Exercise Preconditioning-Induced Early and Late Phase of Cardioprotection Is Associated With Protein Kinase C Epsilon Translocation

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Background: Exercise preconditioning (EP) can provide powerful protection to the heart. Evidence supports the contention that EP directly enhances myocardial tolerance to ischaemia through a protein kinase C (PKC)-mediated mechanism. However, studies investigating the role of isoform-specific PKC after EP are lacking.

Methods and Results: In this study, a male Sprague-Dawley rat model of EP was established (4 periods of 30m/min for 10min exercise then a 10min rest at 0% grade treadmill exercise). Rats were subjected to exhaustive exercise to induce myocardial injury. Chelerythrine (5 mg/kg) was injected before EP to investigate the role of PKC in EP. EP was found to attenuate exhaustive exercise-induced myocardial injury in both of EP's 2 protective phases, especially the latter phase. After EP, PKC ϵ was markedly upregulated, and PKC ϵ was translocated to myocardial intercalated disks, and p-PKC ϵ ^{Ser729} was translocated to the myocardial cytomembrane. Even though PKC ϵ was markedly upregulated and translocated to intercalated disks during exhaustive exercise, p-PKC ϵ ^{Ser729} was mainly distributed in the cytoplasm. A chelerythrine injection before EP did not suppress the activation of PKC and the protection of EP.

Conclusions: These results indicate that PKC ϵ plays an important role in EP-mediated protection of the myocardium during exhaustive exercise-induced myocardial injury, and that a chelerythrine injection during exercise is not suitable for demonstrating the role of PKC ϵ . (*Circ J* 2014; **78**: 1636–1645)

Key Words: Cardioprotection; Exercise preconditioning; Protein kinase C epsilon

I schemia/reperfusion (I/R) is one of the major causes of myocardial injury. Over the years, investigators have studied many approaches regarding the protection of the heart against I/R injury. Specifically, cardioprotection of ischemic preconditioning (IP)¹ and remote IP^{2,3,4} were described as the immediate adaptation of the heart to brief sublethal ischemia but it lacking practical utility for providing protection to human. Recently, researchers have found that, like IP, exercise preconditioning (EP), which here refers to brief episodes of exercise, can also enhance the tolerance of the heart to subsequent ischemic insult.^{5,6} It is demonstrated here that EP has 2 distinct protective phases, the early phase, which occurs immediately after the exercise, and a late phase, which peaks 24h after exercise.^{6–9} Although the powerful cardioprotective effect of EP has been proved, the intracellular mechanisms involved in this EP-conferred cardioprotection remain unclear. Recent evidence supports the contention that acute exercise directly enhances myocardial tolerance to ischemia in the hearts of rats through a protein kinase C (PKC)-mediated mechanism.⁵ However, studies investigating the role of isoform-specific alterations in PKC after EP are still rare. One recent study clearly demonstrated that

the EP-induced early phase of cardioprotection requires PKC δ translocation.⁸ In this study, which was performed by the present team, short-term adaptation of adult rats to EP was found to markedly increase the expression of PKC δ and phosphorylation of PKC δ . However, the PKC ϵ responsible for EP has not been identified. In the present study, it was hypothesized that EP caused an increase in PKC ϵ expression and translocation of PKC ϵ , and that the PKC inhibitor, chelerythrine (CHE), suppressed these events, thus attenuating the EP-mediated cardioprotection.

Methods

Ethical Approval

Adult (8-week-old) male Sprague-Dawley rats (Sippr BK, Shanghai, China) were housed at a constant temperature (22±2°C) on a 12-h light/dark cycle. They were fed ad libitum on standard laboratory rat chow and had free access to tap water. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and was

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approved by the Ethics Committee for Science Research at Shanghai University of Sport.

Experimental Protocol

All animals performed a light exercise familiarization on the treadmill for 5 consecutive days. The velocity on the treadmill was 15 m/min and the exercise duration was for 10–20 min/day. The EP protocol was designed with minor modifications of the protocol described by Domenech et al.¹⁰ and Parra et al.⁶ On the seventh day, animals were randomly assigned to 10 experimental groups (n=25 rats per group):

- **Group C, Control:** rats were placed on the treadmill without movement.
- **Group EE, Exhaustive exercise:** rats were acclimated to the treadmill at 30 m/min with 0% grade till exhausted. Exhaustion was defined as the rat being unable to upright itself when placed on its back. Rats were sacrificed 0.5 h after exhaustion.
- **Group EEP, Early exercise preconditioning:** rats were acclimated to the treadmill at 30 m/min with 0% grade for 4 periods of 10 min each, with intervening periods of rest that were 10 min long. Exercise began and ended with 5-min “warm up” and “cool down” periods at 15 m/min with 0% grade. Rats were sacrificed 0.5 h after exercise.
- **Group EEP+EE, Early exercise preconditioning plus exhaustive exercise:** rats were treated as per Group EEP, where 0.5 h after exercise, rats were acclimated to the treadmill at 30 m/min with 0% grade till exhausted. Rats were sacrificed 0.5 h after exhaustion.
- **Group LEP, late exercise preconditioning:** rats were acclimated to the treadmill at 30 m/min with 0% grade for 4 periods of 10 min each, with intervening periods of rest that were 10 min long. Exercise began and ended with 5-min “warm up” and “cool down” periods at 15 m/min with 0% grade. Rats were sacrificed 24 h after exercise.
- **Group LEP+EE, Late exercise preconditioning plus exhaustive exercise:** rats were treated as per Group LEP, where 24 h after exercise, rats were acclimated to the treadmill at 30 m/min with 0% grade till exhausted. Rats were sacrificed 0.5 h after exhaustion.
- **Group CHE+EEP+EE, CHE plus early exercise preconditioning plus exhaustive exercise:** rats were treated as per Group EEP+EE, but CHE (5 mg/kg) was injected intraperitoneally, 10 min before exercise.
- **Group CHE+LEP+EE, CHE plus late exercise preconditioning plus exhaustive exercise:** rats were treated as per Group LEP+EE, but CHE was injected intraperitoneally 10 min before exercise.

Rats were anesthetized with trichloroacetaldehyde monohydrate (400 mg/kg, intraperitoneally), and then were fixed on an animal operation table in the dorsal position. The abdominal cavity was quickly opened, and the blood was drawn from the inferior caval vein. For histological analysis, the heart was exposed for perfusion fixation, as described previously.⁹ For Western blot analysis,¹¹ the heart was rapidly excised, and the left ventricle free wall was isolated at the level of the near apex. Once the tissue was divided, the pieces were quick frozen in liquid nitrogen.

Detection of Serum Cardiac Biomarkers

Blood samples were centrifuged immediately after collection and the serum was separated. Serum cTnI levels were measured by using automated immunochemiluminescence on an Access 2 immunoassay system (Beckman Coulter, USA). This assay is based on a single-step sandwich principle, with paramagnetic

particles coated as the solid phase and 2 monoclonal cTnI antibodies.⁸ The sensitivity threshold for cTnI was 0.01 g/L.

By using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA), the serum NT-proBNP levels were measured with a rat NT-proBNP ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The sensitivity threshold for NT-proBNP was 1 g/L.

Hematoxylin-Basic Fuchsin-Picric Acid (HBFP) Staining

Rat hearts were measured by HBFP staining, as described previously.⁸ Five visual fields from each section, with 5 sections per group, totaling 25 visual fields, were randomly taken for morphometric analysis using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). The positive area and the integral optical density (IOD) were calculated.

Transmission Electron Microscopy Analysis

After the perfusion fixation, samples for the transmission electron microscopy analysis were taken from the tissue beneath the endocardial surface of the left ventricular anterior free wall at the level of the near apex, and prepared as previously described.⁹ Briefly, the tissue was fixed in paraformaldehyde and postfixed in osmium tetroxide. After osmium fixation, the tissue was dehydrated, embedded, sectioned and stained with uranium acetate-lead citrate. Samples were examined and photographed with a transmission electron microscope (H-800; Hitachi, Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from LV tissue with Trizol. After DNase treatment, 1 mg of total RNA samples was reversed transcribed with oligo primers and SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

Quantification of cardiac gene expression was determined by using real-time polymerase chain reaction (RT-PCR). The RT-PCR was performed using a PKCε (Gene Bank Accession No.: NM_017171.1) specific forward primer (5'-CCAACTCTATTGCTGCTTCCA-3') and reverse primer (5'-CATGAGGTCTCCACCGTTTACA-3') to amplify the product. The samples were normalized using the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Gene Bank Accession No.: NM_017008), using a forward primer (5'-GGAAAGCTGTGGCGTGAT-3') and reverse primer (5'-AAGGTGGAAGAATGGGAGTT-3'). The RT-PCR procedure was performed as previously described.¹²

In Situ Hybridization Protocol

In situ hybridization was performed using RNA probes for the PKCε gene with the PKCε ISH assay kit (Boster Inc, Wuhan, China). The 3 mRNA-probes were:

- (1) 5'-AAGCAACATCCATTCTTCAAGGAGATTGACTGGGT-3'
- (2) 5'-ACCAAGAGAGATGTCAATAACTTTGACCAAGACTT-3'
- (3) 5'-CAGATCAACCAGGAAGAATTCAAAGGCTTCTCCTA-3'

Briefly, after deparaffinization and dehydration, the sections were immersed in hydrogen peroxide for 10 min to block the endogenous peroxidase activity. After pretreatment steps, the target probe was applied and hybridized for 24 h at 40°C. Thereafter, the amplification steps including application of biotinylation-linked digoxin labeling, were performed for 60 min at 37°C prior to DAB-visualization.

Figure 1. Early and late cardioprotection of EP against exhaustive exercise-induced myocardial injury. **(A)** Alterations of serum cTnI levels in the cardioprotection of EP. Significant differences ($P<0.05$) are indicated as follows: from Group C (*), from Group EE (#). **(B)** Alterations of serum NT-proBNP levels in the cardioprotection of EP. Significant differences ($P<0.05$) are indicated as follows: from Group C (*), from Group EE (#), from Group EEP+EE (&), from Group LEP+EE (%). **(C)** Myocardial ischemia detected by HBFP staining. The ischemic cardiomyocytes stained a vivid crimson red color in contrast to the light brown color of non-ischemic tissue. Ischemic cardiomyocytes that were stained crimson red were dominated in Group EE and Group LEP+EE, while only the red patchy stain was seen in Group EEP+EE, Group CHE+EEP+EE and Group CHE+LEP+EE. Cardiomyocytes in Group C (C1&C2), Group EEP and Group LEP displayed a light brown color. Original magnification was $\times 400$. **(D)** Ultrastructural alteration of cardiomyocytes. Exhaustive exercise (EE) led to mitochondrial swelling, break or disappearance of the mitochondrial cristae, a diffused and less coherent Z-line, and wide interfibrillar space. EP alleviated exhaustive exercise-induced damage. The cardiomyocytes in Group EEP+EE and Group LEP+EE demonstrated damage to a lesser extent, involving alleviated intracellular edema and slight mitochondrial swelling. CHE did not abolish the protective effect of EP; similar results were observed in Group CHE+EEP+EE and Group CHE+LEP+EE where only a few vacuolated mitochondria were observed (original magnification $\times 10,000$). C, control; EE, exhaustive exercise; EEP, early exercise preconditioning; EEP+EE, early exercise preconditioning plus exhaustive exercise; LEP, late exercise preconditioning; LEP+EE, late exercise preconditioning plus exhaustive exercise; CHE+EEP+EE, chelerythrine plus early exercise preconditioning plus exhaustive exercise; CHE+LEP+EE, chelerythrine plus late exercise preconditioning plus exhaustive exercise; serum NT-proBNP, serum levels of N-terminal pro-hormone brain natriuretic peptide; HBFP, hematoxylin-basic fuchsin-picric acid.

Immunohistochemistry

Immunohistochemistry was performed, as described previously,⁸ for the observation of PKC ϵ in rat myocardium. Primary antibodies against PKC ϵ or p-PKC ϵ^{Ser729} , diluted at 1:50, were obtained from Santa Cruz (Santa Cruz Biotechnology, CA, USA), and incubated overnight at 4°C prior to detection using the streptavidin-biotin complex kit (Boster Bio-engineering, Wuhan, China), with a diaminobenzidine/peroxidase substrate to produce a brown color. The positive area and the IOD were

calculated in the same way as that for HBFP staining.

Western Blotting

Western blotting was performed with a SDS-PAGE Electrophoresis System, as described previously.⁸ Primary antibodies against PKC ϵ or p-PKC ϵ^{Ser729} were obtained from Santa Cruz (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Results were visualized with the enhanced chemiluminescence method and evaluated by ImageJ software (NIH,

Table 1. Hematoxylin-Basic Fuchsin-Picric Acid (HBFP) Staining Image Analysis Results in Rat Myocardium

Group	n	Positive area (μm^2)	IOD
C	25	0	0
EE	25	55.26 $\pm$ 6.91*	14092.39 $\pm$ 1761.04*
EEP	25	11.27 $\pm$ 4.52	2873.89 $\pm$ 1152.01
EEP+EE	25	33.77 $\pm$ 6.24#	8610.24 $\pm$ 1590.79#
LEP	25	0	0
LEP+EE	25	69.09 $\pm$ 14.79#	17617.76 $\pm$ 3770.28#
CHE+EEP+EE	25	31.17 $\pm$ 6.64	7947.65 $\pm$ 1691.97
CHE+LEP+EE	25	43.65 $\pm$ 8.26\$	11130.16 $\pm$ 2107.04\$

Values are expressed as mean $\pm$ SD. Significant differences ($P<0.05$) are indicated as follows: from Group C (*), from Group EE (#), from Group LEP+EE (\$).

C, control; CHE+EEP+EE, chelerythrine plus early exercise preconditioning plus exhaustive exercise; CHE+LEP+EE, chelerythrine plus late exercise preconditioning plus exhaustive exercise; EE, exhaustive exercise; EEP, early exercise preconditioning; EEP+EE, early exercise preconditioning plus exhaustive exercise; IOD, integral optical density; LEP, late exercise preconditioning; LEP+EE, late exercise preconditioning plus exhaustive exercise.

Figure 2. Changes of myocardial PKC ϵ mRNA expressions in rats. (A) PKC ϵ mRNA levels determined by quantitative real-time polymerase chain reaction (PCR). Significant differences ($P<0.05$) are indicated as follows: from Group C (*), from Group EE (#), from Group EEP+EE (&), from Group LEP+EE (%). (B) Distribution of PKC ϵ mRNA in cardiomyocytes by in situ hybridization. All groups of PKC ϵ mRNA in situ hybridization signal demonstrated a brown granular pattern of distribution. CN, the negative result of none treated with PKC ϵ mRNA probes in control group. Original magnification was $\times 400$. PKC ϵ , protein kinase C epsilon; C, control; EE, exhaustive exercise; EEP+EE, early exercise preconditioning plus exhaustive exercise; LEP+EE, late exercise preconditioning plus exhaustive exercise; CHE+EEP+EE, chelerythrine plus early exercise preconditioning plus exhaustive exercise; CHE+LEP+EE, chelerythrine plus late exercise preconditioning plus exhaustive exercise.

Figure 3. Changes of myocardium PKCε expressions in rats. (A) Myocardial PKCε levels were determined by Western blotting. Values are presented as mean±SD. Significant differences ($P<0.05$) are indicated as follows: from Group C (*), from Group EE (#), from Group EEP (&), from Group EEP+EE (%). (B) Immunohistochemistry staining of PKCε in cardiomyocytes. All groups of PKCε positive immunoreactivity demonstrated a brownish color. C1&C2 demonstrated the positive results of the control group; original magnification was ×1,000. Arrowheads from the other groups indicated PKCε positive immunoreactivity localized to intercalated disks; original magnification was ×400. PKCε, protein kinase C epsilon; C, control; EE, exhaustive exercise; EEP, early exercise preconditioning; EEP+EE, early exercise preconditioning plus exhaustive exercise; CHE+EEP+EE, chelerythrine plus early exercise preconditioning plus exhaustive exercise; CHE+LEP+EE, chelerythrine plus late exercise preconditioning plus exhaustive exercise.

Bethesda, MD, USA).

Statistical Analysis

Data were analysed using a one-way ANOVA (SPSS16.0; SPSS, Chicago, IL, USA) followed by the Student-Newman-Keuls comparison of groups. All results shown are significant ($P<0.05$), unless stated otherwise.

Results

Early and Late Cardioprotective Effect of EP Against Exhaustive Exercise

The mean running distance to exhaustion for Group EE, Group EEP+EE and Group CHE+EEP+EE were $2,657.37\pm975.57$ (n=18), $2,058.00\pm852.28$ (n=20), and $1,756.67\pm833.69$ m (n=16), respectively, and no significant differences were de-

tected between Group EE and Group EEP+EE ($P=NS$). Interestingly, the mean running distance to exhaustion for Group LEP+EE and Group CHE+LEP+EE were $4,590.00\pm1,582.82$ m (n=20) and $4,512.00\pm1,467.93$ m (n=20), respectively, and compared with Group EE, the mean running distance to exhaustion was significantly longer in Group LEP+EE and Group CHE+LEP+EE ($P<0.05$).

Serum cTnI levels (Figure 1A) in Group EE were significantly higher than in Group C ($P<0.05$). Compared with Group EE, serum cTnI levels in Group EEP+EE and Group LEP+EE decreased significantly ($P<0.05$).

Serum NT-proBNP levels (Figure 1B) in Group EE were significantly higher than those in Group C ($P<0.05$). Compared with Group EE, serum NT-proBNP levels in Group EEP+EE decreased significantly ($P<0.05$). No significant differences were found between Group EE and Group LEP+EE ($P=NS$).

Table 2. Protein Kinase C Epsilon (PKCε) Immunoreaction Image Analysis Results in Rat Myocardium

Group	n	Positive area (μm ²)	IOD
C	25	1.57±0.79	401.53±200.34
EE	25	3.48±1.06*	886.62±271.16*
EEP	25	1.52±0.72	387.66±183.40
EEP+EE	25	2.75±1.42#	701.36±362.72#
LEP	25	2.96±1.14*§	753.82±289.70*§
LEP+EE	25	2.16±1.44#	551.55±368.07#
CHE+EEP+EE	25	4.91±2.43&	1252.75±619.28&
CHE+LEP+EE	25	1.67±1.19	426.11±296.52

Significant differences (P<0.05) are indicated as follows: from Group C (*), from Group EE (#), from Group EEP+EE (&), from Group EEP (§).

Abbreviations as in Table 1.

Compared with Group EEP+EE, serum NT-proBNP levels in Group CHE+EEP+EE increased significantly (P<0.05). Compared with Group LEP+EE, serum NT-proBNP levels in Group CHE+EEP+EE decreased significantly (P<0.05).

HBFP stained images and image analysis (Table 1, Figure 1C) showed that the hypoxic and ischemic cardiomyocytes stained a vivid crimson red color in contrast to the light brown color of non-hypoxic and non-ischemic tissue. Cardiomyocytes in Group C and Group LEP displayed a light brown color while crimson red was seen in the majority of cases in Group EE and Group LEP+EE; the positive area and IOD of HBFP staining in Group EE were significantly increased compared with Group C (P<0.05). In Group EEP, only the red spot stain was seen in a small fraction of cardiomyocytes. In Group EEP+EE, Group CHE+EEP+EE and Group CHE+LEP+EE, the red patchy stain was scattered across the cardiomyocytes. The positive area and IOD of HBFP staining in Group EEP+EE were significantly decreased compared with those in Group EE (P<0.05). Nevertheless, the 2 values in Group LEP+EE were significantly increased compared with those in Group EE (P<0.05). Compared with Group LEP+EE, the positive area and IOD of HBFP staining in Group CHE+LEP+EE were significantly decreased (P<0.05), but there were no significant differences in Group CHE+EEP+EE compared with Group EEP+EE.

The ultrastructure of cardiomyocytes from the left ventricular anterior free wall showed that normal cardiomyocytes in Group C were mainly characterized by well-arranged myofibrils, intercalated discs and mitochondria, and cardiomyocytes in Group EEP and Group LEP (Figure 1D), which was similar to those described in Group C. In Group EE, exhaustive exercise led to mitochondrial swelling, break and disappearance of the mitochondrial cristae, a diffused and less coherent Z-line, wide interfibrillar space, and EP-alleviated exhaustive exercise-induced damage. The cardiomyocytes in Group EEP+EE and Group LEP+EE demonstrated damage to a lesser extent; only a few vacuolated mitochondria were observed. Similar results were observed in Group CHE+EEP+EE and Group CHE+LEP+EE.

mRNA Expression of PKCε in the Cardioprotective Effect of EP

The PKCε RT-PCR results (Figure 2A) and in situ hybridization results (Figure 2B) showed that, compared with Group C, PKCε mRNA levels decreased significantly in Group LEP (P<0.05), but there were no significant change in Group EE and Group EEP. Compared with Group EE, PKCε mRNA levels decreased significantly in Group EEP+EE (P<0.05). Compared with Group EEP+EE, PKCε mRNA levels increased significantly in Group CHE+EEP+EE (P<0.05), and

the same result was found between Group LEP+EE and Group CHE+LEP+EE (P<0.05).

Protein Expression of PKCε in the Cardioprotective Effect of EP

Western blot results showed that compared with Group C, PKCε levels increased robustly in Group EE, Group EEP and Group LEP (P<0.05; Figure 3A). Compared with Group EE, PKCε levels decreased significantly in Group EEP+EE (P<0.05). Compared with Group EEP+EE, PKCε levels increased robustly in Group LEP+EE (P<0.05). Compared with Group EEP+EE, PKCε levels increased robustly in Group LEP+EE (P<0.05). In addition, we did not find any significant difference in the PKCε level between Group EEP+EE and Group CHE+EEP+EE; the same result was also found between Group LEP+EE and Group CHE+LEP+EE.

The PKCε immunohistochemistry results revealed that in Group C, PKCε was localized and showed a cytosolic diffuse pattern; this result is highly consistent with previously published data (Figure 3B). Compared with Group C, the pattern of PKCε changed in other groups except for Group LEP+EE, which PKCε was preferentially localized to intercalated disks. Immunoreaction image analysis of PKCε revealed that in comparison with Group C, a positive reaction of PKCε was markedly increased in Group EE, Group EEP and Group LEP (P<0.05); compared with Group EE, a positive reaction significantly decreased in Group EEP+EE and Group LEP+EE (P<0.05). The opposite result was found between Western blot and immunoreaction image analysis where it was shown that in comparison with Group EEP, PKCε was markedly increased in Group LEP (P<0.05). Interestingly, compared with Group EEP+EE, a positive reaction significantly increased in Group CHE+EEP+EE (P<0.05), but the results were adverse between Group LEP+EE and Group CHE+LEP+EE (Table 2).

Protein Expression of p-PKCε^{Ser729} in the Cardioprotective Effect of EP

The p-PKCε^{Ser729} Western blotting results are presented in Figure 4A. Compared with Group C, p-PKCε^{Ser729} levels decreased robustly in Group EEP (P<0.05), but there was no significant change in Group EE and Group LEP. Compared with Group EE, p-PKCε^{Ser729} levels decreased significantly in Group EEP+EE (P<0.05). Compared with Group EEP, p-PKCε^{Ser729} levels increased significantly in Group LEP (P<0.05). Compared with Group EEP+EE, p-PKCε^{Ser729} levels increased robustly in Group LEP+EE (P<0.05). Compared with Group LEP+EE, p-PKCε^{Ser729} levels decreased significantly in Group CHE+LEP+EE (P<0.05).

Figure 4. Changes of myocardium p-PKC ϵ^{Ser729} expressions in rats. **(A)** Myocardial p-PKC ϵ^{Ser729} levels were determined by Western blotting. Values are presented mean $\pm$ SD. Significant differences ($P<0.05$) are indicated as follows: from Group C (*), from Group EE (#), from Group EEP (&), from Group EEP+EE (%), from Group LEP+EE (##). **(B)** Immunohistochemistry staining of p-PKC ϵ^{Ser729} positive immunoreactivity demonstrated a brownish color. C1&C2 demonstrated the positive results of the control group; original magnification was $\times 1,000$. Arrowheads from the other groups indicated p-PKC ϵ^{Ser729} positive immunoreactivity localized to the myocardial cytomembrane; original magnification was $\times 400$. p-PKC ϵ^{Ser729} , phosphorylated-protein kinase C epsilon of Ser729 in the Activation Loop; C, control; EE, exhaustive exercise; EEP, early exercise preconditioning; EEP+EE, early exercise preconditioning plus exhaustive exercise; LEP+EE, late exercise preconditioning plus exhaustive exercise; CHE+EEP+EE, chelerythrine plus early exercise preconditioning plus exhaustive exercise; CHE+LEP+EE, chelerythrine plus late exercise preconditioning plus exhaustive exercise.

The p-PKC ϵ^{Ser729} immunohistochemistry results (**Figure 4B**) revealed that in Group C, the p-PKC ϵ^{Ser729} was localized and showed a cytosolic diffuse pattern, and compared with Group C, the pattern of p-PKC ϵ^{Ser729} changed in Group EEP, EEP+EE, Group LEP, Group LEP+EE, Group CHE+EEP+EE and Group CHE+LEP+EE, in which p-PKC ϵ^{Ser729} was preferentially localized to the cytomembrane. Immunoreaction image analysis of p-PKC ϵ^{Ser729} revealed that there were no significant differences among Group C, Group EE, Group EEP and Group LEP (**Table 3**). However, in comparison with Group EEP, a positive reaction of p-PKC ϵ^{Ser729} was markedly increased in Group LEP ($P<0.05$). Interestingly, compared with Group LEP+EE, a positive reaction significantly increased in Group CHE+LEP+EE ($P<0.05$), which was opposite to the results found with Western blot result.

Discussion

The present study tested 2 hypotheses. The first is that exhaustive exercise-induced myocardial injury can be attenuated by only 1 bout of EP. The second is that EP affects PKC ϵ translocation or activation. The main findings were that EP could induce cardioprotection in direct proportion with the increase of PKC ϵ expression and translocation from cardiomyocytes to myocardial cell membranes.

Early and Late Cardioprotective Effect of EP Against Exhaustive Exercise

Exercise could enhance sustained relaxation to endothelium-dependent agonist stimulation in small arteries of control and ischemic hearts by enhancing nitric oxide contribution; endothelial Ca^{2+} responses have already been proven.^{13,14} Some re-

Table 3. Phosphorylated-Protein Kinase C Epsilon of Ser729 in the Activation Loop (p-PKC ϵ ^{Ser729}) Immunoreaction Image Analysis Results in Rat Myocardium

Group	n	Positive area (μm^2)	IOD
C	25	1.63 $\pm$ 1.33	415.95 $\pm$ 339.90
EE	25	1.04 $\pm$ 0.71	264.49 $\pm$ 182.16
EEP	25	1.47 $\pm$ 1.33	374.41 $\pm$ 339.83
EEP+EE	25	1.32 $\pm$ 1.31	335.52 $\pm$ 334.87
LEP	25	2.25 $\pm$ 1.52*	574.33 $\pm$ 387.23*
LEP+EE	25	1.23 $\pm$ 0.84	313.30 $\pm$ 213.54
CHE+EEP+EE	25	1.04 $\pm$ 0.44	265.69 $\pm$ 113.27
CHE+LEP+EE	25	5.67 $\pm$ 2.48%	1445.21 $\pm$ 632.70%

Significant differences ($P<0.05$) are indicated as follows: from Group EEP (*), from Group LEP+EE (%). Abbreviations as in Table 1.

searchers have found that EP can attenuate myocardial infarction area in rats and dogs;^{5,6} our recent study showed that the 1 bout of EP could attenuate myocardial injury induced by exhaustive exercise, but this cardioprotection was only observed during the early phase of EP.⁸ Therefore, the present study addressed whether EP could attenuate myocardial injury induced by exhaustive exercise during the late phase of EP. In this experiment, serum cTnI increased after rats underwent exhaustive exercise. However, during the early and late cardioprotective phases of EP, the rats that underwent exhaustive exercise showed decreased levels of serum cTnI. Myocardial HBFP staining data also showed that ischemic cardiomyocytes with crimson red dominated in Group EE, which indicated that rats in Group EE had myocardial anoxia or ischemia. But the present study hypothesis was slightly different from previous ideas about HBFP staining involving Group LEP+EE. This phenomenon might have been observed because the rats in Group LEP+EE underwent longer durations of treadmill exercises. In addition, we found that the extent of myocardial morphologic changes was not consistent with the level of serum NT-proBNP. Serum NT-proBNP data showed that Group LEP+EE had greater systolic wall stress during exercise. Similarly, some researchers found that the dynamics of NT-proBNP changes during and after ergometric exercises cannot be used for the diagnosis of exercise-induced heart injury.^{15,16} Therefore, it is reasonable to attribute the increased exercise capacity, at least in part, to an enhanced tolerance of the heart to ischemic insults. However, the severity of myocardial hypoxia and ischemia does not always match the severity of myocardial injury. Our results showed that prolonged exercise did not result in more severe ischemia/hypoxia and ultrastructural damage in cardiomyocytes. Taken together, these data indicate that EP can induce cardioprotection against exhaustive exercise during both the early and late phases of EP.

EP-Induced Early and Late Phase of Cardioprotection Is Associated With PKC ϵ Translocation

EP protects the heart from left ventricular dysfunction, oxidative injury, mitochondrial damage, and cell death.^{7,17} But the molecular mechanisms that mediate the powerful cardioprotective effect of EP remain to be elucidated. Several studies have shown that EP increased the expression of some myocardial proteins, such as heat shock proteins,¹⁸ mitochondrial and sarcolemmal ATP-sensitive potassium channels (mitoK_{ATP} or sarcK_{ATP}),¹⁹ cyclooxygenase-2,²⁰ and endoplasmic reticulum stress proteins,⁷ which can attenuate many types of heart injuries. It has also been demonstrated that, in IP, PKC modulates its downstream proteins and regulates K_{ATP} channels, myocar-

dial metabolism, and subsequent tissue oncosis.²¹ We originally hypothesized that PKC ϵ immunoreactivity might increase after EP, and was based in part on widespread experimental evidence supporting a cardioprotective role for PKC ϵ in a setting of IP, preventing decreases in infarct size after IP.²² The signaling pathways responsible for cardioprotection have been shown to involve regulation of the opening of both sarcK_{ATP} and mitoK_{ATP} channels, the mitochondrial permeability transition pore, and connexin 43(Cx43). Application of ψ eRACK potentiates sarcK_{ATP} during ischemia, which can reduce the rate of entry of Ca²⁺ into the cytosol and the preservation of ATP.²³ Carson and Korzick found that exercise can activate PKC ϵ in a manner similar to IP, even though 1 bout of exercise can lead to decreases in total PKC ϵ .²⁴ However, phosphorylated PKC ϵ (p-PKC ϵ ^{Ser729}, phosphorylation of Ser729 in the activation loop) in the myocardium was increased, and increases in p-PKC ϵ ^{Ser729} might activate its substrates to evoke cardioprotection.

The present study results show that EP can urge PKC ϵ translocated to intercalated disks (including immediately after EP and 24 h after EP). With PKC ϵ translocated to intercalated disks, the exhaustive-exercise-induced myocardial injury was significantly attenuated by the early phase of EP, indicating that intercalated disks might be downstream targets of PKC ϵ during the early phase of EP. This result was the same as those found by other studies, which showed that, upon activation, PKC ϵ translocated to multiple subcellular sites such as intercalated disks.^{25,26} Enhanced binding of PKC ϵ to Cx43, a major gap junction (GJ) protein, might be the cause of PKC ϵ translocating to intercalated disks.²⁷ Phosphorylation of Cx43 by PKC ϵ plays a crucial role in δ -opioid-induced suppression of GJ permeability in ischemic myocardium.²⁸ During the late phase of EP, PKC ϵ did not translocate to intercalated disks, but myocardial injury was still significantly attenuated, as it was during the early phase of EP. The cause of this might be related to PKC ϵ phosphorylation. The activity and intracellular localization of members of the PKC family are controlled by phosphorylation at 3 highly conserved sites in the catalytic kinase domain. Phosphorylation of the COOH-terminal hydrophobic priming site Ser729 of PKC ϵ increases kinase activity, and the absence of phosphorylation at this priming site reduces kinase activity.²⁹ These all indicate that p-PKC ϵ ^{Ser729} plays an important role in cardioprotection against I/R injury. The present study results showed that EP could cause p-PKC ϵ ^{Ser729} to translocate to the cytoplasmic membrane (both immediately after EP and 24 h after EP). With p-PKC ϵ ^{Ser729} localized at the cytoplasmic membrane, the exhaustive exercise-induced myocardial injury was significantly attenuated by EP, especially during the late phase. However, after exhaustive exercise, no translocation of p-PKC ϵ ^{Ser729}

took place, and myocardial injury was sustained. From these results, which indicated that p-PKC ϵ^{Ser729} was key to induced cardioprotection, it was found that p-PKC ϵ^{Ser729} could phosphorylate cytoplasmic membrane proteins such as CX43 and that the phosphorylation of CX43 could decrease cardiomyocyte GJ permeability, prevent the spreading of injury between conjoined cells and finally enhance resistance to ischemic injury.³⁰ Phosphorylation by PKC ϵ opens mitoK_{ATP} channels, preserving mitochondrial function and generating local ROS,³¹ which can further activate PKC ϵ in a positive feedback mechanism and confer cardioprotection.¹⁹

A significant quantitative difference in PKC ϵ and p-PKC ϵ^{Ser729} was observed between the early and late phases of EP. The present data show that PKC ϵ and p-PKC ϵ^{Ser729} were significantly higher in Group LEP+EE than in Group EEP+EE, but there was little difference between Group EEP and Group LEP. These results indicate that longer periods of treadmill exercise might lead to increases in PKC ϵ and p-PKC ϵ^{Ser729} . However, exhaustive exercise also induced high PKC ϵ levels and translocation of PKC ϵ to intercalated disks, but p-PKC ϵ^{Ser729} remained mainly in the cytoplasm and was distributed in a diffuse pattern. The reason why exhaustive exercise induced the translocation of PKC ϵ to intercalated disks is still unknown, but these results suggest that p-PKC ϵ^{Ser729} is essential to the cardioprotection that it confers.

It was originally hypothesized that PKC ϵ mRNA might also increase following EP, in part based on experimental evidence that the important role of PKC ϵ in a setting of preventing infarct size reduction followed IP. On the contrary, the present study results show that EP downregulated PKC ϵ mRNA levels (both immediately after EP and 24 h after EP). This was consistent with the results from a study by Carson and Korzick where the research showed that significant decreases were found in myocardial PKC ϵ mRNA levels immediately and 24 h after 1 bout of acute exercise.²⁴ This and the assessments of PKC ϵ protein expression, which showed protein PKC ϵ to be highly expressed during EP, showed that PKC ϵ mRNA undergoes downregulation upon sustained stimulation. The molecular mechanisms underlying the downregulation of PKC are still unclear, but studies showed that chronic activation of PKC eventually results in the complete dephosphorylation and degradation of the enzyme by a ubiquitin/proteasome-dependent mechanism, which we here refer to as downregulation.^{32,33} Therefore, cardioprotection afforded by EP might be mainly attributable to the translation of PKC ϵ and not to the transcription of PKC ϵ .

The Opposite Effect of CHE

CHE is a non-specific catalytic inhibitor of PKC,³⁴ which was found to attenuate PKC phosphorylation and translocation from cytosol to the membrane or nucleus.³⁵ During exercise, CHE suppressed the activation of 3 exercise-induced PKC isoforms (PKC α , PKC δ , and PKC ϵ) and attenuated the exercise-mediated reduction of myocardial injury size during ischemia-reperfusion injury.¹⁸ However, recent studies by the present team have shown CHE to have no effect on the cardioprotective effects of EP, and CHE did not attenuate the expression of PKC δ or p-PKC δ^{Thr507} proteins during EP.⁸ In fact, the influence of CHE on the cardioprotective effect is still a point of controversy. A study has found that the pharmacological inhibition of PKC before ischemia induced cardioprotection by reducing intracellular sodium overload via increases in Na-K-ATPase activity.³⁶ The observation was consistent with the present study results, which showed that CHE did not abolish the translocation of PKC ϵ or p-PKC ϵ^{Ser729} , and unexpectedly increased the expression of PKC ϵ and p-PKC ϵ^{Ser729} during both protective

phases of EP. Moreover, CHE did not attenuate the protective effect of EP, which adversely had a protective effect on EP. These phenomena might be associated with the mechanisms independent of PKC inhibition, and other signal transduction events during EP should also be evaluated as possible causes of the effects described above.

Conclusions

In summary, the current study results demonstrate that EP can attenuate exhaustive exercise-induced myocardial injury in the early and the late phases of cardioprotection, especially during the late phase of EP. After EP, PKC ϵ was markedly upregulated, PKC ϵ was translocated to myocardial intercalated disks, and p-PKC ϵ^{Ser729} was translocated to the myocardial cytoplasmic membrane. No translocation of p-PKC ϵ^{Ser729} was observed in the hearts of rats after exhaustive exercise. These results show that the translocation of p-PKC ϵ^{Ser729} plays an important role in EP-conferred cardioprotection. Injection of the PKC inhibitor, CHE, before EP failed to attenuate the cardioprotective effect of EP, suggesting that a CHE injection during exercise might not be an appropriate means of demonstrating the role of PKC ϵ .

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