Sodium 4-phenylbutyrate protects against spinal cord ischemia by inhibition of endoplasmic reticulum stress

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Objective: Delayed paraplegia after operation on the thoracoabdominal aorta is considered to be related to vulnerability of motor neurons to ischemia. Previous studies have demonstrated the relationship between neuronal vulnerability and endoplasmic reticulum (ER) stress after transient ischemia in the spinal cord. The aim of this study was to investigate whether sodium 4-phenylbutyrate (PBA), a chemical chaperone that reduces the load of mutant or unfolded proteins retained in the ER during cellular stress, can protect against ischemic spinal cord damage.

Methods: Spinal cord ischemia was induced in rabbits by direct aortic cross-clamping (below the renal artery and above the bifurcation) for 15 minutes at normothermia. Group A (n = 6) was a sham operation control group. In group B (n = 6) and group C (n = 6), vehicle or 15 mg/kg/h of sodium 4-PBA was infused intravenously, respectively, from 30 minutes before the induction of ischemia until 30 minutes after reperfusion. Neurologic function was assessed at 8 hours, and 2 and 7 days after reperfusion with a Tarlov score. Histologic changes were studied with hematoxylin-eosin staining. Immunohistochemistry analysis for ER stress-related molecules, including caspase12 and GRP78 were examined.

Results: The mean Tarlov scores were 4.0 in every group at 8 hours, but were 4.0, 2.5, and 3.9 at 2 days; and 4.0, 0.7, and 4.0 at 7 days in groups A, B, and C, respectively. The numbers of intact motor neurons at 7 days after reperfusion were 47.4, 21.5, and 44.9 in groups A, B, and C, respectively. There was no significant difference in terms of viable neurons between groups A and C. Caspase12 and GRP78 immunoreactivities were induced in motor neurons in group B, whereas they were not observed in groups A and C.

Conclusion: Reduction in ER stress-induced spinal cord injury was achieved by the administration of 4-PBA. 4-PBA may be a strong candidate for use as a therapeutic agent in the treatment of ischemic spinal cord injury. (J Vasc Surg 2010; 52:1580-6.)

Clinical Relevance: Spinal cord injury following surgical repair of thoracic or thoracoabdominal aorta is a disastrous complication. Previous studies have demonstrated the relationship between neuronal vulnerability and endoplasmic reticulum (ER) stress after transient ischemia of spinal cord. Sodium 4-phenylbutyrate (4-PBA) is a low molecular weight fatty acid that has been approved for clinical use as an ammonia scavenger in children with urea cycle disorders and for treatment of sickle cell disease and thalassemia. A number of investigators have recently reported utilization of 4-PBA as a chemical chaperone to reverse the mislocalization and/or aggregation of proteins associated with human disease. The 4-PBA of therapeutic dose has low toxicity and uniquely penetrates well into cerebrospinal fluid. In this study, we investigated whether intravenous administration of 4-PBA is beneficial to protecting the spinal cord against ischemic damage in a rabbit model. Spinal cord ischemia was induced by direct aortic cross-clamping while 4-PBA of therapeutic dose was infused intravenously. In this simple and less invasive model, reduction in ER stress-induced spinal cord damage was achieved by intravenous 4-PBA. Our results indicate that 4-PBA of therapeutic dose can be a promising strategy, which is clinically feasible without significant adverse effects. Although further investigations are mandatory before it is applied to the clinical practice, such as optimal timing or route for administration 4-PBA, intravenous administration of 4-PBA may be a new candidate as a therapeutic agent for protecting the spinal cord against ischemic damage in thoracoabdominal aortic surgery.

Paraplegia caused by ischemic spinal cord injury is one of the most serious complications of surgery performed on the thoracic or thoracoabdominal aorta, with a reported prevalence of 3% to 23% in different series.1-5 Various adjunctive procedures for spinal cord protection have been introduced, including cerebrospinal fluid drainage, distal perfusion of the aorta, or use of steroid, barbiturate, or free-radical scavengers, and have successfully reduced the incidence of postoperative paraplegia in recent years. However, postoperative paraplegia remains a devastating complication, and its occurrence is still somewhat unpredictable. Patients undergoing thoracic aneurysm repair who awake without neurologic deficit immediately after the operation may eventually develop paraplegia.4,5 The exact mechanism of such a delayed vulnerability is not fully understood. In a rabbit spinal cord ischemia model, delayed and selective motor neuron death after transient ischemia has been reported, and it was greatly associated with activated apoptotic signals.6
There is evidence to suggest that the activation of apoptosis is initiated by cell-surface receptors or by mitochondrial stress.7 Recently, another apoptotic-regulatory pathway involved in endoplasmic reticulum (ER) stress has received attention. In the case of mild ER stress, cells develop a self-protective, signal transduction pathway termed the unfolded protein response, which includes induction of molecular chaperones in the ER such as glucose regulated protein 78 (GRP78), translational attenuation, and ER-associated protein degradation.8 However, if the damage is severe, the unfolded protein response ultimately triggers the apoptosis pathway.10 One of the important molecules relevant to cell death is caspase12 which is regarded to be a representative cell death signal in ER stress.11 Sodium 4-phenylbutyrate (4-PBA) is a low molecular weight fatty acid that has been approved for clinical use as an ammonia scavenger in children with urea cycle disorders and for treatment of sickle cell disease and thalassemia on the basis of its capacity to activate transcription of β- and γ-globin.12,13 4-PBA has been found to prolong life and contribute to therapy for spinal muscular atrophy by altering the pattern of gene expression. In the last few years, a number of studies have described the use of 4-PBA as a chemical chaperone to reverse the mislocalization and/or aggregation of proteins associated with human disease. 4-PBA can act as a chemical chaperone by reducing the load of mutant or mislocated proteins retained in the ER.14-16 Evidence obtained in recent years has demonstrated that endoplasmic reticulum (ER)-mediated cell death plays an important role in the mechanisms underlying ischemia-reperfusion neuronal damage. Therefore, targeting the ER may provide a therapeutic approach for blocking the pathologic process induced by spinal cord ischemia. However, to our knowledge, no pharmacologic approach for treating spinal cord ischemia-induced ER dysfunction has been reported. In this study, we investigated the protective effects of 4-PBA after transient spinal cord ischemia, apoptotic signaling of caspase12, and induction of GRP78.

MATERIALS AND METHODS

Animal preparation. Animal care and all procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. This study protocol was approved by the Research Facilities for Laboratory Animal Science of the Hiroshima University School of Medicine.

Anesthesia and monitoring. We used Japanese female white rabbits (2.5-3.0 kg) in this experiment. General anesthesia was induced with an intramuscular injection of ketamine 50 mg/kg. An ear vein catheter was inserted for the continuous intravenous infusion of lactated Ringer solution. Cefazolin (10 mg/kg) was injected through the catheter. After a tracheostomy and endotracheal intubation, the rabbits were ventilated mechanically with a fraction of inspired oxygen of 1.0 and isoflurane (1.5%-2.0%) to induce sufficient anesthesia. Adequacy of ventilation was monitored by blood gas analysis. The arterial blood pressure was measured by a catheter (JMS cut-down tube; C3, Hiroshima, Japan) placed into the right femoral artery. Electrocardiogram was also monitored continuously. The operation was performed at normothermia. The rectal temperature was continuously monitored and kept at 38.0 ± 0.5°C during the operation using a heating blanket.

Surgical procedures. With animals in the right lateral decubitus position, a flank skin incision parallel to the spine was made on the left side. From a retroperitoneal approach, the abdominal aorta was exposed below the left renal artery and above the iliac bifurcation, and was encircled. After the intravenous administration of heparin (100 U/kg), the abdominal aorta just distal to the left renal artery and just proximal to the iliac bifurcation was cross-clamped and isolated for 15 minutes to produce spinal cord ischemia. To determine the clamping time, we conducted preliminary experiments. Fifteen rabbits were divided into three groups with clamping times of 10, 12, and 15 minutes (n = 5 for each group). Only the abdominal aorta just distal to the left renal artery was clamped. At 7 days after reperfusion, the groups clamped for 15 minutes were all paraplegic.

For the formal experiments, female domesticated white rabbits, weighing 2.5 to 3.0 kg, were divided into three groups: sham operation control group (group A, with only aortic exposure but without transient ischemia), transient ischemia and treatment with vehicle group (group B), and transient ischemia and treatment with 4-PBA group (15 mg/kg/h, group C). Saline solution (vehicle, 1 mL/kg body weight) or 4-PBA (4 mg/mL in saline vehicle) was administered intravenously from 30 minutes before induction of ischemia until 30 minutes after reperfusion. Total operation time ranged from 1.5 hours to 2.5 hours, and the total amount of 4-PBA administration ranged from 46.9 mg to 56.3 mg. After 15 minutes, the aortic clamp was removed. The abdomen was closed, and all catheters were withdrawn. The animals were allowed to recover from anesthesia and extubated, and routine postoperative care was carried out. Immediately after euthanasia, the spinal cord was quickly removed through a laminectomy at L4 and the bone around the spinal cord was chopped off. All samples were frozen in powdered dry ice and stored at −80°C.

Neurologic and histopathologic studies. The neurologic status in groups A, B, and C (n = 6 in each group) was assessed at 8 hours, and 2 and 7 days after reperfusion by an observer blinded to the protocol used for each animal. The motor function of the hind limbs was graded by using the Tarlov score, in which 0 indicates no movement of the hind limbs, 1 indicates perceptible movement of the joints of the hind limbs, 2 indicates good movement of the joints but an inability to stand, 3 indicates the ability to stand and walk, and 4 indicates a complete recovery.17 The animals (n = 6 in each group) were then euthanized after the completion of the neurologic evaluation at 7 days. Sectioned specimens from the lumbar spinal cord at the L4 level were stained with hematoxylin and eosin and were
studied for ischemic pathology by using standard light microscopy. Neuronal ischemic injury was evaluated at a magnification of ×200 by an observer blinded to the treatment groups. Normal motor neurons in the anterior horn of the spinal cord (anterior to a line drawn through the central canal perpendicular to the anterior median fissure) were counted for each animal and averaged. Ischemic neurons were defined by the following criteria: (1) shrinkage of the cell body, (2) an eosinophilic cytoplasm with a loss of Nissl granules, (3) triangular and pyknotic nuclei, and (4) a homogenizing change of the neuron.

Caspase12 and GRP78 immunohistochemistry. Immunohistochemistry was used to evaluate the changes in expression of caspase12 and GRP78, at 8 hours and 1 day after reperfusion in groups A, B, and C (n = 3 for each group at each time point). After endogenous peroxidase activity was quenched by exposing the slides to 0.3% H2O2 and 10% methanol for 30 minutes, the spinal cord sections were blocked in 2% normal horse serum for 2 hours at room temperature. Next, they were incubated with primary antibodies for 20 hours at 4°C. The primary antibodies used were as follows: rat monoclonal anti-caspase12 antibody (SC-12747; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) diluted 1:50 and goat polyclonal anti-GRP78 antibody (SC-1050; Santa Cruz Biotechnology) diluted 1:200. The slides were rinsed in PBS and incubated for 3 hours with biotinylated anti-rat IgG (PK-6104; Vector Laboratories) at 1:200 dilution in PBS containing 0.018% normal horse serum, respectively. Subsequently, they were incubated with avidin-biotin-horseradish peroxidase complex (PK-6104 and PK-6105; Vector Laboratories) at 1:200 dilution in PBS containing 0.3% H2O2 and 0.018% normal horse serum, respectively. Subsequently, the slides were rinsed in PBS and incubated for 3 hours with biotinylated antirat IgG (PK-6104; Vector Laboratories, Burlingame, Calif) and biotinylated antigoat IgG (PK-6105; Vector Laboratories) at 1:200 dilution in PBS containing 0.018% normal horse serum, respectively. Subsequently, they were incubated with avidin-biotin-horseradish peroxidase complex (PK-6104 and PK-6105). The slices were colonized with DAB/H2O2 solution and counterstained with hematoxylin. To ascertain specific binding of antibody for protein, a set of sections were stained in a similar way without primary antibody.

Statistical analysis. All results were expressed as means ± SD. Statistical significance was assumed at a P value of <.05. The Mann-Whitney U test, one-way analysis of variance, and post hoc tests with the Fisher protected least significant difference test were used to adequately identify which group differences accounted for the significant P value.

RESULTS

Physiologic status. There were no significant changes in the distal mean arterial pressures and heart rates between the groups during the operation. All 18 animals survived for 7 days without significant intra- or postoperative complications.

Neurologic outcomes. Fig 1 shows the neurologic outcomes of rabbits in each group. The Tarlov scores in groups A, B, and C were 4.0 ± 0.0, 4.0 ± 0.0, and 4.0 ± 0.0 at 8 hours; 4.0 ± 0.0, 2.5 ± 1.2, and 3.9 ± 0.4 at 2 days; and 4.0 ± 0.0, 0.7 ± 0.8, and 4.0 ± 0.0 at 7 days after reperfusion, respectively. While all sham-operated control animals (group A) demonstrated normal neurologic function, the group B rabbits presented with progressive deterioration of neurologic function, as was anticipated from the results of the preliminary experiments. Five out of six rabbits in group B demonstrated severe paraparesis by 7 days. In group C, neurologic function slightly deteriorated at 2 days but recovered by 7 days. There was no significant difference in Tarlov score between groups A and C across all time points. The score was significantly higher in group C than in group B at 2 and 7 days (P < .0001).

Histopathologic assessment. Fig 2 shows the typical histopathologic findings of the spinal cord from a group B rabbit with paraplegia and a group C rabbit with normal neurologic function in comparison with those in group A. In group B (Fig 2, B), the motor neurons in the anterior horn were shrunken or had pale homogenous cytoplasm compared with those in group A, consistent with ischemic change. In group C (Fig 2, C), no apparent histologic changes were detected. The number of motor neurons with a normal appearance in the anterior horn of the spinal cord at 7 days after reperfusion was 47.4 ± 5.7, 21.5 ± 6.0, and 44.9 ± 7.9 in groups A, B, and C, respectively (Fig 3). The number of normal neurons was significantly less in group B than in groups A and C (P < .0001). There was no significant difference in the number of normal neurons between groups A and C (P = .019).

Histochemical study. Immunohistochemistry results for caspase12 and GRP78 are shown in Figs 4 and 5. In the sham-operated animals, there was no evidence of caspase12 and GRP78 expression in any cells of the spinal cord. In group B, caspase12 and GRP78 were present in motor neurons after 8 hours of reperfusion (Fig 4, A and Fig 5, A) but were apparently reduced at 1 day (Fig 4, B and Fig 5, B). In group C, expression of caspase12 and GRP78 in motor neurons was not observed at either 8 hours or 1 day (Fig 4, C and D, and Fig 5, C and D).

DISCUSSION

This study has demonstrated that the peripheral administration of 4-PBA at therapeutic doses prevents both im-
mediate and delayed neuronal damage of the spinal cord following an ischemic insult. Neurologic function was preserved with little histologic change observed in motor neurons, most probably due to suppression of ER stress.

When cells are exposed to stress, ER function is disrupted. Because the ER regulates the folding and quality control systems of membrane and secretory proteins, ER stress leads to an accumulation of unfolded proteins in the ER lumen and ER-mediated cell death can be induced.14 In ER stress, several signaling pathways from the ER such as the unfolded protein response (UPR) are simultaneously activated. Accumulation of unfolded proteins in the ER is sensed by inositol-requiring protein 1 (IRE1), a serine/threonine kinase with an endoribonuclease domain in the ER membrane, and transcription induction of chaperone genes such as glucose-regulated proteins (GRPs) including GRP78 is promoted to re-fold the unfolded and accumulated proteins.19,20 Caspase12, the first identified ER-associated member of the caspase family, is regarded as a representative molecule implicated in the cell death pathway seen in ER stress.11

ER stress has been reported to contribute to ischemia-reperfusion injury in the brain and spinal cord by inducing apoptosis of neuronal cells.8,10,21,22 We speculated that targeting the ER-associated apoptotic pathway might be an effective way to minimize neuronal cell damage, and explored the effect of an ER stress inhibitor, 4-PBA, on minimizing the ischemic injury using a rabbit spinal cord ischemia model.

A low molecular weight fatty acid, 4-PBA, can act as a chemical chaperone, assisting with protein folding and thus relieving the ER stress of the cell.16,23-25 In the present study, 4-PBA administration was associated with decreased expression of caspase12 and GRP78 as well as the number of apoptotic cells, suggesting it has beneficial effects on attenuating ER stress. It appears to effectively reduce the neuronal damage, which progresses after reperfusion. Treatment with 4-PBA 30 minutes before and after ischemia notably improved the neurologic outcome. We used the 4-PBA dose of 15 mg/kg/h, based on phase 1 and pharmacokinetic studies of 4-PBA by peripheral infusion.26,27 It has low toxicity and good penetrations into the cerebrospinal fluid.28 It has been clinically used for treatment of urea cycle disorders in children, sickle cell disease, thalassemia, and cystic fibrosis.29 Our study has suggested that 4-PBA at therapeutic doses can be a potential agent for preventing spinal cord ischemia. We found that 4-PBA seems to widely suppress ER stress-mediated apoptotic signaling under conditions of ischemia. Considering the findings of the present study, it is reasonable to conclude that the protective effect of 4-PBA on ER stress-mediated injury may involve the activity of chemical chaperone.
There are two patterns of neuronal injury due to ischemia: immediate and delayed.\(^{30}\) The former is often caused by a severe ischemic insult and rapidly leads to necrotic neuronal cells. The latter is caused either by apoptosis\(^{6,31,32}\) or necrosis.\(^{30,33,34}\) Disruption of the infrarenal abdominal aortic blood flow for 15 minutes causes immediate neuronal injury by 2 days and delayed paraplegia by 7 days.\(^{6,31-34}\) In the current study, 4-PBA prevented not only immediate neuronal injury but also delayed paraplegia. In the 4-PBA-treated groups, many intact motor neurons were preserved, whereas in the negative control group, the immunohistochemical findings showed expression of caspase12 in motor neurons. To date, 14 caspases have been discovered. They are supposed to be activated in a multiple cascade fashion, activated by caspase9,\(^{35}\) an initiator caspase. Under appropriate stress signals, caspase12 is not only activated within the ER but also translocates to the cytosol, where it can activate caspase9 in a noncytochrome-c-dependent manner.\(^{36}\) Activation of caspase 12 is considered to be one of the potential mechanisms by which the ER contributes to apoptosis. Expression of caspase12 precedes the appearance of neuronal damage and might, therefore, be implicated in the activation of apoptosis.

There are a few points to consider when 4-PBA is applied to clinical situation. First, histologic examination shows that there is some loss of motor neurons in the 4-PBA-treated groups, indicating that the protective effect of 4-PBA is not perfect. One strategy that may enhance the effectiveness of 4-PBA without increasing its adverse effects is topical injection into the clamped segment of the aorta or combined use of 4-PBA with topical/core cooling. Although preservation of the feeding artery to the spinal cord is essential, such an artery cannot always be identified preoperatively. The protective effect of 4-PBA administered intravenously or the above-mentioned integrated “spinoplegia” protocol may be a potential option in such instances.

Second, the spinal cord anatomy and circulation in rabbits differ significantly from humans. The main source of blood in the rabbit spinal cord is a segmental supply from the abdominal aorta.\(^{37}\) Thus, the lumbar spinal cord of rabbits becomes ischemic by clamping of the infrarenal aorta. Despite the different anatomy, rabbits are often used as the experimental model of spinal cord ischemia because of the simplicity and reproducibility of the model. Some studies have occluded the infrarenal aorta with an intra-aortic balloon.\(^{6,31,32}\) Although this procedure is easy and less invasive, it is unclear whether aortic occlusion is complete or not. We chose a direct clamping of the aorta through a retroperitoneal approach to precisely determine
the ischemic time of the spinal cord for the reproducible development of paraplegia. On this basis, the current study showed the beneficial effect of 4-PBA.

Third, the drug toxicity of intravenous administration of 4-PBA and the peak time of ER stress at the onset of ischemia are other concerns that will likely limit the clinical application of 4-PBA, although reported side effects are not considerable.26 An additional experiment that examines the timing of injection, injection method and dosage of 4-PBA administration would be required before any clinical applications.

In conclusion, administration of 4-PBA intravenously is feasible and effective without apparent neuronal damage of the spinal cord in a rabbit model. This study indicated that this method can potentially be a useful adjunct for spinal cord protection during thoracic and thoracoabdominal aortic operations.

The authors thank Mr Kazunori Iwase and Ms Emi Fukuda for their excellent technical assistance.

AUTHOR CONTRIBUTIONS

Conception and design: TM, KOrihashi, MH
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Final approval of the article: TS
Statistical analysis: TM, KOrihashi
Obtained funding: Not applicable
Overall responsibility: TM

REFERENCES