⁺ channels to impaired CBF autoregulation in rat pial artery. On the other hand, FPI has been observed to be associated with the generation of superoxide on the cerebral cortex, which is at least one mechanism contributing to altered reactivity of cerebral arterioles (2, 3, 10, 16). In addition, it was revealed that transgenic mice overexpressing copper-zinc (Cu/Zn) superoxide dismutase (SOD) showed significantly attenuated neurological deficits following traumatic brain injury (5), suggesting that oxidative stress plays a deleterious role. Reduced NADP (NADPH) oxidase has recently been demonstrated in adventitial smooth muscle cells (4, 20) and is considered a major source of superoxide in vascular cells (12). However, its participation in FPI-induced CBF autoregulatory dysfunction is unknown.

Adenoviral vectors have been used to achieve efficient transfer and expression of recombinant genes in different vasculatures in both ex vivo and in vivo experiments, raising the possibility of this approach to treat vascular disorders (21, 27). Recent results (29, 30) have described the perivascular adenoviral transfection of reporter gene encoding β-galactosidase to rabbit, mice, and rat cerebral vasculatures.

On the basis of these results, it is hypothesized that acute elevation of intracellular superoxide anion after brain injury results in activation of tyrosine kinase-linked inhibition of K⁺ channels and impairment of autoregulatory vasodilation in response to acute hypotension and to K⁺ channel openers in the rat pial artery. Thus the present study was designed to test the
involvement of NAD(P)H oxidase-derived superoxide anion in the development of the functional impairment of vasodilatory responses. To address this hypothesis, we employed diphenyleneiodonium (DPI), an NAD(P)H oxidase inhibitor, and the recombinant adenovirus encoding human Cu/Zn SOD. We thereby proved their effectiveness in the recovery of the pial arteries to vasodilate in response to CGRP and levcromakalim and acute hypotension in association with restoration of CBF autoregulation in the rats subjected to FPI.

MATERIALS AND METHODS

Preparation of animals. The animal experimentation committee of the College of Medicine, Pusan National University, approved the experimental design of this study and the guiding principles for research. Male Sprague-Dawley rats (250–300 g) were anesthetized with urethane (1 g/kg ip) and placed on a heating pad to maintain a constant rectal temperature (37 ± 0.5°C). After a tracheostomy was performed, the animal was mechanically ventilated with room air with the use of a respirator (model 683, Harvard Apparatus; South Natick, MA). Catheters were placed in carotid and femoral arteries for measurement of blood pressure and for withdrawing and sampling of the arterial blood, respectively. The blood was collected before and after installation of a cranial window for blood gas and pH analysis (STAT Profile 3, Nova Biomedicals; Boston, MA). FPI was induced as previously described (14). In brief, a 4.5-kg pendulum was used to strike a piston that was connected to the female Leuer-Loc fitting, which had been implanted over the exposed dura of the rat cerebral cortex. The intensity of the blow was adjusted to 2.0–2.5 atm.

Measurement of vessel diameter and CBF. The details for the measurements of pial arterial diameter and CBF have been published previously (14). Briefly, after the closed cranial window was installed over the contralateral side of parietal cortex to the side of injury, the image of pial arterioles was visualized through the cranial window and captured with a charge-coupled device videocamera (model VDC 3900, Sanyo) through a stereomicroscope (model SMZ-2T, Nikon). The caliber was measured with the use of a width analyzer (model C3161, Hamamatsu Photonics; Hamamatsu, Japan) after the image was fed to a television monitor.

CBF to the pial artery was measured with the use of laser-Doppler flowmetry (Laserflo BPM2, Vasamedics; St. Paul, MN) equipped with a 1-mm-diameter needle probe (model P-433-5, Vasamedics). After the dura mater was carefully sectioned, the probe was placed near and lateral to the pial artery in the open window and advanced into the cerebrospinal fluid (CSF) ~0.2 mm above the cortical surface. The changes in CBF were monitored during controlled hypotension. Stepwise hypotension was achieved by bleeding of the arterial blood into the reservoir. Blood (1 ml) was withdrawn every 2 min while the systemic arterial blood pressure was being monitored until it lowered to ~50%. In the case of infusion, the reservoir blood was infused back 1 ml every 2 min. The laser-Doppler flowmetry outputs were regarded as arbitrary units and the changes in CBF were expressed as a percentage of the baseline CBF.

Three hours after FPI, the vasodilatory responses of the pial artery to vasodilators (0.001–0.1 µmol/l CGRP and 0.1–10 µmol/l levcromakalim) and to stepwise hypotension were determined. The changes in the pial arterial blood flow in response to stepwise hypotension and to its reverse of blood pressure were also measured. Thereafter, we reexamined the vasodilatory and autoregulatory responses in rats injected with adenovirus vector-encoding human Cu/Zn SOD (AdCMV-SOD) or treated with DPI (1 µmol/l). In the present experiment, AdCMV-SOD was administered via cisterna magna 3 days before FPI, and DPI was applied 30 min before application of vasodilators and hypotensive procedure. Alterations in pial arterial diameter and blood pressure were expressed as percent changes in the baseline diameter and mean arterial blood pressure, respectively. The intracranial pressure was maintained constant at 5–6 mmHg throughout the experiment by adjustment of the height of the free end of plastic tube connected to the outlet of the window. Only one arteriole was observed under the window in each rat.

Measurement of NAD(P)H oxidase activity. In a separate experimental system, cerebral vasculature including basilar artery, cerebral arteries (anterior, middle, and posterior cerebral arteries), and pial arterioles was isolated and homogenized in 50 mmol/l phosphate buffer, and NAD(P)H-induced production of superoxide in the vascular homogenates was measured as an index for NAD(P)H oxidase activity as described previously (24). Namely, the vascular homogenates were placed in 200 µl of HEPES buffer containing lucigenin (5 µmol/l) and placed into a luminometer (Miorolun LB96P, Berthold). After dark adaptation, both NADH (100 µmol/l) and NADPH (100 µmol/l) were added to the vial. Counts were recorded every 30 s for 15 min, and the respective background counts were subtracted. Chemiluminescence was expressed as counts per second per milligram of protein. In some experiments, inhibitors were added to the samples 10 min before readings.

Preparation of adenoviral vector and in vivo gene transfer. Replication-deficient recombinant adenoviral vector (serotype 5, produced in HEK-293 cells) driven by the cytomegalovirus immediate early promoter was used to transfer the gene to the cerebral vasculature. AdCMV-SOD and AdCMV Escherichia coli β-galactosidase (AdCMVLacZ) genes were obtained from Dr. John Engelhardt (Gene Therapy Core Center, University of Iowa). The DNA constructs of replication-deficient adenovirus comprise almost a full-length copy of the adenoviral genome, in which a Cu/Zn SOD and LacZ expression cassette is incorporated at the site of E1 region deletions. For each vector, high-titer adenoviral vector stocks were prepared by double-cesium gradient purification, and viral titer [plate-forming units (pfu/ml)] was determined by standard methods.

To transfer the gene to the cerebral vasculature in vivo, rats were anesthetized with thiopental sodium (50 mg/kg ip) and a 27-gauge needle was aseptically inserted into the cisterna magna as previously described (23). An equal volume (100 µl) of CSF was removed before injection of viral suspension (100 µl of 1 × 10⁹ pfu/ml) to avoid an increase in intracranial pressure.

Immunohistochemistry for Cu/Zn SOD. For immunohistochemical analysis for Cu/Zn SOD, serial 5-µm-thick frozen sections of the pial artery were adhered to poly-L-lysine-coated slides, allowed to dry in room air, and fixed in acetone. After treatment with H₂O₂ (0.6%) and bovine serum albumin (2%), the preparations were incubated in the goat anti-rat Cu/Zn SOD and LacZ expression cassette is incorporated at the site of E1 region deletions. For each vector, high-titer adenoviral vector stocks were prepared by double-cesium gradient purification, and viral titer [plate-forming units (pfu/ml)] was determined by standard methods.

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line (purple) and examined for positive staining of Cu/Zn SOD (gray-black) by light microscopy. Immunoreactivity for Cu/Zn SOD was quantitated by ImagePro Plus Imaging software (Media Cybernetics; Silver Spring, MD) and the stain density was expressed as pixels per squared micrometer of tissue.

Drugs. CGRP and DPI were purchased from Sigma (St. Louis, MO). CGRP was dissolved in 0.1% bovine serum albumin to make a stock solution of 0.01 mmol/l. Levromakalim, a K⁺ channel opener, was generously donated by the Korea Research Institute of Chemical Technology (Daejon, Korea) and dissolved in ethanol-polyethylene glycol (50:50% vol/vol) to make a stock solution of 1 mmol/l.

Statistical analysis. Results were expressed as means ± SE. Student’s t-test was used for analyzing values between the data of vehicle- and inhibitor-treated groups [concentration giving 25% maximal response (EC25) and lower limit of concentration ranges used in this study caused little change in systemic arterial blood pressure. Vasodilation induced by either CGRP or levromakalim was markedly blunted after FPI with significantly increased EC25 values of 0.113 ± 0.024 μmol/l (P < 0.001) and 41.35 ± 13.23 μmol/l (P < 0.05), respectively. In turn, these blunted vasodilations to vasodilators were largely restored under suffusion with artificial CSF containing 1 μmol/l DPI, with significantly decreased EC25 values of 0.004 ± 0.002 μmol/l (P < 0.01) and 0.85 ± 0.24 μmol/l (P < 0.05), respectively (Fig. 2). The pial arterial diameter was little changed under suffusion with artificial CSF containing 1 μmol/l DPI.

AdCMVSOD-mediated transgene expression on the pial artery. In the pial artery from the vehicle-treated group, positive staining for Cu/Zn SOD was observed in the endothelium but not in the media or adventitia. Positive staining for Cu/Zn SOD was observed in both endothelial and adventitial cells in vessels from the AdCMVSOD-treated group (Fig. 3). The amount of Cu/Zn SOD expression in the endothelium in AdCMVSOD-treated group was not different from those in vehicle-treated group. Transgene expression in the adventitial cells was observed 1 day after and maximized 3 days after intracisternal administration of AdCMVSOD (100 μl of 1 × 10¹⁰ pfu/ml).

Effect of AdCMVSOD-mediated gene transfer on vascular reactivity. In rats treated with AdCMVSOD (100 μl of 1 × 10¹⁰ pfu/ml, intracisternal administration),

### Table 1. Physiological variables: MABP, blood gas, and pH analysis before and after FPI

<table>
<thead>
<tr>
<th></th>
<th>Before FPI</th>
<th>After FPI</th>
<th>AdCMVSOD + FPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>25</td>
<td>43</td>
<td>8</td>
</tr>
<tr>
<td>MABP, mmHg</td>
<td>118.5 ± 9.3</td>
<td>110.4 ± 6.8</td>
<td>103.4 ± 9.1</td>
</tr>
<tr>
<td>P₂O₂, mmHg</td>
<td>32.5 ± 5.7</td>
<td>37.2 ± 6.2</td>
<td>35.5 ± 6.7</td>
</tr>
<tr>
<td>P₂O₂, mmHg</td>
<td>103.9 ± 10.4</td>
<td>101.0 ± 4.3</td>
<td>96.8 ± 7.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.39 ± 0.05</td>
<td>7.42 ± 0.04</td>
<td>7.38 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. FPI, fluid percussion injury; MABP, mean arterial blood pressure; AdCMVSOD, adenovirus encoding human copper-zinc superoxide dismutase.
the FPI-induced blunted vasodilation to CGRP or levcromakalim was significantly restored with decreased EC25 values of 0.011 ± 0.003 μmol/l (P < 0.01) and 1.81 ± 0.28 μmol/l (P < 0.05), respectively (Fig. 4).

Effect of AdCMVSOD-mediated gene transfer on autoregulatory vasodilation. Changes in pial arterial diameter in response to a stepwise hypotension were plotted as a function of changes in systemic mean arterial blood pressure, and mean slope was calculated from linear regression. Three hours after FPI, the pial artery showed reduced vasodilation to lowering of blood pressure, as evidenced by reduced slopes of regression lines (slope for vasodilation, −1.99 ± 0.27 in control to −0.43 ± 0.14, P < 0.001, and that for vasoconstriction, from −1.60 ± 0.17 to −0.58 ± 0.14, P < 0.001; n = 6). Markedly blunted autoregulatory vasodilation to hypotension after FPI was largely recovered after pretreatment with 1 μmol/l DPI (slope for vasodilation to −1.25 ± 0.19, P < 0.01, and that for vasoconstriction to −0.93 ± 0.13; n = 5). Application of AdCMVSOD (100 μl of 1 × 10^10 pfu/ml) 3 days before FPI prevented reduction in the autoregulatory vasodilation of the pial microvessels to hypotension with the restored slopes for vasodilation to −1.35 ± 0.19 (P < 0.01; n = 5) and for vasoconstriction to −1.11 ± 0.13 (P < 0.05; n = 5) (Fig. 5). Suffusion with artificial CSF containing DPI (<1 μmol/l) showed little effect on the pial arterial diameter.

Effect of AdCMVSOD-mediated gene transfer on lower limit of CBF autoregulation. Mean arterial blood pressure was 118.5 ± 9.3 mmHg under resting conditions. CBF to the pial arteries was well preserved...
despite a decrease in mean arterial blood pressure up to 50–60 mmHg. When blood pressure levels decreased further, CBF fell steeply depending on the fall in blood pressure thereafter. The lower limit of autoregulation, defined as the blood pressure where CBF decreased by 10% of the baseline, was 55.7 ± 3.5 mmHg in the control group, which was shifted to 81.9 ± 3.6 mmHg after FPI (P < 0.001). After application of AdCMVLacZ, the lower limit of CBF autoregulation was little affected. However, after pretreatment with AdCMVSOD (100 μl of 1 × 10^10 pfu/ml) 3 days before FPI, the lower limit of CBF autoregulation was significantly restored to 64.6 ± 1.6 mmHg (P < 0.01), comparable to the level with DPI treatment (60.3 ± 2.1 mmHg, P < 0.001) (Fig. 6).

**DISCUSSION**

The major findings of this study were the following: 1) FPI caused a significant increase in the activity of NAD(P)H oxidase in the cerebral vasculature, 2) vasodilations of the pial artery to CGRP and levromakalim were significantly suppressed in association with impairment of autoregulatory vasodilation in response to acute hypotension, and 3) these impaired vasodilations in FPI rats were significantly restored with the recovery of lower limit of CBF autoregulation, after pretreatment with recombinant AdCMVSOD and DPI, an NAD(P)H oxidase inhibitor.

Cerebral autoregulation may be altered in systemic diseases, including hypertension, diabetes mellitus, and some disorders of central nervous system. Maintenance of CBF has been considered to be very important from a therapeutic point of view, according to a report...
by Heiss et al. (13) that the severity of neurotic symptoms in the chronic stage of cerebral infarction correlated with CBF levels. On the other hand, FPI causes a partial or complete impairment of the capacity of cerebral vessels to autoregulate CBF (9, 11). The magnitude of vasodilatory reserves available for autoregulation is significantly decreased in FPI, and the brain may be more vulnerable to the effects of secondary insults, such as hypotension (6). In line with these facts, the morbidity and mortality of severe head trauma increase when hypotension accompanies the brain injury (26). Consistent with the results of Prat et al. (25), in that loss of cerebral autoregulation occurred during the first 4 h after injury, our (14) previous data clearly showed that cerebral autoregulatory dysfunction was maximized 3 h after brain injury. Considering these results, immediate therapeutic approaches to preserve the autoregulatory vasodilation (CBF autoregulation) in response to hypotension appear to be very important in reducing the morbidity and mortality of patients after brain trauma.

Although the mechanism(s) contributing to posttraumatic changes in CBF autoregulation is still unknown, there is evidence that oxygen free radicals play a role in brain injury. Brain injury in cats has been reported (16) to enhance superoxide production, and sustained dilation and abnormal responsiveness of the pial arterioles were observed after injury. Furthermore, in transgenic mice overexpressing human Cu/Zn SOD, significant attenuation of acute injuries (i.e., brain edema and increased blood-brain barrier permeability) and chronic neurological deficits were demonstrated (5). Thus it was concluded that FPI-induced superoxide generation on the cerebral cortical surface might contribute to impaired reactivity of cerebral arterioles (10, 15). Consistent with these earlier reports, our results clearly showed that rats exposed to FPI exhibited enhanced NAD(P)H oxidase activity in the cerebral vasculature. Furthermore, both FPI-induced activation of vascular NAD(P)H oxidase and reduced vasodilatory hemodynamics to CGRP and levromakalim and to acute hypotension were significantly suppressed by treatment with DPI, an NAD(P)H oxidase inhibitor. These results suggest the role of NADPH oxidase-derived oxygen free radicals in the FPI-induced CBF autoregulatory dysfunction. However, it remains unclear about the involvement of other mechanisms, including endothelin-1, prostanoids, opiates, and other excitatory neurotransmitters.

Ellison et al. (8) and Muir et al. (18) have reported that SOD could restore the posttraumatic cortical blood flow in rats, suggestive of the role of oxygen radicals in cerebrovascular abnormality in brain trauma. According to the results of Spranger et al. (28), SOD activity was significantly lower in patients with ischemic stroke and the endogenous antioxidants were depleted as a consequence of an excessive production of oxygen free radicals. SOD has also been demonstrated to improve outcome after traumatic brain injury in phase II clinical trials (19). Although SOD has been reported (22) to be effective in preventing alterations in CBF after brain trauma, native SOD has a plasma half-life of ~6 min in rats. Furthermore, the entry of exogenous SOD into the brain is normally very limited by the blood-brain barrier, although the amount of SOD that enters the brain after moderate FPI is increased severalfold due to the altered vascular permeability (32). Therefore, it is not easy to maintain therapeutic concentration of SOD in the targeted tissues after the bolus injection.

Adenoviral vectors have been widely used to achieve efficient transfer and expression of recombinant genes in different vasculatures both in ex vivo and in vivo experiments, thereby raising the possibility of their use to treat vascular disorders (21, 27). It has recently been demonstrated (7, 23, 29) that intracisternal administration of AdCMVlacZ effectively transfers recombinant genes to the cerebral vasculatures of rat, mice, and rabbit. In the present study, after intracisternal administration of recombinant AdCMV-SOD, expression of Cu/Zn SOD was demonstrated 1 day later and maximized after 3 days in the adventitial cells of pial artery.

Considering the fact that an NAD(P)H oxidase in adventitial cells is a major source of superoxide in the vascular cells (12), periadventitial transfer of the Cu/Zn SOD gene is expected to prevent oxygen radical-mediated alterations in vascular function. In our results, recombinant adenovirus-mediated transfer to the cerebral vasculature of Cu/Zn SOD cDNA has prevented alterations in CBF autoregulation with restoration of vasodilation to vasodilators. These results suggest that alterations in CBF autoregulation and vascular reactivity to vasodilators were ascribed to the production of an NAD(P)H oxidase-derived superoxide anion. Furthermore, recombinant adenovirus-mediated transfer of Cu/Zn SOD cDNA to cerebral vasculature may have the therapeutic potential to minimize the oxidative injury caused by oxygen free radicals until the endogenous free radical scavenger system recovers. This approach may contribute dramatically to mortality reduction and to improved outcome following traumatic brain injury.

In summary, a FPI-induced NAD(P)H oxidase-derived superoxide anion is at least one mechanism underlying the FPI-induced cerebral autoregulatory dysfunction, and such impaired function of the pial artery can be prevented by recombinant adenovirus-mediated transfer of the Cu/Zn SOD gene to the cerebral vasculature.

We thank Dr. John Engelhardt, Gene Therapy Core Center, University of Iowa, for the generous donation of adenoviral vectors containing transgenes.

This study was supported in part by grants from the Korea Science and Engineering Foundation (to C.D. Kim and K. W. Hong), Korea Research Foundation, Ministry of Health and Welfare, and Research Institute of Genetic Engineering (all to K. W. Hong).

REFERENCES


