Protective effects of cold spinoplegia with fasudil against ischemic spinal cord injury in rabbits

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Objective: Paraplegia remains a serious complication after surgical repair of thoracoabdominal aortic aneurysms. The aim of this study was to evaluate the neuroprotective efficacy of fasudil, a Rho kinase (ROCK) inhibitor, by reducing the number of infiltrating cells in the ventral horn and increasing the induction of eNOS against ischemic spinal cord injury in rabbits.

Methods: Eighteen Japanese white rabbits were divided into three groups: saline (group 1, n = 7, 4°C) and fasudil (group 2, n = 6, 4°C) were immediately infused into the isolated segmental lumbar arteries over 30 seconds after aortic clamping. Group 3 (n = 5) was the sham-operated group. Hind limb function was evaluated 4 and 8 hours, and 1 and 2 days after 15 minutes of transient ischemia. Cell damage was analyzed by hematoxylin and eosin staining and temporal profiles of endothelial nitric oxide synthase immunoreactivity were performed. The number of intact motor neuron cells and infiltrating cells in the ventral horn were compared.

Results: Two days after reperfusion, group 2 and group 3 showed better neurologic function, a greater number of intact motor neuron cells, and a smaller number of infiltrating cells in the ventral horn than group 1. The induction of endothelial nitric oxide synthase (eNOS) was prolonged up to 2 days after reperfusion in group 2.

Conclusion: These results indicate that fasudil has neuroprotective effects against ischemic spinal cord injury in rabbits by reducing the number of infiltrating cells in the ventral horn and prolonging the expression of eNOS. (J Vasc Surg 2010; 51:445-52.)

Clinical Relevance: Paraplegia or paralysis caused by spinal cord ischemia remains a devastating and unpredictable complication after descending and thoracoabdominal aortic surgery. This study has revealed that fasudil has a neuroprotective effect against ischemic spinal cord injury in rabbits. Inhibition of the Rho/Rho kinase pathway by fasudil reduces the number of infiltrating cells in the ventral horn and prolongs the expression of eNOS. In the near future, Rho kinase may be an important therapeutic target for paraplegia induced by spinal cord ischemia.

Paraplegia or paralysis caused by spinal cord ischemia remains a devastating and unpredictable complication after descending and thoracoabdominal aortic surgery. The reported incidence of paraplegia ranges from 2.9% to 23% for operations on the thoracic aorta. Despite numerous surgical techniques, including systemic deep hypothermia, distal aortic perfusion, cerebrospinal fluid drainage, intercostals artery implantation, use of motor sensory or somatosensory-evoked potentials, and pharmacologic interventions, the complication still cannot be prevented completely.

The mechanism of acute spinal cord dysfunction is believed to result from its ischemic damage during cross-clamping. Ischemia can occur when there is permanent exclusion of the essential intercostal arterial blood supply to the spinal cord or temporary interruption of the spinal cord blood flow. To investigate the mechanisms of the vulnerability of spinal motor neurons to ischemia, we created a rabbit model of spinal cord ischemia in which we statistically analyzed cell damage.

Rho kinase (ROCK), a target protein of the small guanosine triphosphate (GTP)-bound form of Rho, is a serine/threonine kinase. Previous studies suggested that ROCK or increased ROCK activity is involved in the pathogenesis of various vascular lesions such as smooth muscle contraction, cell (neutrophil and monocyte) migration, and endothelial injury through the downregulation of endothelial nitric oxide synthase (eNOS) activity and production of O²⁻ in neutrophils and vessels. Therefore, it has been suggested that ROCK plays an important role in pathologic conditions, including cerebral and coronary vasospasm, hypertension, vascular inflammation and remodeling and arteriosclerosis.

Nitric oxide (NO), constitutively produced by eNOS, regulates cerebral blood flow and vascular tone, and protects against ischemic stroke by increasing collateral flow to the ischemic area. Indeed, eNOS-deficient mice develop larger cerebral infarctions after middle cerebral arterial occlusion. In contrast, augmentation of endothelial NO production has been shown to decrease the cerebral infarct area probably by enhancing collateral blood flow. Thus, enhanced eNOS activity could have beneficial effects on cerebrovascular disease. ROCK inhibitors reverse the
downregulation of eNOS under hypoxic conditions.\(^\text{11}\) However, the physiologic relevance of eNOS regulation by ROCK is unknown.

Fasudil is a ROCK inhibitor (RKI) that has shown clinical efficacy on cerebral vasospasm in patients after clipping surgery for aneurysmal subarachnoid hemorrhage.\(^\text{12}\) Recently, it was reported that fasudil improved neurologic functions significantly reduced the size of the infarct area and prevented the accumulation of neutrophils in rat and mouse models of microembolization stroke.\(^\text{10,13}\) In animal models, fasudil and hydroxyfasudil had efficacy in the treatment of acute ischemic stroke.\(^\text{13}\) However, the possibility that fasudil may have a neuroprotective effect against spinal cord injury in ischemia has not yet been examined. The aim of this study, therefore, was to investigate the neuroprotective effect of fasudil by reducing the number of infiltrating cells in the ventral horn and increasing the induction of eNOS against ischemic spinal cord injury in rabbits.

MATERIALS AND METHODS

Animal care. This experiment was reviewed by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Kyushu University, and was carried out under the “Guidelines for Animal Experiments” of the Faculty of Medicine, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Animal models. Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing from 2.5 to 3.0 kg were used for this study. The rabbits were anesthetized with intramuscular administration of ketamine at a dose of 50 mg/kg and maintained with 2% halothane inhalation. A continuous intravenous infusion line was secured at the marginal ear vein with another 24-gauge catheter. An arterial line was secured in the car artery with a 24-gauge catheter to monitor the proximal arterial pressure. The rectal temperature was kept at 38°C by using a heating lamp. A median laparotomy was performed, and the abdominal aorta just distal to the left renal artery was exposed from just inferior to the left renal artery to above the bifurcation. After systemic heparinization (200 U/kg), a 5F catheter was introduced into the central portion of the abdominal aorta to above the bifurcation. To measure the temperature of the spinal cord, a needle-tip thermistor (PTN-201; Unique Medical Co Ltd, Tokyo, Japan) was inserted into the central portion of the spinal cord (L4) with puncture of the dura.\(^\text{15}\) The spinal cord temperature and the rectal temperature were simultaneously recorded. The temperature was recorded from just before cross-clamping to 15 minutes after declamping. The postoperative neurologic assessment was not performed for these 10 rabbits because the traumatic damage to the spinal cord resulting from the insertion of a needle tip thermistor would have affected the neurologic outcome in the hind limbs.

Neurologic assessment. Neurologic function was assessed at 4 and 8 hours, and 1 and 2 days after reperfusion. Two investigators without knowledge of the treatment independently graded the neurologic function. The motor function of the hind limbs was graded by using the modified Tarlov scale (5 = normal hop, 4 = weak hop, 3 = sits alone, 2 = sits with assistance, 1 = slight movement, 0 = no movement).

Histopathology and the number of infiltrating cells in the ventral horn. All animals were killed after the completion of the neurologic evaluation at 2 days after reperfusion. Spinal cords between L2 and L3 were quickly harvested. Sectioned specimens from the lumbar spinal cord at the level of L2 or L3 were then fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. The sections were cut into 5-μm-thick slices and stained with hematoxylin and eosin and examined by light microscopy to evaluate the number of normal motor neurons and infiltrating cells in the ventral horn (group 1: n = 7, group 2: n = 6, group 3: n = 5). The number of intact large motor neuron cells in the ventral gray matter region was counted by an observer who was unaware of animal group or neurologic outcome examined each slide. In cases with pyknotic nuclei, eosinophilic cytoplasm, or (absent nuclear hematoxylin staining) large motor neuron cells were considered “necrotic or dead”. When the cells demonstrated basophilic striping (containing Nissl substance), motor neuron cells were considered “viable or alive”. The number of infiltrating cells in the ventral horn of the spinal cord was counted for each slide as the sum of the cell count on five fields at ×400 magnification.\(^\text{16}\)

Immunohistochemistry. We also performed an immunohistochemical study to investigate changes in the expression of eNOS at 2 days after reperfusion in groups 1,
2, and 3 (n = 3 each). A mouse monoclonal anti-eNOS antibody (SA-258, 1:200 dilutions; BIOMOL International LP, Exeter, UK) was used for the immunohistochemistry. Spinal cord sections were rinsed in 0.1 M phosphate-buffered saline (PBS) for 20 minutes, and blocked in 2% normal horse serum for 2 hours at room temperature. Then they were incubated with primary antibodies in 10% normal horse serum or 10% normal rabbit serum containing 0.018% normal horse and rabbit serum. Subsequently they were incubated with avidin-biotin-horseradish peroxidase complex (PK-6102; Vector Laboratories). Sections were colored with DAB/H2O2 solution, and the peroxidase complex was counterstained with hematoxylin. To determine specific binding of antibody for the protein, a set of sections were stained in a similar way without the primary antibody. Staining was categorized in four grades in the following manner: no staining (−), slight staining (+), moderate staining (++), or dense staining (+++).

**Statistical analysis.** All results were presented as mean ± standard deviation. Statistical analyses of the neurologic score and the cell number were performed by using one-way analysis of variance and Bonferroni’s multiple comparison test to adequately identify which group differences resulted in a significant P-value (<.05). In regard to cooling efficiency of the spinal cord, the random-coefficient growth curve model was applied for the analysis of repeated measurements.

**RESULTS**

**Physiologic status.** No significant differences were noted in the rectal temperature or the mean arterial pressure of the proximal and the distal aorta among groups during operation (P > .05; Table 1).

**Cooling efficiency.** In both groups, the spinal cord temperature decreased rapidly after saline or saline plus fasudil (0.1 mg/kg) were infused into the isolated aortic segment. In group 1, the spinal cord temperature decreased by a maximum 2.1 ± 0.51°C. In group 2, the spinal cord temperature decreased by a maximum 2.5 ± 0.75°C. There was no significant difference in the spinal cord temperature between the two groups at each time point.

**Neurologic outcome.** The individual neurologic scores of the three groups at 4 and 8 hours, and 1 and 2 days after reperfusion are shown in Fig 1. The animals in the sham-operated group showed no neurologic deficits, and their neurologic scores were 5.0 ± 0.2 when observed 4 and 8 hours, and 1 and 2 days after reperfusion (Fig 1). The Tarlov scores were 3.9 ± 0.4 and 4.2 ± 0.4 at 4 hours; 3.9 ± 0.4 and 4.3 ± 0.5 at 8 hours; 2.6 ± 1.8 and 4.5 ± 0.8 at 1 day; and 1.7 ± 1.9 and 4.3 ± 1.2 at 2 days after reperfusion for groups 1 and 2, respectively. The score was significantly higher in group 2 than group 1 at 1 and 2 days (P = .022 and P = .014, respectively). However the scores at 4 and 8 hours were not significantly different between groups 1 and 2. The scores at all time points after surgery were significantly higher in group 3 than group 1 (P < .001, P < .001, and P = .003, respectively). Moreover, the score was significantly higher in group 3 than in group 2 at 4 hours (P = .03).

**Histopathology and the number of infiltrating cells in the ventral horn.** The histologic appearance of lumbar spinal cord stained with hematoxylin-eosin is shown in Fig 2, A, and the results of counting viable motor neurons are summarized in Fig 2, B. In group 1, about half of motor neurons in the ventral horn were damaged, and the number of normal motor neurons was significantly smaller than in group 2 and group 3 at 2 days after reperfusion (P = .014 and P = .008, respectively). The number of infiltrating cells in the ventral horn in group 1 was significantly greater than group 2 and group 3 at 2 days after reperfusion (113.7 ± 10.0 cells vs 79.5 ± 10.5 cells and 85 ± 7.0 cells in 5 fields; P < .001 and P < .001, respectively, Fig 3). However the number of infiltrating cells in the ventral horn at 2 days after reperfusion was not significantly different between groups 2 and 3 (Fig 3).

**Immunohistochemical study.** Table II shows a summary of the immunohistochemical analysis for each group. The motor neurons of sham controls and group 1 were slightly positive for eNOS (Fig 4, A, B). The eNOS immunoreactivity was strongly induced in the motor neurons of group 2 at 2 days after reperfusion (Fig 4, C).

**DISCUSSION**

In the present study, we demonstrated that fasudil in cold saline, which was infused into the isolated aortic segment, can reduce the neurologic damage of spinal cord ischemia-reperfusion injury in rabbits. The neuroprotective effect of fasudil was induced by reducing the number of infiltrating cells in the ventral horn and increasing the induction of eNOS. We previously reported that an RKI...
improved cardiac function after 24-hour heart preservation. The present study provides evidence for the first time that fasudil reduces neurologic injury in a rabbit model of spinal cord ischemia.

Animal models. Isaka et al\(^\text{15}\) reported that the groups which were clamped, only the abdominal aorta just distal to the left renal artery for 12 and 15 minutes were all paraplegia. In the present study, we used this animal model to investigate the neuroprotective effect of fasudil as spinoplegia infused into an isolated aortic segment after cross-clamping.

The role of ROCK and the relationship between RKI and eNOS. A recent study demonstrated the substantial involvement of intracellular signaling pathways mediated by small GTP-binding proteins (G proteins), such as Rho, Ras, Rab, Sarl/Arf, and Ran families.\(^\text{18}\) The effector domains of RhoA, RhoB, and RhoC (collectively referred to here as Rho) have the same amino acid sequence, and these G proteins seem to have similar intracellular targets.\(^\text{18}\) Rho is known to modulate Ca\(^{2+}\) sensitization of vascular smooth muscle cells and is thought to act by inhibiting myosin phosphatase activity.\(^\text{18}\)

ROCK, a target protein of the small GTP-bound form of Rho, is a serine/threonine kinase that is activated when bound to the active GTP-bound form of Rho. Previous studies suggested that ROCK or increased ROCK activity is involved in the pathogenesis of various vascular lesions such as smooth muscle contraction, cell (neutrophil and monocyte) migration, and endothelial injury through the down-regulation of eNOS activity and production of O\(^2-\) in neutrophils and vessels.\(^\text{3-5}\) Recently, it was demonstrated that fasudil and hydroxyfasudil, selective ROCK inhibitors, increased eNOS expression, and NO production in vitro and in vivo under ischemic conditions, and led to increased collateral blood flow, decreased cerebral infarction size, and improved neurologic deficit score after cerebral ischemia.\(^\text{10}\) In another experimental study, it was also reported that increased production of eNOS had a neuroprotective effect in the spinal cord after transient ischemia in rabbits.\(^\text{19}\)

Although we tried to examine the Western blot analysis for eNOS in the spinal cord after reperfusion, we could not detect a band of eNOS. However, in the present study, we demonstrated that the group which was treated with fasudil showed more prolonged eNOS immunoreactivity at 2 days after reperfusion and higher neurologic scores at 1 and 2 days after reperfusion than the group which was not treated with fasudil. Thus, in the present study, it is suggested that prolonged expression of eNOS by treatment with fasudil under ischemic condition improved collateral flow and microcirculation, and had a neuroprotective effect.

The relationship between RKI and inflammatory responses. Activated neutrophils have been reported to contribute to ischemia-reperfusion injury in the brain and...
Activated neutrophils may increase tissue damage through free radicals, elastase, and cytokines. A previous study demonstrated that fasudil and hydroxyl fasudil prevented the inflammatory responses in the post-ischemic brain by inhibiting neutrophil and monocyte infiltration and inhibiting the production of $\text{O}_2^{-}$ in neutrophils and vessels. Another study has demonstrated that long-term concomitant treatment with fasudil markedly suppresses angiotensin II-induced upregulation of all nicotinamide adenine dinucleotide phosphate oxidase subunits. In the present study, we could not evaluate the production of reactive oxygen species (ROS) in the spinal cord of all groups, but the number of infiltrating cells in the ventral horn was significantly reduced in the fasudil-treated group compared to the saline group. These results were compatible with the previous reports. Fasudil has a neuroprotective effect through suppression of migration of infiltrating cells.

**Hypothermic effect.** According to previous reports, hypothermia with systemic cooling or local cooling has been demonstrated to protect the spinal cord motor neurons during transient ischemia. Other studies introduced various methods of local cooling. None of these methods has been commonly used in clinical applications because of problems of complexity and risk of each method. Systemic deep hypothermia is used clinically and is effective against neuronal ischemia, but systemic deep hypothermia is associated with a high incidence of cardiac dysfunction and coagulopathy. Mild hypothermia has been shown to confer protection against histopathologic and metabolic derangements that follow ischemic injury. In the present study, mild hypothermia is maintained, and the actual temperature of the spinal cord of rabbits is 2°C to 4°C colder than the pre-ischemic temperature during ischemia. In the present study, we also demonstrated that mild hypothermia, even 2°C colder than normal, confers neuroprotective effects on the spinal cord motor neurons in the early phase after reperfusion.

**Spinoplegia.** We have previously reported that RKI improves cardiac function after 24-hour heart preservation. In that report, the most effective dose of RKI was

![Histologic findings in the spinal cord at 2 days after reperfusion](image)
Although we performed preliminary experiment of 1 mg/kg fasudil supplant into the spinoplegia, such as our previous study which did perform a dose response curve of fasudil in a cardiac model, this experiment did not improve neurologic function and produced profound systemic vasodilation and instability. Therefore, in the present study, we justified the use of a single dose of 0.1 mg/kg fasudil which is a dose to minimize changes in the systemic pressure of the rabbits. Spinoplegia of 10 mL or 20 mL infused into segmental lumbar arteries has already been reported. Thus, in the present study, spinoplegia of 5 mL/kg (from about 12.5 mL to 15 mL) was infused into segmental lumbar arteries. There was no significant difference in the amount of spinoplegia between groups 1 and 2.

Spinoplegia was first described in the report by Svensson et al. They demonstrated that segmental infusion of a single dose of cold crystalloid with lidocaine, termed “spinoplegia”, was effective in reducing the severity of ischemia during aortic cross-clamping. Previous studies demonstrated the effect of various spinoplegia against ischemic spinal cord injury. In this experiment, we established a local cooling model and investigated the reduction of ischemic damage to spinal cord motor neurons under regional mild hypothermia and infusion of fasudil. There was no significant difference in the direct temperature of the spinal cord at each time point between the two groups infused with fasudil or just saline; however, no postoperative neurologic assessment was performed.

A previous study demonstrated that a group of rabbits which was infused with 37°C saline into the isolated aortic arteries...
segment after cross-clamping showed a significantly lower neurologic score than another group which was infused with 3°C saline. In the present study, the neurologic scores of the fasudil-treated group and the saline group at 4 and 8 hours, showed comparatively high scores and these were not significantly different between the two groups. These results showed that local cooling of the spinal cord, which was established with infusion of cold reagent, has a neuroprotective effect at an early stage after reperfusion. At 2 days after reperfusion, however, the fasudil-treated group showed better neurologic function than the saline group (P = .014), a greater number of intact motor neuron cells than the saline group (P = .014), and a smaller number of infiltrating cells in the ventral horn than the saline group (P < .001). These results demonstrated that fasudil improves collateral flow and microcirculation in the spinal cord under ischemic conditions, and reduces the inflammatory cells (neutrophil and monocyte) migration, thus having a neuroprotective effect against spinal cord ischemic injury in rabbits. Fasudil is a promising additive agent in spinoplegia.

LIMITATIONS

Although we clearly demonstrated the protective effects of cold spinoplegia with fasudil against ischemic spinal cord injury in rabbits, these findings have some limitations. First, the animal model used in this study is invasive and complicated. Another control group, which was only clamped and not perfused with any agent, showed a significantly lower neurologic score at the early stage after reperfusion than other groups infused with cold agent (data not shown). Most animals of the positive control, which was only clamped and not perfused with any agent, died within 2 days after reperfusion. In the present study, we used this animal model to investigate the neuroprotective effect of fasudil as spinoplegia infused into an isolated aortic segment after cross-clamping. The use of a less invasive animal model of transient spinal cord ischemia could obtain more data about whether the neuroprotective effects of fasudil are equal when infused intravenously after or during transient spinal cord ischemia. Second, Western blot analysis could not identify the activity of eNOS, and so we only showed more prolonged eNOS immunoreactivity and a smaller number of infiltrating cells in the ventral horn of fasudil group at 2 days after reperfusion. Finally, as we could not measure the collateral flow or microcirculation in the spinal cord after reperfusion, we are unable to explain the actual mechanism of the neuroprotective effect of fasudil. Thus, further studies regarding the expression of eNOS and measurement of the collateral flow or microcirculation in the spinal cord after transient ischemia are called for.

CONCLUSIONS

The mechanisms of Rho/Rho kinase pathway-mediated spinal cord ischemic injury and infiltrating cell-mediated spinal cord ischemic injury are not fully understood. Inhibition of the Rho/Rho kinase pathway by fasudil can alleviate ischemic motor damage by reducing the number of infiltrating cells in the ventral horn and prolonging the expression of eNOS. The Rho/Rho kinase pathway, eNOS, and infiltrating cells may affect the survival and death-promoting events in spinal cord ischemic injury. This study has revealed that fasudil has a neuroprotective effect against ischemic spinal cord injury in rabbits. Inhibition of the Rho/Rho kinase pathway by fasudil reduces the number of infiltrating cells in the ventral horn and prolongs the expression of eNOS.

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