Original Article

Remote Ischemic Preconditioning Enhances the Expression of Genes Encoding Antioxidant Enzymes and Endoplasmic Reticulum Stress-Related Proteins in Rat Skeletal Muscle

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Purpose: Ischemic preconditioning (IPC), including remote IPC (rIPC) and direct IPC (dIPC), is a promising method to decrease ischemia-reperfusion (IR) injury. This study tested the effect of both rIPC and dIPC on the genes for antioxidant enzymes and endoplasmic reticulum (ER) stress-related proteins. **Materials and Methods:** Twenty rats were randomly divided into the control and study groups. In the control group (n=10), the right hind limb was sham-operated. The left hind limb (IscR) of the control group underwent IR injury without IPC. In the study group (n=10), the right hind limb received IR injury after 3 cycles of rIPC. The IscR received IR injury after 3 cycles of dIPC. Gene expression was analyzed by Quantitative real-time polymerase chain reaction from the anterior tibialis muscle. **Results:** The expression of the antioxidant enzyme genes including glutathione peroxidase (GPx), superoxide dismutase (SOD) 1 and catalase (CAT) were significantly reduced in IscR compared with sham treatment. In comparison with IscR, rIPC enhanced the expression of GPx, SOD2, and CAT genes. dIPC enhanced the expression of SOD2 and CAT genes. The expression of SOD2 genes was consistently higher in rIPC than in dIPC, but the difference was only significant for SOD2. The expression of genes for ER stress-related proteins tended to be reduced in IscR in comparison with sham treatment. However, the difference was only significant for C/EBP homologous protein (CHOP). In comparison with IscR, rIPC significantly up-regulated activating transcription factor 4 and CHOP, whereas dIPC up-regulated CHOP. **Conclusion:** Both rIPC and dIPC enhanced expression of genes for antioxidant enzymes and ER stress-related proteins.

Key Words: Ischemic preconditioning, Reperfusion injury, Muscle, Skeletal

INTRODUCTION

Ischemia-reperfusion (IR) injury, which is a phenomenon caused by reperfusion after ischemia, is an additional regional and systemic harmful insult beyond the damage caused by ischemia. IR injury can occur in a wide variety of clinically important situations such as trauma, orthopedic surgery, flap surgery, stroke, ischemic heart disease, organ transplantation, and vascular surgery [1,2]. Intense IR injury has both local and systemic effects because of the physiological, biochemical, and immunological changes that occur during the ischemia and reperfusion periods [3,4]. The IR sequelae may lead to the loss of organ or limb function or even to life-threatening complications [4,5].

Since Murry et al. [6] reported the protective effect of ischemic preconditioning (IPC) on the myocardium in a canine model, there have been many basic and clinical studies aimed at understanding the pathophysiology of IR injury and reducing IR injury by pharmacological and mechanical conditioning [7,8]. To reduce IR injury, various agents have been tested, but most of them have not been found to be applicable in clinical practice [9]. However, IPC has been proven to be beneficial in animal experiments and it has been successfully translated into clinical practice in some reports [10-12]. IPC is defined as brief episodes of IR applied by mechanical intervention before prolonged ischemia of the organ. Previous studies have suggested that IPC is not a local reaction but a systemic phenomenon mediated by neurogenic and humoral pathways [13,14]. Direct IPC (dIPC), which is performed at the target organ, has potential disadvantages because it causes direct stress in the target organ and mechanical trauma to the vascular structure, which might limit its clinical application. On the other hand, remote IPC (rIPC), which is performed at a remote site, might overcome these limitations of dIPC.

Although the mechanisms underlying IR injury are complex and not thoroughly understood, reactive oxygen species play an important role in the IR injury mechanism and antioxidant enzymes have protective effects from IR injury [15,16]. We examined three antioxidant enzymes, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT). The main biological role of GPx is to protect the organism from oxidative damage. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. SOD is an enzyme that alternately catalyzes the dismutation of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) [17]. Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is also damaging, but less so, and is degraded by other enzymes such as CAT. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. CAT is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species [18].

IR injury disturbs the cellular energy, redox status and Ca^{2+} concentration, which causes endoplasmic reticulum (ER) stress. The production of ER stress-related proteins increases in response to the ER stress in order to protect the organ from injury [19,20]. One of the components of the ER stress-mediated apoptosis pathway is the C/EBP homology protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 [21]. CHOP has now been shown to be induced mainly by ER stress. CHOP is normally expressed at low levels and is localized to the cytoplasm. Cellular stress triggers an upregulation of CHOP levels and accumulation in the nucleus where it can act as either a transcriptional repressor or activator. Protein misfolding in the ER leads to cell death through PERK-mediated phosphorylation of eIF2α [21]. Activating transcription factor 4 (ATF4) and CHOP are key transcription factors downstream of p-eIF2α, and have been demonstrated that they interact to directly induce genes encoding protein synthesis and the unfolded protein response. Several stress conditions such as hypoxia, anoxia, and glucose deprivation result in ER stress, initiating the unfolded protein response pathway that increases the synthesis of ATF4. Glucose-regulated protein 78 kDa (GRP78) is known as binding immunoglobulin protein or heat shock 70 kDa protein 5 (HSPA5). GRP78 is a chaperone located in the lumen of the ER that binds newly synthesized proteins as they are translocated into the ER, and maintains them in a state competent for subsequent folding and oligomerization. GRP78 is also an essential component of the translocation machinery and plays a role in retrograde transport across the ER membrane of aberrant proteins destined for degradation by the proteasome. Its synthesis is markedly induced under conditions that lead to the accumulation of unfolded polypeptides in the ER [22].

There have been few studies regarding the effect of rIPC on the antioxidant enzymes and ER stress-related proteins. The purposes of this study were to test the hypotheses that IPC increases the expression of genes for antioxidant enzymes and ER stress-related proteins to reduce the deleterious effect of IR injury in rat myocytes and that rIPC is as efficient as dIPC in inducing changes in the expression of these genes in rat myocytes.

MATERIALS AND METHODS

1) Animals and anesthesia

Twenty 8-week-old male Sprague-Dawley rats weighting 250-300 g were kept in a non-stimulating environment for 1 week prior to the experiment and fed a regular diet and water ad libitum. Rats were fasted overnight prior to the procedure with free access to water.

Each rat was placed in an anesthesia induction cage and ventilated with a mixture of 3% isoflurane and oxygen. After induction, the rat was anesthetized with a mixture of 0.03 mg/g zolazepam/tiletamine (Zoletil; Virbac, Carros, France) and 0.01 mg/g xylazine (Rompun; Bayer Healthcare LLC, Shawnee Mission, KS, USA) given as an intraperitoneal injection. The rat was considered anesthetized when it was unresponsive to mechanical stimulation and the palpebral reflex was absent. A half dose of the initial anesthetic mixture was administered additionally 60 min after initial anesthesia.

2) Surgical procedures

A rat was placed on a constantly heated table in the supine position with limbs immobilized by adhesive tape. Bilateral groins were incised and common iliac arteries were isolated. Inferior epigastric arteries were ligated and cut to prevent collateral circulation. Hind limb ischemia was induced by clamping the common iliac artery with a vascular clamp (SukitaClip; Mizuho Medical Ca., Bunkyo-ku, Japan) and by wrapping a rubber tourniquet around the proximal thigh to completely occlude collateral circulation. IR injury was established according to a method described previously [16].

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Keimyung University School of Medicine, Daegu, Korea (KM-2014-17) and the experiments were performed according to the National Institutes of Health guidelines on the use of experimental animals.

3) Experimental design

Rats were divided into control and study groups. Hind limbs of both groups were assorted as in Fig. 1.

In the control group (n=10), the right hind limb was sham operated without ischemia (sham) and the left hind limb was subjected to ischemia for 60 min after 30 min anesthesia without IPC (IscR). Both hind limbs had a 120 min reperfusion period before their muscles were harvested.

In the study group (n=10), three preconditioning cycles were performed on the left hind limb. Thus, rIPC and dIPC were done in the right and left hind limbs respectively. Each cycle of IPC consisted of 5 min ischemia and 5 min reperfusion using a vascular clamp and a rubber tourniquet. Immediately after preconditioning, both hind limbs had 60 min ischemia followed by 120 min reperfusion.

At the end of reperfusion, the anterior tibialis muscle was harvested from the hind limbs. For histologic analysis, parts of the muscle were fixed in buffered 10% formalin
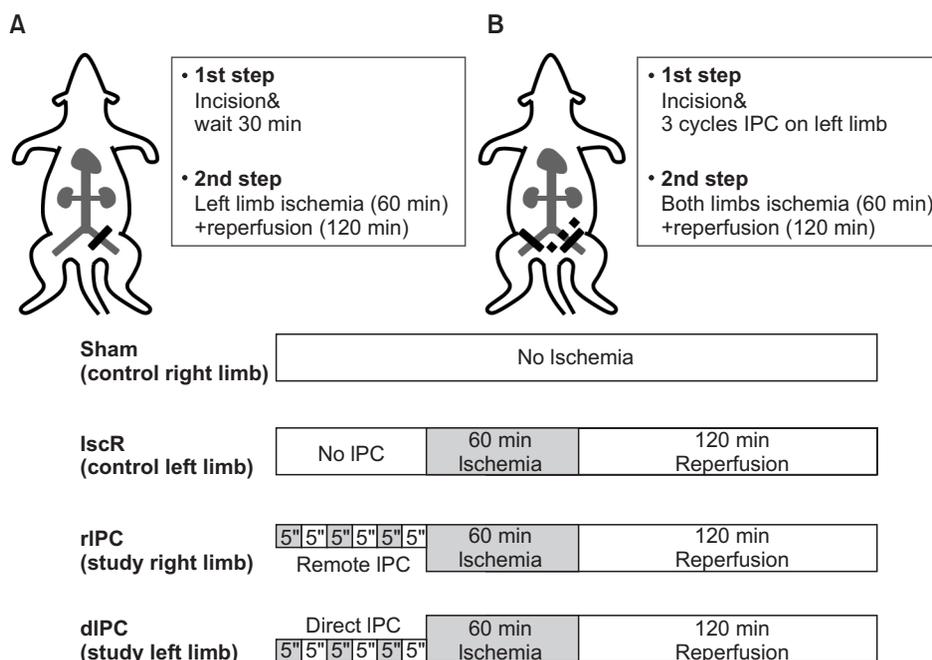

Fig. 1. Experimental design of the study. (A) Control group: right hind limb underwent sham operation without ischemia and left hind limb underwent 60 min ischemia and 120 min reperfusion without IPC. (B) Study group: animals underwent 3 cycles of rIPC in the right hind limb and 3 cycles of dIPC in the left hind limb, followed by 60 min ischemia and 120 min reperfusion in both limbs. IscR, ischemia-reperfusion; IPC, ischemic preconditioning; rIPC, remote IPC; dIPC, direct IPC.

and embedded in paraffin wax. Sections (5 μm) were cut and stained with hematoxylin and eosin. The rest of the muscle was frozen in liquid nitrogen-cooled isopentane and stored at -80°C until analysis of mRNA expression.

4) Quantitative real-time polymerase chain reaction analysis

Each anterior tibialis muscle was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using an Ultra-Turrax T25 homogenizer (Staufel, Germany) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was performed in a volume of 20 μL containing 2.5 mM MgCl$_2$, 0.5 μM forward and reverse primers, 2 μL of Power SYBR Green PCR master mix (Applied Biosystems), and 2 μL of cDNA using a Real-Time PCR 7500 system. The reactions were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 35 s [23]. Relative quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method; the data were presented as fold changes compared with the control [24]. The expression level of the gene of interest was normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific primers used for qPCR are listed in Table 1.

5) Statistical analysis

IBM SPSS Statistics ver. 20.0 software (IBM Co., Armonk, NY, USA) was used for statistical analysis. Means were compared between groups using Wilcoxon's rank sum test. Results are shown as mean±standard error. The differences were considered significant for P-value less than 0.05.

RESULTS

1) Histologic evaluation of IR injury

Sham-treated skeletal muscle had intact muscle cell membranes, no swelling, and normal structure without significant morphologic changes. However, the IscR, rIPC, and dIPC limbs showed damaged muscle cell membranes, blurring of cross-striations, separation of fibers, focal necrosis, increased infiltration of inflammatory cells, and centralization of nuclei (Fig. 2).

2) Effect of IPC on expression of antioxidant enzyme genes

We examined the expression levels of genes encoding the following antioxidant enzymes: GPx, SOD1, superoxide dismutase 2 (SOD2), and CAT. In comparison with sham treatment, IscR reduced the levels of GPx, SOD1, and CAT transcripts by 51.5% (P<0.01), 34.3% (P<0.05), and 41.9% (P<0.01), respectively (Table 2, Fig. 3). In comparison with IscR, rIPC significantly enhanced the expression of GPx (108.3%, P<0.01), SOD2 (164.8%, P<0.001), and CAT (126.0%, P<0.01) genes, whereas dIPC significantly increased the expression of SOD2 (66.7%, P<0.05) and CAT (120.0%, P<0.01). The expression of GPx, SOD1, and CAT genes was consistently higher in rIPC than in dIPC, but the difference was only significant for SOD2, which was higher in rIPC (37.1%, P<0.05) (Table 2, Fig. 3).

3) Effect of IPC on the expression of ER stress genes

To determine whether IPC induces the expression of genes for ER stress-related proteins, we measured the expression levels of genes for ATF4, CHOP, and GRP78.

Similar to the antioxidant enzyme genes, the expression of genes for ER stress-related proteins tended to decrease

Table 1. Primers used for quantitative real-time polymerase chain reaction amplification

Gene	Forward primers (5'→3')	Reverse primers (5'→3')
ATF4	GTT GGT CAG TGC CTC AGA CA	CAT TCG AAA CAG AGC ATC GA
CHOP	CCA GCA GAG GTC ACA AGC AC	CGC ACT GAC CAC TCT GTT TC
GRP78	AAC CCA GAT GAG GCT GTA GCA	ACA TCA AGC AGA ACC AGG TCA C
GPx	GCC GAG TGT GGT TTA CGA AT	GGC TGC AAA CTC CTT GAT TT
SOD1	AGA TGA CTT GGG CAA AGG TG	CAA TCC CAA TCA CAC CAC AA
SOD2	CTG GAC AAA GGT GAG CCC TA	GAA CCT TGG ACT CCC ACA GA
CAT	TTA TGG CCT CCG AGA TCT TTT C	ACC TTG TCA GGT CAA ATG GA
GAPDH	AGT TCA ACG GCA CAG TCA A	TAC TCA GCA CCA GCA TCA CC

ATF, activating transcription factor; CHOP, C/EBP homologous protein; GRP, glucose-regulated protein; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
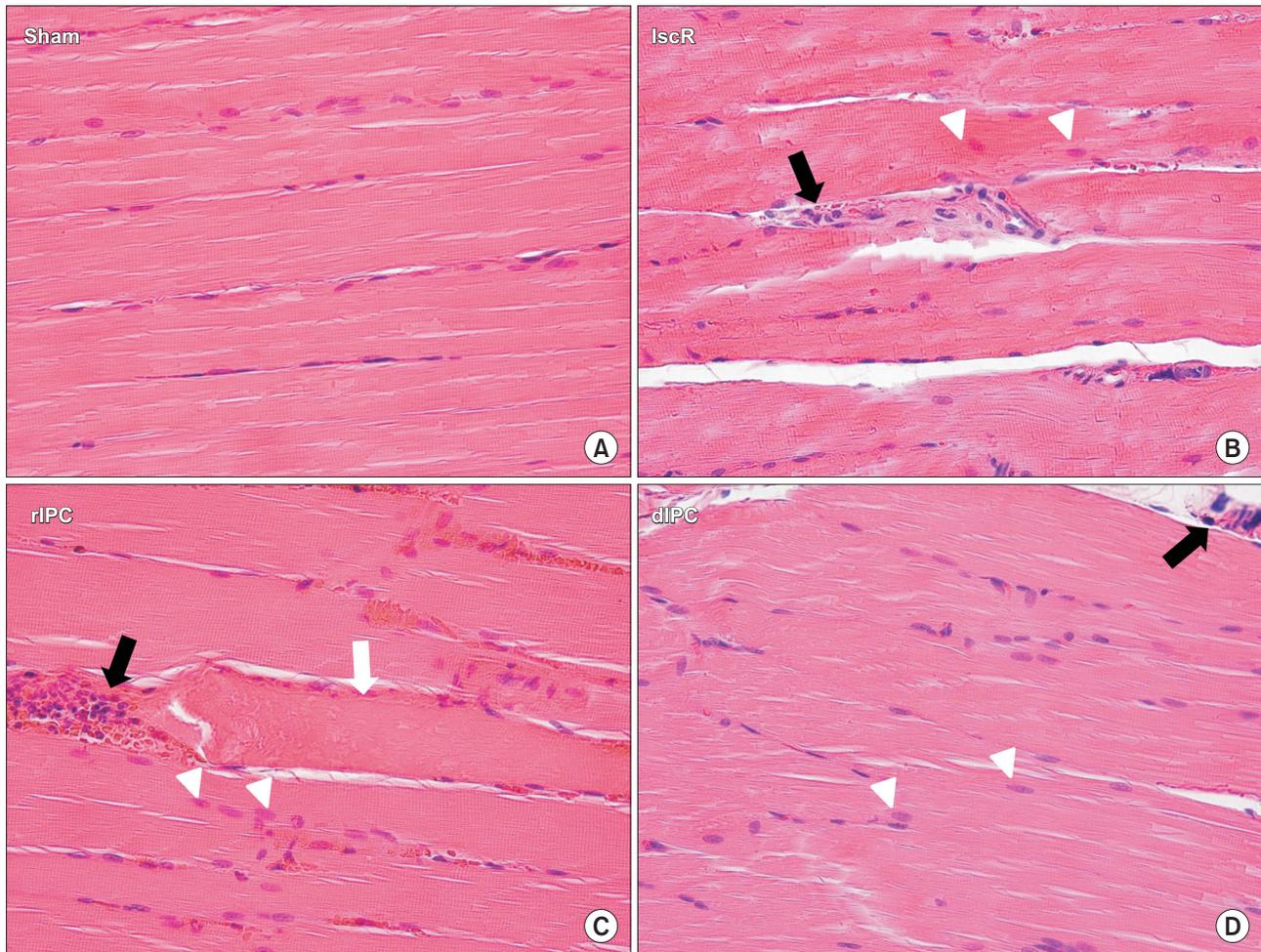
Fig. 2. Photomicrographs of the anterior tibialis muscle (H&E stain, ×400). (A) Normal skeletal muscle architecture. (B-D) Cross-striation blurring, muscle fiber separation, centralization of nuclei (white arrowheads), focal myocyte necrosis (white arrow), and infiltration of inflammatory cells (black arrows) were found upon ischemia-reperfusion injury with or without preconditioning. IscR, ischemia-reperfusion; rIPC, remote ischemic preconditioning; dIPC, direct ischemic preconditioning.

Table 2. Relative mRNA expression levels of antioxidant enzymes and ER stress-related proteins

Gene	Sham	IscR	rIPC	dIPC
GPx	0.99±0.26	0.48±0.23^a	1.00±0.58^b	0.90±0.58
SOD1	0.99±0.19	0.65±0.30^c	1.86±2.00	0.96±0.37
SOD2	0.81±0.44	0.54±0.26	1.43±0.79^{d,e}	0.90±0.20^{f,g}
CAT	0.86±0.26	0.50±0.27^h	1.13±0.48^i	1.10±0.35^j
ATF4	0.85±0.29	0.54±0.25	1.23±1.03^k	0.83±0.35
CHOP	1.07±0.36	0.66±0.28^l	1.27±0.55^m	1.20±0.41^n
GRP	0.92±0.45	0.50±0.25	1.09±0.87	0.74±0.26

Results are expressed as means±standard error of the mean.

ER, endoplasmic reticulum; IscR, ischemia-reperfusion; rIPC, remote IPC; dIPC, direct IPC; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; ATF, activating transcription factor; CHOP, C/EBP homologous protein; GRP, glucose-regulated protein.

Percentage changes are indicated for significant differences between treatment groups. ^{a}P<0.01 compared with sham (−51.5%) ((0.48/0.99)−1)×100%. ^{b}P<0.01 compared with IscR (108.3%)((1.00/0.48)−1)×100%. ^{c}P<0.05 compared with sham (−34.3%)((0.65/0.99) −1)×100%. ^{d}P<0.05 compared with sham (76.5%)((1.43/0.81)−1)×100%. ^{e}p<0.001 compared with IscR (164.8%)((1.43/0.54)−1)×100%. ^{f}P<0.05 compared with IscR (66.7%)((0.90/0.54)−1)×100%. ^{g}P<0.05 compared with rIPC (-37.1%)((0.90/1.43)−1)×100%. ^{h}P<0.01 compared with sham (-41.9%)((0.50/0.86)−1)×100%. ^{i}P<0.01 compared with IscR (126.0%)((1.13/0.50)−1)×100%. ^{j}P<0.01 compared with IscR (120.0%) ((1.10/0.50)−1)×100%. ^{k}P<0.01 compared with IscR (127.8%)((1.23/0.54)−1)×100%. ^{l}P<0.05 compared with sham (-38.3%)((0.66/1.07) −1)×100%. ^{m}P<0.05 compared with IscR (92.4%)((1.27/0.66)−1)×100%. ^{n}P<0.05 compared with IscR (81.8%)((1.20/0.66)−1)×100%.
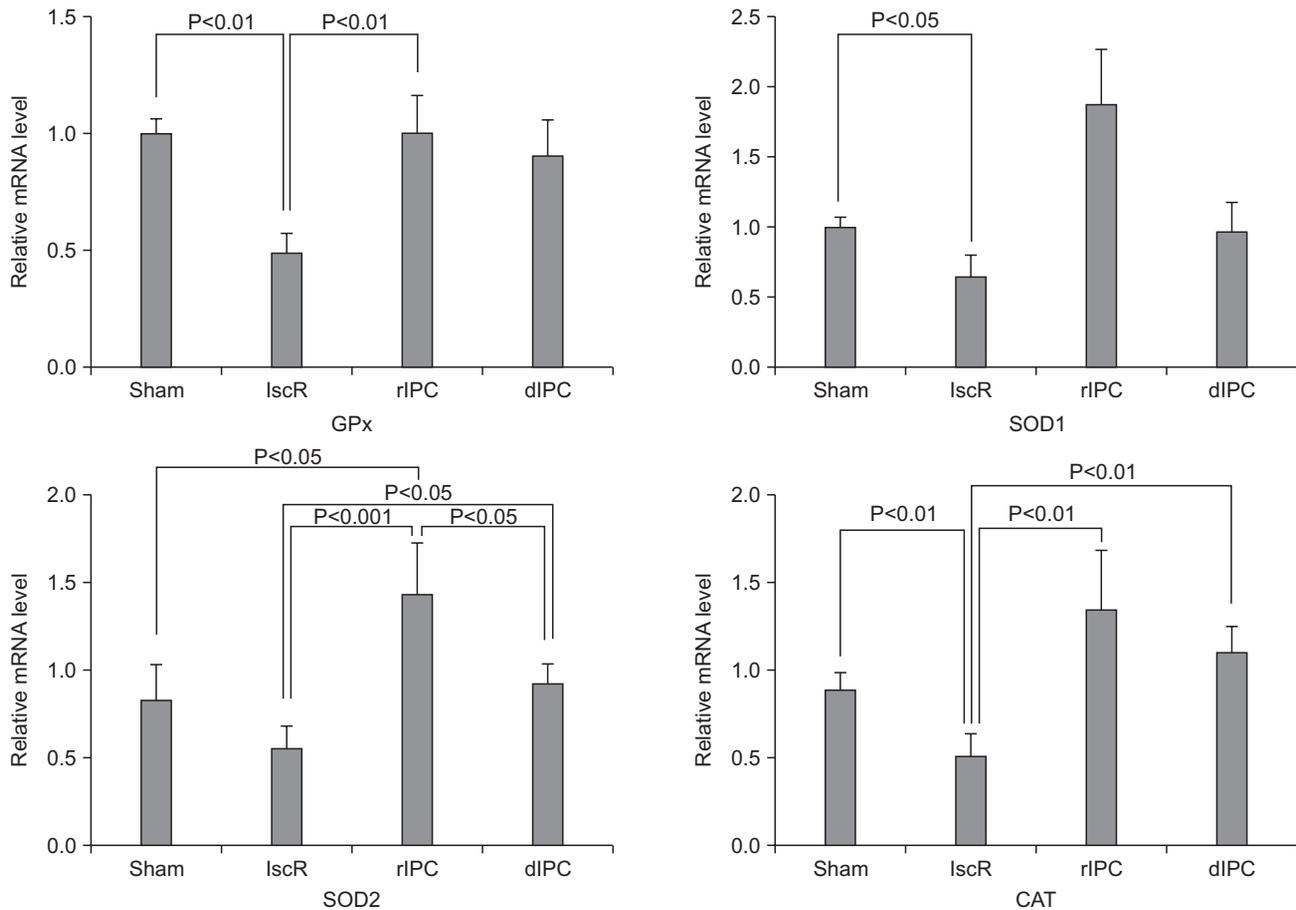
Fig. 3. Effects of ischemia-reperfusion (IscR), remote ischemic preconditioning (rIPC), and direct ischemic preconditioning (dIPC) on the gene expression of antioxidant enzymes. GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

in IscR in comparison with sham treatment. However, the difference was only significant for CHOP (−38.3%, P<0.05). rIPC significantly up-regulated ATF4 (127.8%, P<0.01) and CHOP (92.4%, P<0.05) genes, whereas dIPC increased the expression of CHOP (81.8%, P<0.05) in comparison with IscR. The expression of genes for ER stress-related proteins was slightly higher in rIPC than in dIPC, but the differences were not significant (Table 2, Fig. 4).

DISCUSSION

IR injury plays a crucial role in complications after vascular surgery, such as aortic surgery and extremity revascularization surgery. Paradoxically, both the lack of oxygen during ischemia and the replenishment of oxygen during reperfusion can cause tissue injury. Increased levels of reactive oxygen species have been suggested to be key players in IR injury. To combat the oxidative stress, cells possess antioxidant defense machinery that includes three major antioxidant enzymes, SOD, GPx, and CAT [18]. To

assess the status of the antioxidant defense, we analyzed the expression levels of the antioxidant enzyme genes. We found that IR injury decreases their expression, in line with previous IPC studies in animal models [4].

The present study revealed that the expression levels of genes for antioxidant enzymes tended to be increased after IPC. The expression of GPx, SOD2, and CAT genes was significantly increased by rIPC, whereas the expression of SOD2 and CAT genes was significantly increased by dIPC. These findings suggest that IPC may increase the levels of antioxidant enzymes, which play a role in myocyte protection against IR injury. Khanna et al. [25] argued that reactive oxygen species related to IR injury and sub-threshold generation of reactive oxygen species by pharmaco-mechanical preconditioning may have a protective effect by activating redox signaling. On the other hand, Mansour and colleagues detected no significant change in SOD and GPx in the rat skeletal muscle IPC model [4]. Even though previous data have been inconsistent, numerous reports have demonstrated that IPC increases
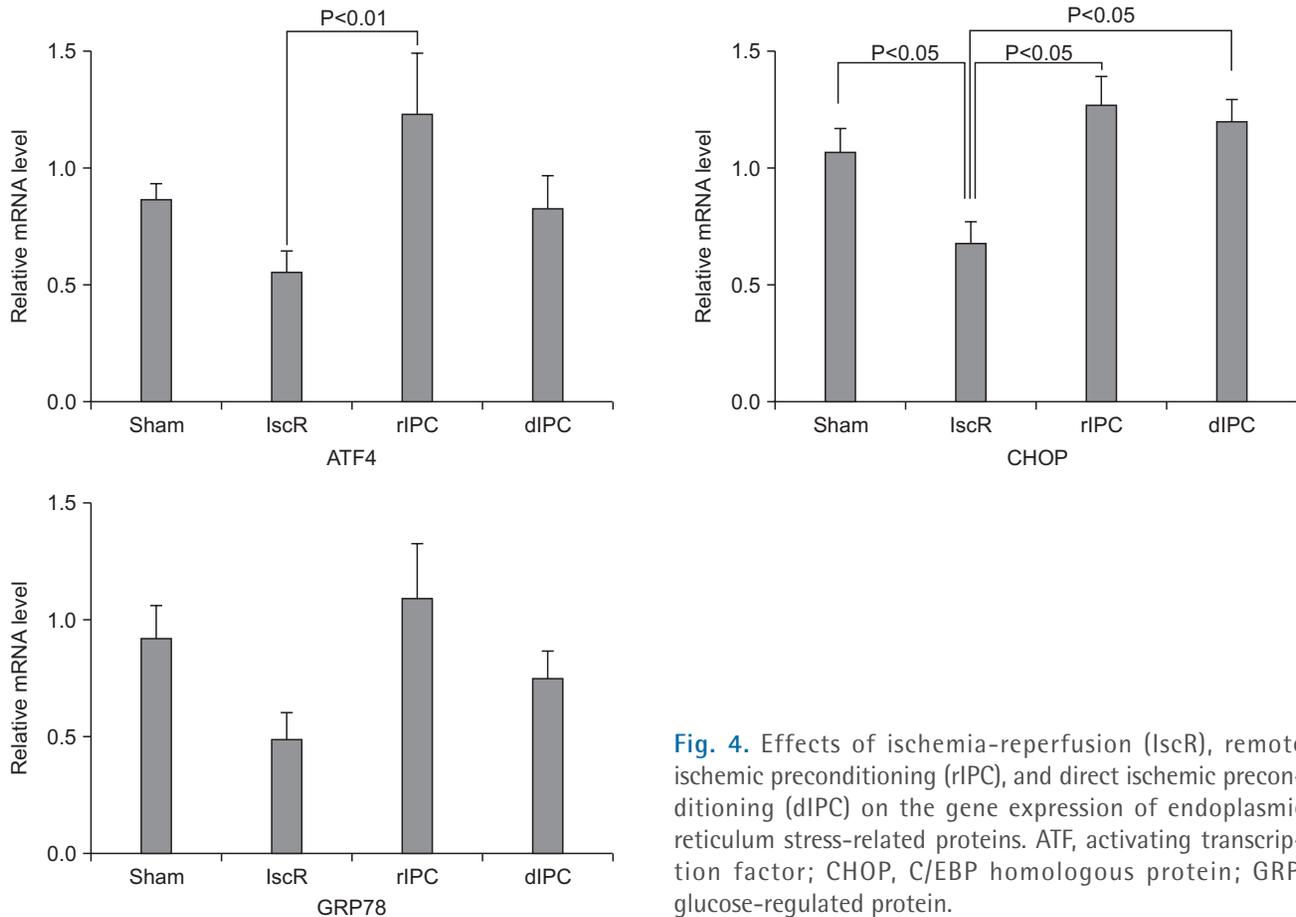
Fig. 4. Effects of ischemia-reperfusion (IscR), remote ischemic preconditioning (rIPC), and direct ischemic preconditioning (dIPC) on the gene expression of endoplasmic reticulum stress-related proteins. ATF, activating transcription factor; CHOP, C/EBP homologous protein; GRP, glucose-regulated protein.

the levels of antioxidant enzymes, which may reduce the harmful effect of IR injury [18,26].

Both IPC treatments also tended to up-regulate expression levels of genes for ER stress-related proteins. The expression of ATF4 and CHOP genes was increased significantly by rIPC, and CHOP expression was also significantly increased by dIPC. The ER is the site of synthesis and folding of secretory proteins. Disturbance of ER homeostasis alters protein folding and induces ER stress. The ER senses the stress and responds to it through translational attenuation, up-regulation of genes for ER chaperones and related proteins, and degradation of unfolded proteins by a quality-control system [21].

GRP78 is one of the ER chaperone proteins that facilitate degradation of harmful unfolded and misfolded proteins to restore ER function and to protect the organism [21]. In this study, the expression of the GRP78 gene was higher in both IPC treatments than in sham treatment, although the differences did not reach statistical significance. Recent studies have suggested that GRP78 plays a critical cytoprotective role in IPC and GRP78 up-regulation is induced by IPC or pharmacologic preconditioning, thus contributing to the protective effect against IR injury [22].

If ER function is severely impaired because of excessive or prolonged exposure to stress, the affected cells may undergo apoptosis. The ATF4-CHOP pathway is the main pro-apoptotic pathway during ER stress [20]. In this study, IR injury of the skeletal muscle decreased, whereas IPC clearly enhanced the expression of ATF4 and CHOP genes. This may suggest that IPC activates the ATF4-CHOP pathway to protect the organism by eliminating damaged cells in response to severe impairment of ER function.

These findings point to a role for ER stress response in skeletal muscle protection from reperfusion injury by IPC. However, caution is needed in interpretation of these results because the ER stress response may be both protective and deleterious in various tissues, depending on the balance between survival and apoptotic pathways [20,22].

Although there were no statistically significant differences between rIPC and dIPC, the expression of genes for antioxidant enzymes and ER stress-related proteins tended to be higher in rIPC than in dIPC. A possible explanation for this difference between rIPC and dIPC is that short-term IPC might lead to additional ischemic burden to the target organ and might be deleterious. Since the first evidence of rIPC was reported about 20 years ago, this simple

method has been the focus of extensive experimental and clinical research [27]. Controversies exist concerning the potential beneficial effects of rIPC in clinical trials. Remote Preconditioning Trialists' Group et al. [28] have shown a protective effect of rIPC during cardiac surgery, but some studies have failed to demonstrate a significant beneficial effect of rIPC [10]. Nevertheless, rIPC might deserve a wider use in clinical practice such as vascular and transplantation surgery, because it is relatively easy to perform, is non-invasive, and causes no harm to the ischemic target organ.

One issue that remains to be resolved is that the optimal number, duration, and timing of rIPC cycles are unknown. Additional studies are needed to evaluate variations in these parameters and the optimal choice of application sites such as the arm, leg, or internal organs.

We used the anterior tibialis muscle for histologic and gene expression analyses. The anterior tibialis muscle is a long and narrow muscle in the anterior compartment of the hind limb. This muscle is well demarcated and easy to harvest and fix the specimen in the same shape. In addition, most of the anterior tibialis muscle consists of fast-twitch fibers, which are more vulnerable to IR injury than slow-twitch fibers [29].

This study had limitations. First, the reperfusion time we used was only 120 min. Thus, our results reflect only the early response of rIPC to IR injury. We could not extend the reperfusion time because some of the experimental animals died after prolonged reperfusion (more than 120 min) in pilot experiments. Second, to obtain more reliable results, the levels of antioxidant enzymes and ER stress-related proteins should be measured directly by enzyme-linked immunosorbent assay and western blotting.

CONCLUSION

The expression levels of genes for antioxidant enzymes and ER stress-related proteins tended to be increased after both rIPC and dIPC. rIPC may be more efficient than dIPC in enhancing the expression of genes for antioxidant enzymes and ER stress-related proteins.

ACKNOWLEDGEMENTS

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