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Original Article

Ischemic preconditioning protects motor neurons against ischemic injury by inducing endoplasmic reticulum stress, upregulating GRP78 and inhibiting caspase-12 activation

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Abstract: It has been established that ischemic preconditioning (IPC) via brief ischemia/reperfusion (I/R) induces resistance to the lethal cell ischemia. The aim of the present study was to elucidate the involvement of endoplasmic reticulum stress (ERS) in the IPC mediated protection against ischemic motor neuron injury. A rat model of spinal cord ischemia was established by clamping the infrarenal aorta. A total of 96 male Wistar rats were allocated at random into four groups: Sham, IPC, I/R and IPC+I/R. IPC consisted of 2 cycles of 5 min of ischemia and 5 min of reperfusion. In the I/R group, the aortic occlusion was continued for 45 min and then aorta patency was restored. The spinal cord was removed at 4, 8, 24 or 48 h, following 45 min of transient ischemia. Neurological function was evaluated prior to sacrifice, and histological changes were assessed using hematoxylin and eosin (HE) staining. A terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay was conducted to detect apoptotic cells. Immunohistochemistry (IHC) and western blot analysis were used to assess the expression of glucose regulated protein 78 (GRP78) and caspase 12. The results indicated that IPC markedly reduced motor neuron death during the subsequent ischemia. Elevated protein expression of GRP78 was observed, with a peak at 4 h of reperfusion in the IPC+I/R group, correlating with the ischemic tolerance time window. The results of the western blot and immunohistochemical analyses indicated that GRP78 expression was associated with the mitigation of caspase-12 activation. These results suggested that IPC induced ERS, upregulated GRP78 and inhibited the activation of caspase-12 in motor neurons, thus protecting the spinal cord against I/R injury in rats.

Keywords: Endoplasmic reticulum stress, spinal cord ischemia, ischemic preconditioning, glucose regulated protein 78, caspase-12

Introduction

Paraplegia is a devastating complication that may occur during procedures that require transient occlusion of the thoracoabdominal aorta. The exact mechanism underlying this phenomenon is not fully understood. Animal studies have suggested that the activation of the apoptotic machinery served a crucial role in the motor neuron death that occured as a result of spinal cord ischemia [1, 2]. It is increasingly evident that a variety of organelles, including the mitochondria, endoplasmic reticulum (ER), Golgi apparatus and lysosomes, are involved in the intrinsic pathway of apoptosis [3-6]. The observed induction of ER molecular chaperones following brain ischemia suggests that ER stress (ERS) is activated in response to ischemia [7]. Sakurai et al [8] reported the possible involvement of ERS in neuronal injury; therefore, ERS associated apoptosis may play a pivotal role in neuronal death.

Secretory proteins are synthesized and folded in the ER, and the disturbance of ER homeostasis can negatively affect protein folding and lead to ERS. The ER is able to sense the stress and initiate a response through translational attenuation, the upregulation of genes for ER chaperones, such as glucose regulated protein 78 (GRP78) and GRP94, and their associated proteins, and the degradation of unfolded pro-

teins by a quality control system, known as the unfolded protein response [9-12]. The response to ERS comprises three phases: adaptation, alarm and apoptosis. The adaptation phase improves the ability of the cell to manage serious injury [13]; however, when the ER function is severely impaired, apoptotic signals are released by the organelle. Caspase-12, which was the first member of the caspase family found to be associated with the ER, is activated by ERS [9, 14, 15].

A brief period of nonlethal ischemia, known as ischemic preconditioning (IPC), prevents neuronal death caused by subsequent severe ischemia [16]. The ischemic tolerance induced by IPC may be associated with ERS reduction given the crucial role of ERS in neuron ischemia and apoptosis. GRP78, which is a representative molecular chaperone in the ER [17], may thus be implicated in the establishment of ischemic tolerance in the spinal cord following IPC. In the present study, it was speculated that IPC would protect motor neurons against ischemic injury by inducing ERS, upregulating GRP78 and inhibiting the activation of caspase-12 in motor neurons; therefore, the induction of GRP78 and caspase-12 was investigated in motor neurons that had undergone IPC.

Materials and methods

Animals

The handling and care of all animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Shandong University (Jinan, China). A total of 96 male Wistar rats, weighing 300-350 g, were obtained from the Shandong University Medicine Analysis Center (Jinan, China). The rats were given free access to food prior to the experimental procedure and exhibited normal neurological function prior to anesthesia. The rats were allocated at random into the sham, IPC, ischemia/reperfusion (I/R), and IPC+I/R groups (n=24 per group).

Surgical procedures

The animals were anesthetized via an intramuscular injection of ketamine (20 mg/kg). A short incision was made in the neck region of each rat, and a 24 gauge catheter of suitable length was inserted into the left common carotid artery. A pressure transducer (PowerLab; AD Instruments, Pty Ltd, Castle Hill, Australia) was used to monitor the proximal mean aortic blood pressure (PAP), and a temperature probe was inserted into the rectum to enable core body temperature to be continuously monitored throughout the procedure. Body temperature was maintained at 37±1°C using a heating pad. A vertical midline incision was made in the upper abdomen to expose the peritoneal cavity, and the abdominal aorta was exposed and isolated. In the IPC group (n=24), following the intravenous administration of heparin (150 IU/kg; Wanbang Biopharmaceuticals Co, Ltd., Jiangsu, China), the IPC was induced by 2 cycles of 5 min of ischemia and 5 min of reperfusion. This was achieved by placing microvascular clamps on the abdominal aorta between the left renal artery and the iliac bifurcation. In the I/R group (n=24), the aortic occlusion was continued for 45 min and aorta patency was restored until sacrifice. In the IPC+I/R group (n=24), the reperfusion interval between IPC and I/R manipulations was 30 min. In the sham group, the rats underwent the surgery without any IPC or I/R manipulations. The aortic isolated ischemic segment pressure (IASP) was monitored using a 24 gauge catheter throughout the occlusion period in all groups, with the exception of the sham group, and was maintained at <15 mmHg. Following the completion of the surgery, the animals were allowed to recover in individual cages at 25°C.

Neurological assessment

All animals were closely monitored following the aortic occlusion. Neurological function was assessed according to a 15 point spinal cord performance scale, as previously described [18]. Neurological status was assessed at 24 and 48 h after surgery in a blinded fashion.

Sample preparation and histological analysis

Animals were sacrificed via an overdose of sodium pentobarbital (50 mg/kg) at 4, 8, 24 or 48 h after reperfusion. Immediately following sacrifice, spinal cord tissue was rapidly removed using a 1 ml syringe plunger, and tissue samples were frozen in liquid nitrogen and stored at -80°C for western blot analysis. All animals undergoing histological, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) and immunohistochemical analy-

ses were anesthetized using sodium pentobarbital (50 mg/kg) and perfused intracardially with 150 ml normal saline, followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.44). The spinal samples were fixed in 10% formalin solution over 24 h. The samples were cut transversely at approximately the L2 or L3 level and embedded in paraffin. Spinal cord samples embedded in paraffin were sectioned at 5 μm for HE staining and immunohistochemistry (IHC). The sections were analyzed using a computer assisted color image analysis system (Image Pro® Plus, version 5.1; Media Cybernetics, Inc., Rockville, MD, USA).

To quantify the histological analysis, the number of intact large motor neuron cells in the ventral gray matter region was counted in 5 sections per animals. In the HE stained sections, cells were considered dead if the cytoplasm was diffusely eosinophilic and viable if the cells demonstrated basophilic stippling (i.e., contained Nissl substance).

IHC

Tissue expression of caspase-12 and GRP78 was assessed immunohistochemically using rat monoclonal anti-caspase-12 (cat. no. sc-21747) and goat polyclonal anti-GRP78 (cat. no. sc 1050) antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following deparaffinization, 30% methanol and 0.3% hydrogen peroxide in PBS were used to quench endogenous peroxidase activity. The slides were then boiled in citrate buffer using microwaves. Nonspecific binding was blocked using 5% bovine serum albumin, and the slides were incubated with primary anti-caspase-12 (1:200) and anti-GRP78 (1:400) antibodies overnight at 4°C. Following incubation, the sections were washed in PBS and then incubated with a peroxidase conjugated polymer targeting rat (1:100; ZB 2307) and goat (1:200, ZB 2306) immunoglobulin (Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China) for 30 min. The sections were rinsed with PBS and subsequently exposed for 7 min to 3,3' diaminobenzidine (DAB) (ZLI 9032; Zhongshan Golden Bridge Biotechnology, Co., Ltd.). Following DAB exposure, the slides were rinsed in water and counterstained with hematoxylin. Immunohistochemical staining of the samples was performed at different times by the same technician. An observer, unaware of animal group and neurological outcome, examined each slide.

TUNEL assay

Cell apoptosis was examined using a commercially available TUNEL assay kit (KGA 703; Nanjing KeyGen Biotech, Co., Ltd., China). In brief, the sections were deparaffinized, digested with proteinase K (20 μg/ml) at room temperature for 15 min and incubated in PBS for 5 min. Following incubation, each section was covered with a terminal deoxynucleotidyl transferase (TDT) enzyme solution (45 μl equilibration buffer, 1 μl biotin 11 dUTP and 4 μl TDT enzyme) and incubated for 1 h at 37°C in a humidified chamber. To terminate the enzymatic reaction, the sections were immersed in stop buffer and then gently rinsed with PBS. Streptavidin horseradish peroxidase (HRP) solution (50 μl), containing 0.25 μl streptavidin HRP and 49.75 μl PBS, was applied to each of the sections, which were subsequently incubated at room temperature for 30 min in the dark. The slides were washed in PBS and exposed for 57 min to DAB, prior to being rinsed in water and counterstained with hematoxylin for examination using light microscopy.

Western blot analysis

Protein from the experimental rat spinal cords was extracted for western blot analysis. The tissue samples were homogenized in a lysis buffer containing 0.1 M NaCl, 0.01 M Tris HCl (pH 7.5), 1 mM EDTA and 1 μg/ml aprotinin. The samples were then centrifuged at 7,000 × g for 15 min at 4°C. A Bradford assay (Bio Rad Laboratories, Inc., Hercules, CA, USA) was performed on the supernatants to determine the protein concentration in each sample. 60 μg protein in loading buffer was used for 14% SDS PAGE. Proteins from the gel were transferred to nitrocellulose membranes (LC2006; Invitrogen Life Technologies, Carlsbad, CA, USA), which were run for 50 min at 120 V. To prevent nonspecific protein binding to the nitrocellulose membrane, the membrane was pre incubated with blocking buffer (5% nonfat dry milk, 2.7 mM KCl, 137 mM NaCl, 8 mM $NaHPO_4$, 1.4 mM KPO_4 and 0.1% Tween 20) for 2 h at room temperature. Subsequently, the membranes were incubated with the monoclonal anti β actin (1:1,000 in blocking buffer; Zhongshan Golden

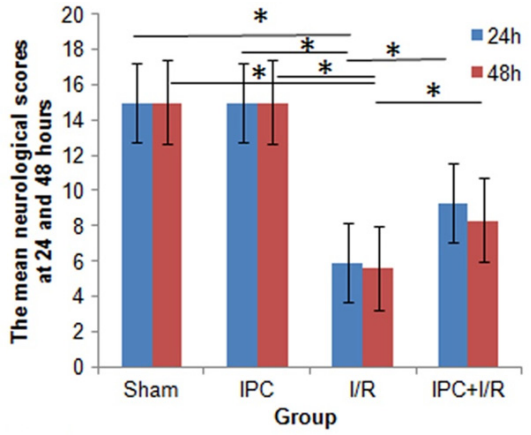

Figure 1. Mean neurological performance scores of the sham, IPC, I/R and IPC+I/R groups at 24 and 48 h after surgery. *p<0.01 I/R vs. sham, IPC and IPC+I/R groups. IPC, ischemic preconditioning; I/R, ischemia/reperfusion.

Bridge Biotechnology, Co., Ltd.), anti-GRP78 (1:500) and anti-caspase-12 (1:1,000) antibodies overnight at 4°C. The membranes were then incubated with secondary HRP conjugated anti rabbit (ZB 2010), anti-rat and anti-goat (1:1,000) immunoglobulin for 1 h at room temperature. The reaction was visualized using chemiluminescence (ECL Enhanced Chemiluminescence kit; GE Healthcare Life Sciences, Chalfont, UK). The film was scanned using an imaging densitometer (FluorChem HD IS 9900; Alpha Innotech, San Leandro, CA, USA), and the optical density was quantified using Multi Analyst software (Bio Rad Laboratories, Inc.).

Statistical analysis

Values are expressed as the mean ± standard deviation. Statistical analysis was performed using one-way ANOVA (Physiological variables, Neurological outcome) and two-way ANOVA (HE staining, IHC, TUNEL assay and Western blot analysis) with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). The comparison of neurological scores within a group was performed using a paired sample t test. p<0.05 was considered to indicate a statistically significant difference.

Results

Physiological variables

Physiological variables were recorded throughout the experimental procedure (data not shown). The rectal temperature did not differ among the groups throughout the surgery (p=0.66). No significant difference was noted among the experimental groups, with the exception of the sham group, in terms of the mean IASP during aortic occlusion (p=0.60). During the occlusion and reperfusion periods, PAP was maintained at 75±5 mmHg.

Neurological outcome

The mean neurological scores are shown in **Figure 1**. At 24 and 48 h, the mean neurological performance scores of the sham, IPC and IPC+I/R groups were significantly higher than those of the I/R group. Significant differences were noticed in neurological outcome between the I/R and IPC+I/R groups at 48 h. These results suggest that IPC enhanced the recovery of motor function at 24 and 48 h.

HE staining, IHC and TUNEL assay

HE staining was used to analyze the degree of ischemic cell injury (**Figure 2A**). In the I/R group, after 45 min of ischemia on the second day of reperfusion, ~20% of the motor neuron cells in the ventral gray matter had died, compared with ~15% in the IPC+I/R group; however, small motor neurons and intermediate neurons survived the ischemia. No obvious cell damage was observed in the sham or IPC groups.

The immunohistochemical analysis of GRP78 and caspase-12 expression in spinal cord sections from the I/R and IPC+I/R groups is shown in **Figure 2B** and **2C**. The spinal cords in the two groups showed evident immunoreactivity at 8 h, but reduced immunoreactivity at 24 h. The immunoreactivity of GRP78 in the IPC+I/R group was increased compared with that in the I/R group at equivalent reperfusion times. The immunoreactivity of caspase-12 in the I/R and IPC+I/R groups was more marked at 8 h than that at 24 h. In the IPC group, the immunoreactivity was low at all time points, while the immunoreactivity in the IPC+I/R group was lower than that in the I/R group at equivalent reperfusion times.

TUNEL staining of the spinal cord sections is shown in **Figure 2D** (only I/R and IPC+I/R groups are shown). An increased number of positive large motor neurons was noted in the I/R and IPC+I/R groups compared with the IPC

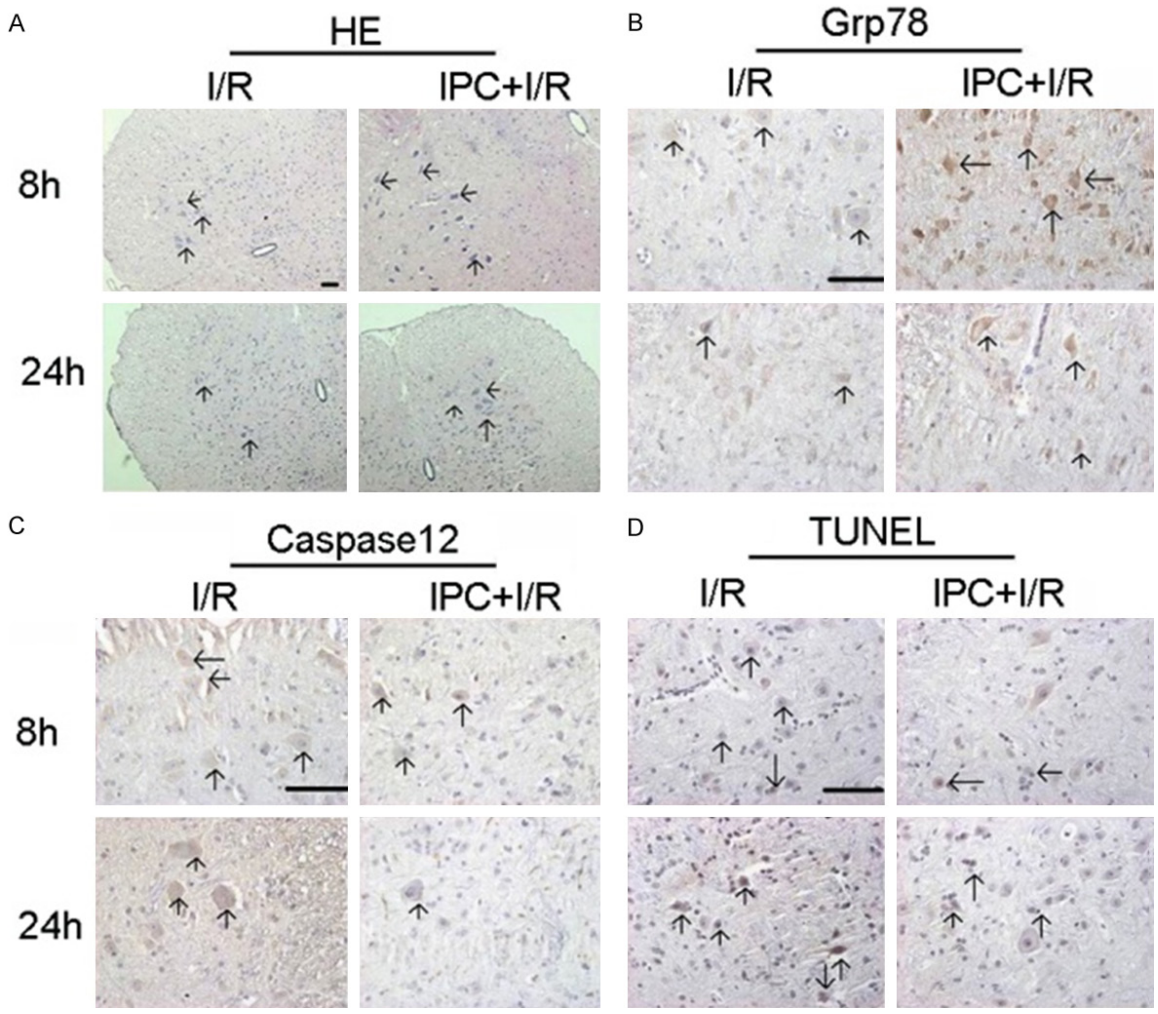

Figure 2. Histological, TUNEL and immunohistochemical analyses of the IPC and IPC+I/R groups at 8 and 24 h after surgery (scale bar, 50 μm). (A) HE staining of sections from spinal cords (arrows represent intact motor neurons). (B and C) Immunoreactive (B) GRP78 and (C) caspase 12 in sections from spinal cords. Arrows represent motor neurons that express immunoreactive GRP78 and caspase 12. (D) TUNEL staining of sections from spinal cords (arrows represent TUNEL-positive motor neurons). HE, hematoxylin and eosin; I/R, ischemia/reperfusion; IPC, ischemic preconditioning; GRP78, glucose-regulated protein 78; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

group at the same reperfusion time; however in the IPC+I/R group, there were fewer apoptotic motor neurons than in the I/R group at the corresponding time points. Quantitative analysis of the HE, IHC and TUNEL investigations is shown in **Figure 3**.

Western blot analysis

Representative results of the western blot assay for the I/R and IPC+I/R groups are shown in **Figure 4**. With regard to the antibody against GRP78, a strong band was detectable in samples from the IPC, I/R and IPC+I/R groups, and a less evident band was observed in the sham group. At 8 h, a band with a molecular weight of 78 kDa was the most evident band in the IPC and IPC+I/R groups. In the I/R group, however, the strongest band was observed at 4 h. With antibody against caspase-12, two bands, representing pro caspase-12 (50 kDa) and cleaved caspase-12 (30 kDa), were detectable in samples from the I/R and IPC+I/R groups. The bands of caspase-12 in the IPC+I/R group were less marked than those in the I/R group at the same time point. Quantitative analysis indicated that GRP78 expression was significantly increased after 8 h

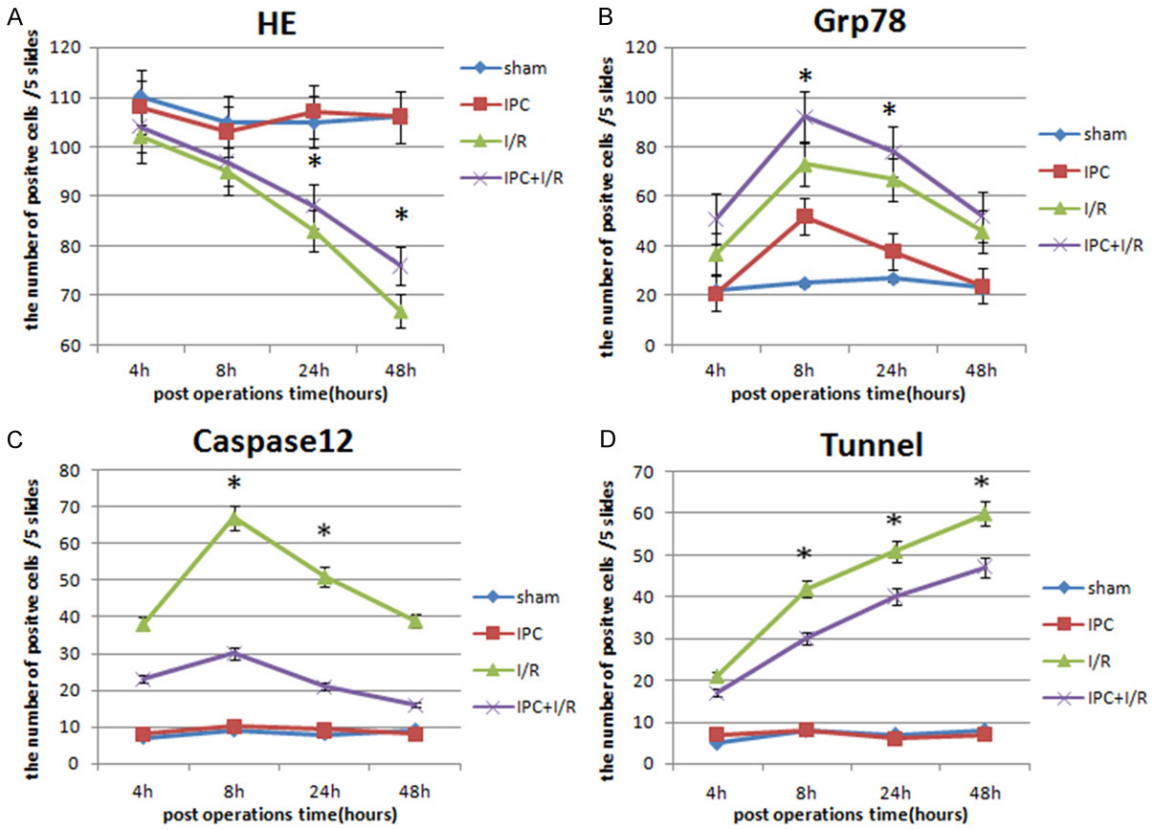

Figure 3. Quantitative analysis of (A) HE, (B and C) immunoreactive (B) GRP78 and (C) caspase-12 and (D) TUNEL staining of the sham, IPC, I/R and IPC+I/R groups at 4, 8, 24 and 48 h after surgery (*$p<0.05$ I/R group vs. IPC+I/R group for parts A-D). HE, hematoxylin and eosin; IPC, ischemic preconditioning; I/R, ischemia/reperfusion; GRP78, glucose regulated protein 78; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

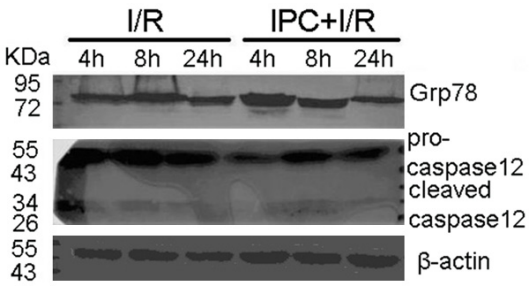

Figure 4. Representative western blot for GRP78, pro-caspase-12, cleaved caspase-12 and β-actin in the I/R and IPC+I/R groups at 4, 8 and 24 h after surgery. I/R, ischemia/reperfusion; IPC, ischemic preconditioning; GRP78, glucose-regulated protein 78.

of reperfusion in the IPC and I/R groups, and at 4 h in the IPC+I/R group (**Figure 5**); however, the expression of caspase-12 (pro caspase-12 and cleaved caspase-12) was significantly enhanced at 8 h in the I/R and IPC+I/R groups.

Discussion

The results of the present study indicate that IPC provides protection against ischemic injury in the spinal cord. A number of previous studies have investigated the effects of IPC, which appears to be protective in numerous organs, including the heart, brain and spinal cord [19-21]. Although the results of the present study have shown the beneficial effects of IPC in reducing spinal cord injury in an animal model, the mechanisms underlying this IPC mediated protection remain unclear. A previous study suggested that IPC upregulates myocardial GRP78 expression in vitro and in vivo [22]. It was suggested that the GRP78 expression induced by preemptive IPC contributed to the protection of cardiomyocytes against ischemic injury [22]. A different study aimed to elucidate the role of ERS in delayed neuronal cell death (DND) following cerebral ischemia in a rat model [23], in which the results indicated that

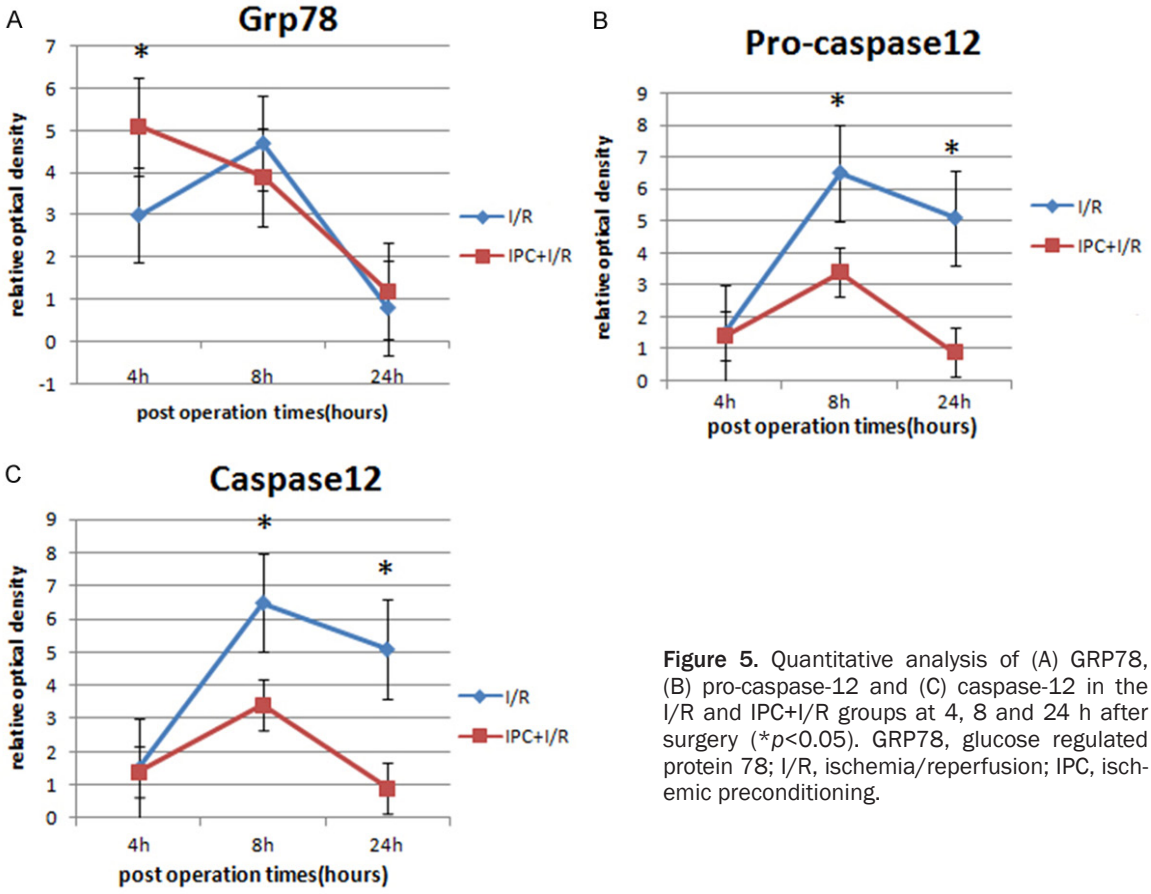

Figure 5. Quantitative analysis of (A) GRP78, (B) pro-caspase-12 and (C) caspase-12 in the I/R and IPC+I/R groups at 4, 8 and 24 h after surgery (*p<0.05). GRP78, glucose regulated protein 78; I/R, ischemia/reperfusion; IPC, ischemic preconditioning.

ERS was involved in DND and was reduced by IPC, and that ERS reduction by IPC was mediated by ER molecular chaperone induction [23].

In addition to the congruity with these previous studies regarding the association between IPC and ERS, our study further reveals how the apoptosis involves in the protective effectiveness of IPC on neuron ischemia.

In the present context, I/R is the most probable cause of ERS. ERS induced biochemical abnormalities and morphological alterations in the ER during the development of I/R induced cerebral and spinal injury have been found in numerous studies [24-26]. Furthermore, a prior study demonstrated that spinal ischemia upregulated the expression of ER chaperones, including GRP78 [8]. The results of the present study, showing that the expression of GRP78 was enhanced as a result of reperfusion, were consistent with these previous findings. The upregulation of GRP78 promotes correct protein folding, and the protective role of GRP78 has been previously observed in an in vitro study in which GRP78 depleted cells exhibited increased susceptibility to tunicamycin treatment and excitotoxicity [27]. We believe that the increased expression of GRP78 in the present study is an indicator of both the occurrence of ERS and the neuroprotective process initiated in response to I/R. In the IPC+I/R group, the peak amplitude of GRP78 expression was at 4 h, which was several hours prior to the I/R group peak, suggesting that IPC protects cells against ischemia by inducing ER chaperones.

The development of ERS in the cell cycles occurs through the phases of adaptation, alarm and apoptosis [13]. IPC is likely to induce motor neurons into the adaptive first stage. Following the occurrence of serious stress, such as I/R, in the spinal cord, motor neurons in the adaptation stage will exhibit improved tolerance to I/R compared with pre-adaptation phase cells. The results of the neurological and histopathological analyses in the present study support this hypothesis.

Caspase-12, a member of the interleukin 1β converting enzyme family, was the first member of the caspase family to be identified as an ER associated protein [28]. Caspase-12 activation is a potential mechanism by which the ER may contribute to apoptosis [14]. Previous studies showed that caspase-12 was activated in neurons on the ischemic side between 5 and 23 h of reperfusion, following a transient 1 h period of middle cerebral artery occlusion in the mouse brain [29, 30]. In the present study, caspase-12 expression was clearly induced after 8 h of reperfusion in motor neurons, which subsequently died; however, the spinal cord tissues that underwent IPC prior to the 45 min of ischemia exhibited reduced levels of activated caspase-12 compared with spinal cord tissues that underwent number of apoptotic motor neurons detected in the IPC+I/R group. The adaptation phase induced by IPC may improve spinal cord cell tolerance following lethal ischemia, leading to an increased number of apoptotic cells.

In conclusion, the present study has provided the first evidence that IPC induced ERS leads to upregulated GRP78 and inhibits the activation of caspase-12 in rat motor neurons. The protection of spinal cord tissue against I/R damage in a rat model by IPC suggests that the ER is a potential therapeutic target for the treatment of ischemic damage in the spinal cord.

Limitation: This study didn't investigate the upstream and downstream of the caspase-12 induced apoptosis signal pathway, such as the level of Ire1/TRAF2, m-calpain, Ca^{++} and caspase-9,3 and others [13, 31-33], which we will investigate in the future studies.

Disclosure of conflict of interest

None.

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