

# Exercise preconditioning-induced late phase of cardioprotection against exhaustive exercise: possible role of protein kinase C delta

Zhe Hao · Shan-Shan Pan · Yu-Jun Shen · Jun Ge

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**Abstract** The objective of this study was to investigate the late cardiac effect of exercise preconditioning (EP) on the exhaustive exercise-induced myocardial injury in rats and the role of protein kinase C (PKC) in EP. Rats were subjected to a run on the treadmill for four periods of 10 min each at 30 m/min with intervening periods of rest of 10 min as an EP protocol. The exhaustive exercise was performed 24 h after EP. PKC inhibitor chelerythrine (CHE) was injected before EP. The results showed that EP increased the running ability of rats, and alleviated the exhaustive exercise-induced injury in cardiomyocytes, but pretreatment with PKC inhibitor CHE did not abolish the late phase cardioprotection of EP. A significant increase of PKC $\delta$ , both at the protein level and the mRNA level in the left ventricular myocardium of rats, accompanied by its activated form (phosphorylated on Thr507, p-PKC $\delta^{\text{Thr507}}$ ) translocated to intercalated disks and was found in the late phase of EP. This circumstance was not attenuated by CHE. These results suggested that a high level of PKC $\delta$  might be involved in cardioprotection against myocardial damage, but if activated PKC $\delta$  at reperfusion took on a key role in cardioprotection was still an outstanding question.

**Keywords** Exercise preconditioning · Exhaustive exercise · Myocardial injury · Protein kinase C delta

## Abbreviations

CHE	Chelerythrine
cTnI	Cardiac troponin I
Cx43	Connexin 43
EP	Exercise preconditioning
HBFP	Hematoxylin–basic fuchsin–picric acid
I/R	Ischemia/reperfusion
ISH	In situ hybridization
LV	Left ventricle
PKC	Protein kinase C

## Introduction

The cardiovascular benefits of regular physical exercise have been well documented. However, more and more evidence showed that impairments in myocardial function and elevations in highly specific cardiac biomarkers after prolonged exercise, suggested that strenuous physical exertion might also result in potential subclinical myocardial damage [1–4]. Therefore, developing a pragmatic countermeasure to reduce strenuous physical exercise-induced potential myocardial injury is vital [5, 6]. Recent studies demonstrated that, exercise preconditioning (EP), a single bout of moderately intense exercise, can render the heart more tolerant to a subsequent ischemic insult [7]. EP has two cardioprotective phases: an early phase, which occurs immediately after the exercise [8, 9], and a late phase, which peaks at 24 h post-exercise. It has been proposed that the protective effect is initiated by activation of intracellular signaling pathways similar to those induced by ischemic preconditioning [10, 11]. Several studies suggest that protein kinase C (PKC) activation plays an important role in the mechanism of EP

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[12–15]. The PKC family of isozymes has three major subgroups: the conventional calcium dependent ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ), the novel calcium independent ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ , and possibly  $\mu$ ), and the atypical PKCs ( $\zeta$  and  $\tau/\lambda$ ). Translocation of PKC isoform (s) from the cytosolic to the particulate fraction results in it binding to a specific substrate [16], and phosphorylation of a threonine residue in the PKC activation loop has an essential role in the maturation of this enzyme [17]. Translocated and specific PKC isoforms during myocardial ischemia are believed to participate in several functions including the opening of mitoKATP channels or the induction of gene expression, which finally afford a cardioprotective phenotype [18, 19].

In our previous study, we have shown that EP attenuated the exhaustive exercise-induced myocardial injury in rats in the early phase. Moreover, p-PKC $\delta^{\text{Thr507}}$  in EP cardiomyocytes was primarily localized to intercalated disks and nuclei. This indicated that an appropriate activation and translocation of PKC $\delta$  may represent a mechanism whereby EP induced an early phase of cardioprotection [20]. However, whether EP can exert the same cardioprotective effect on exhaustive exercise-induced myocardial injury in the late phase is unknown. In the present study, we observed the cardioprotective effect of EP against exhaustive exercise-induced myocardial injury in the late phase according to the evaluation of serum cTnI, serum MDA, and myocardial HBFP staining. In addition, we observed the phosphorylation and translocation of PKC $\delta$  and PKC $\delta$  mRNA expression according to the immunohistochemistry, western blotting, in situ hybridization, and quantitative real-time PCR. In order to confirm the role of PKC $\delta$  in the late phase of EP, a PKC inhibitor chelerythrine (CHE) was used.

## Methods

### Animals

Adult (8-week-old) male Sprague–Dawley rats (Sippr BK, Shanghai, China) were housed in standard cages maintained at constant temperature and humidity with a 12:12 h light/dark cycle and were fed and watered ad libitum. All animal care and experimental procedures were conducted in accordance with 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 approved by the Ethics Committee for Science Research of the Shanghai University of Sport.

### Experimental protocol

All animals underwent a light exercise familiarization on the treadmill for five consecutive days. The velocity on the

treadmill was 15 m/min and the exercise duration was 10–20 min/day. On the 7th day, animals were randomly assigned to five experimental groups:

Group C Control; rats were placed on the treadmill without belt movement.

Group EE Exhaustive exercise; rats ran to exhaustion on the treadmill (0 % grade) at a speed of 30 m/min and were sacrificed 0.5 h after the exhaustion. Exhaustion was defined as the rat being unable to upright itself when placed on its back.

Group LEP Late exercise preconditioning; rats were allowed to run on the treadmill (0 % grade) for four periods of 10 min each at 30 m/min with intervening periods of rest of 10 min, and were sacrificed 24 h after exercise. Exercise began and ended with 5 min “warm up” and “cool down” periods at 15 m/min.

Group LEP + EE Late exercise preconditioning plus exhaustive exercise; rats were treated as group LEP, but ran to exhaustion 24 h after exercise, and were sacrificed 0.5 h after the exhaustion.

Group CHE + LEP + EE CHE plus late exercise preconditioning plus exhaustive exercise; rats were treated as group LEP + EE, but CHE was injected intraperitoneally 10 min before exercise.

Animals were anesthetized with trichloroacetaldehyde monohydrate (400 mg/kg, intraperitoneally), and samples were taken as previously described [20]. Briefly, after the blood was drawn from the inferior caval vein, the heart was exposed for perfusion fixation for histology analysis, or rapidly excised for western blot and quantitative real-time PCR analysis.

In this study, we shared the same data sets in control (C) and exhaustive exercise (EE) group with our previous study [20], our previous study described the major finding of our systematic researches on the early cardiac effect of EP against myocardial injury.

### Detection of serum cardiac biomarkers

Blood samples were centrifuged immediately after collection and the serum was separated. Serum cTnI levels were measured by 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 two monoclonal cTnI antibodies [21]. The sensitivity threshold for cTnI was 0.01 g/L.

Using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA), the serum MDA levels were measured with a rat MDA ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

### Hematoxylin–basic fuchsin–picric acid (HBFP) staining

After the heart was postfixed, embedded in paraffin, sectioned and mounted, HBFP staining was performed as previously described [22]. The ischemic/hypoxic myocardial fibers stain a crimson red color under the light microscope (Olympus, Tokyo, Japan). For the histological analysis, five visual fields from each section, with five sections per group, totaling 25 visual fields, were randomly taken using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). The positive area and the integral optical density (IOD) were calculated.

### Immunohistochemistry

For immunohistochemistry [20], sections were deparaffinized and rehydrated, immersed in hydrogen peroxide to block the endogenous peroxidase activity, and then digested with pepsin for antigen retrieval. After unspecific binding was blocked with serum, sections were incubated with primary antibodies specific to PKC $\delta$  or p-PKC $\delta^{\text{Thr507}}$  (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:200 overnight at 4° C followed by detection using the strept actividin-biotin complex kit (Boster Bio-engineering, Wuhan, China) with diaminobenzidine/peroxidase substrate to produce a brown-colored signal, then counterstained with hematoxylin.

### In situ hybridization

In situ hybridization was performed according to previously described approaches [23–25], and an in situ hybridization detection kit for PKC $\delta$  mRNA (Boster Bio-engineering, Wuhan, China) was used. Briefly, sections were deparaffinized and rehydrated, immersed in hydrogen peroxide and digested with pepsin, incubated with a pre-hybridization solution, and then incubated with the hybridization mixture containing digoxigenin-labelled oligonucleotide probe at 42° C for 48 h. The following probe sequences for PKC $\delta$  mRNA (Accession number NM\_133307.1) detection were used (1:1:1 mixture): 5'-TGG AA G TCA ACA TTC GAC GCC CAC ATC TAT GAA GG-3' (Location of nucleotides: 525–557), 5'-GAC ATG CCT CAC CGA TTC AAG GTC TAT AAC TAC AT-3' (Location of nucleotides: 1044–1076), and 5'-AGG CTT CAC CCC TTT TTC AAG ACT ATC AAC TGG AA-3' (Location of nucleotides: 2148–2180). Following hybridization, the sections were washed in standard saline citrate (SSC), incubated with a solution to block nonspecific binding sites, and then strept actividin-biotin complex/diaminobenzidine detection procedures were performed

resulting in a brown staining product. As a negative control, the probe was omitted.

### Western blotting

Western blotting was performed with a SDS-PAGE Electrophoresis System as described previously [20]. Briefly, 50  $\mu$ g protein samples were resuspended in a reduced sample buffer, and then electrophoresed on 10 % SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. They were then sequentially probed with primary antibodies against PKC $\delta$  or p-PKC $\delta^{\text{Thr507}}$  (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase antibody was used to normalize the loading. After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Results were visualized with the enhanced chemiluminescence method and evaluated by ImageJ software (NIH, Bethesda, MD, USA).

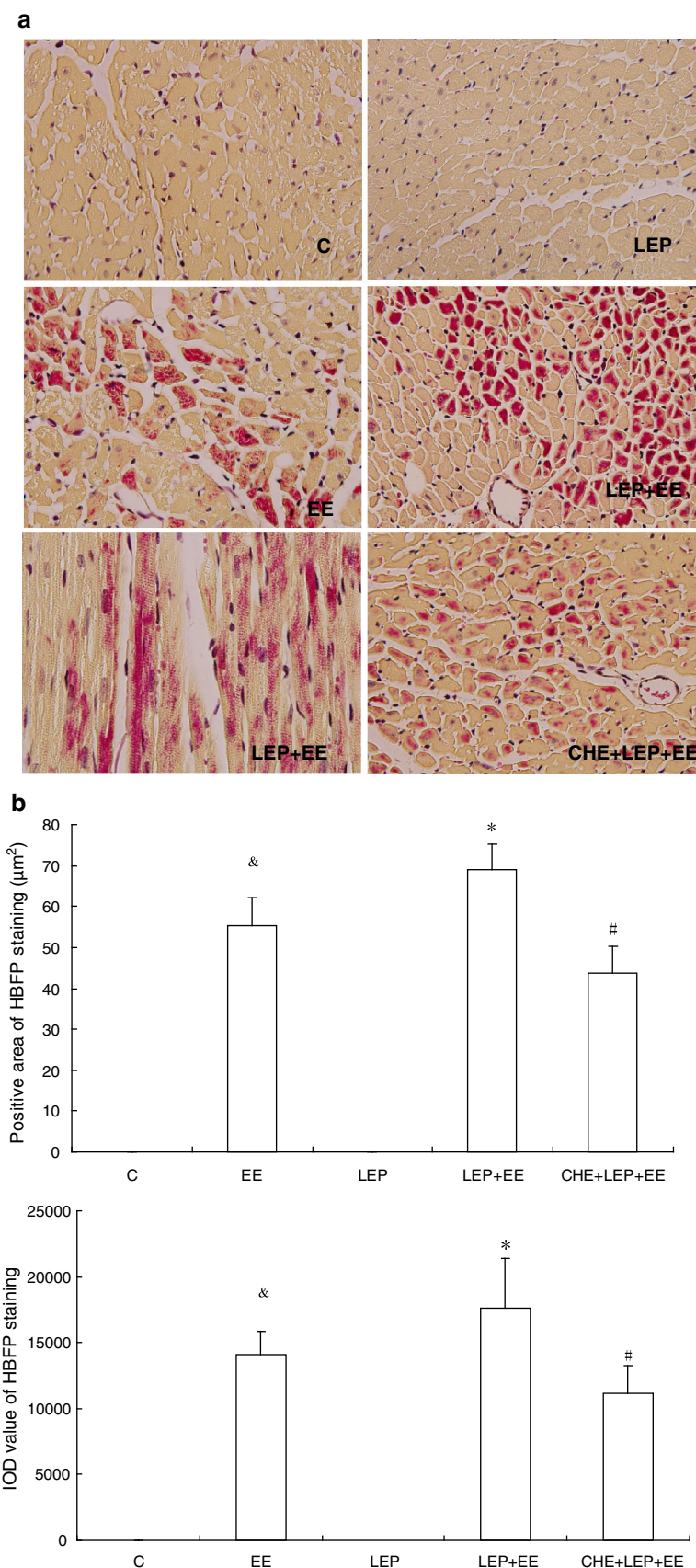
### Quantitative real-time PCR

Total RNA was isolated using acid guanidinium isothiocyanate-phenol–chloroform extraction and ethanol precipitation [26]. RNA concentration and purity were determined using spectrophotometry, and the integrity was examined by agarose gel electrophoresis. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples followed by gel electrophoresis analysis. First strand cDNA was synthesized for 60 min at 50° C in a 20  $\mu$ l reaction containing total RNA, Oligo [27]<sub>18</sub>, dNTPs, 5  $\times$  first strand buffer, dithiothreitol (DDT), RNase inhibitor, Superscript III reverse transcriptase (Invitrogen, Carlsbad, USA) and PCR-grade water. The qRT-PCR was performed on an ABI 7,900 Realtime PCR system (Applied Biosystems, CA, USA) using SYBR Green I as previously described [28]. GAPDH was used as an internal reference. Primers used were: PKC $\delta$ , 5'-GAA GGA GGC ACT CAC CAC AGA-3' (forward) and 5'-TGC AGG TCC AGC CAG AAC T-3' (reverse); GAPDH, 5'-GGA AAG CTG TGG CGT GAT-3' (forward) and 5'-AAG GTG GAA GAA TGG GAG TT-3' (reverse). Reactions contained 2  $\mu$ l cDNA, 0.5  $\mu$ l of each of the 10  $\mu$ M forward and reverse primers, 1  $\mu$ l dNTPs, 0.6  $\mu$ l MgCl<sub>2</sub>, 0.2  $\mu$ l 2  $\times$  ROX Reference Dye, 1  $\mu$ l 10  $\times$  PCR buffer, FastStart Taq DNA Polymerase, SYBR Green I, and PCR-grade water in a final volume of 10  $\mu$ l. The protocol was initiated by incubation at 95° C for 3 min followed by 40 cycles (15 s at 95° C, 20 s at 59° C and 20 s at 72° C). Melting curve analysis after the final cycle confirmed the presence of a single PCR product. The mRNA content of PKC $\delta$  and GAPDH was calculated from the cycle threshold values by using a standard curve constructed from a serial dilution of aliquots

**Fig. 1** Late cardiac effect of EP on exhaustive exercise.

**a** Myocardial ischemia detected by HBFP staining. Ischemic cardiomyocytes with crimson red dominated in group EE. The red staining was markedly increased in group LEP + EE (both in transection and longitudinal section) and decreased in group CHE + LEP + EE (original magnification,  $\times 400$ ).

**b** Quantitative analysis of HBFP staining. The positive area and IOD of HBFP staining in group EE were significantly higher than those in group C ( $P < 0.05$ ). The positive area and IOD of HBFP staining in group LEP + EE were significantly higher than those in group EE and group CHE + LEP + EE ( $P < 0.05$ ). Values are mean  $\pm$  SD, significant differences ( $P < 0.05$ ) are indicated as follows: from group C (&), from group EE (\*), and from group LEP + EE (#)





of cDNA pooled from all the samples [29], and the relative amount of PKC $\delta$  mRNA was normalized to GAPDH.

### Statistical analysis

All the results are expressed as mean  $\pm$  SD, and values were compared using a one-way analysis of variance (SPSS 10.0; SPSS, Chicago, IL). Upon confirmation of a significant main effect, individual differences were determined with post hoc analysis. A value of  $P < 0.05$  was considered significant.

## Results

### Late cardiac effect of EP on exhaustive exercise

The mean distance running to exhaustion in group EE, group LEP + EE, and group CHE + LEP + EE were  $2657.37 \pm 975.57$  m ( $n = 19$ ),  $4,590.00 \pm 1,582.82$  m ( $n = 20$ ), and  $4,512.00 \pm 1,467.93$  m ( $n = 20$ ), respectively. The running distance in group LEP + EE significantly increased compared with group EE ( $P < 0.05$ ), and no significant differences were found between group LEP + EE and group CHE + LEP + EE ( $P = \text{NS}$ ).

HBFP staining of cardiomyocytes of the left ventricular free wall at the level of the near apex and image analysis are shown in Fig. 1a. The ischemic cardiomyocytes stained a vivid crimson red color in contrast to the light brown color of nonischemic tissue. Cardiomyocytes in group C and LEP displayed a light brown color while crimson red was seen in the majority of cases in group EE. Contrary to our hypothesis, EP aggravated the exhaustive exercise-induced ischemia and hypoxia in cardiomyocytes, resulting in markedly enhanced red staining in group LEP + EE, which was attenuated by pretreatment with CHE (group CHE + LEP + EE). The quantitative analysis (Fig. 1b) showed that the positive area and IOD of HBFP staining in group LEP + EE were significantly higher than those in group EE ( $69.09 \pm 14.79$  vs.  $55.26 \pm 6.91$   $\mu\text{m}^2$ ,  $17,617.76 \pm 3,770.28$  vs.  $14,092.39 \pm 1,761.04$ ,  $P < 0.05$ ). Compared with group LEP + EE, the two values decreased significantly in group CHE + LEP + EE ( $43.65 \pm 8.26$  vs.  $69.09 \pm 14.79$   $\mu\text{m}^2$ ,  $11,130.16 \pm 2,107.04$  vs.  $17,617.76 \pm 3,770.28$ ,  $P < 0.05$ ). Positive area and IOD of HBFP staining in group C and group LEP were both 0.

Serum cTnI levels (Table 1) in group EE were significantly higher than those in group C ( $P < 0.05$ ). Compared with group EE, serum cTnI levels in group LEP + EE decreased significantly ( $P < 0.05$ ). Serum cTnI levels in group CHE + LEP + EE were lower than those in group LEP + EE; however, this reduction was not statistically significant ( $P = \text{NS}$ ).

**Table 1** Alterations of serum cTnI levels in the late cardiac effect of EP

Group	<i>n</i>	cTnI ( $\mu\text{g/L}$ )
C	20	$0.02 \pm 0.01$
EE	18	$3.87 \pm 5.04^*$
LEP	20	$0.02 \pm 0.01$
LEP + EE	19	$0.83 \pm 0.63^\#$
CHE + LEP + EE	19	$0.38 \pm 0.30$

Values are mean  $\pm$  SD, significant differences ( $P < 0.05$ ) are indicated as follows: from group C (\*), from group EE (#)

**Table 2** Alterations of serum MDA levels in the late cardiac effect of EP

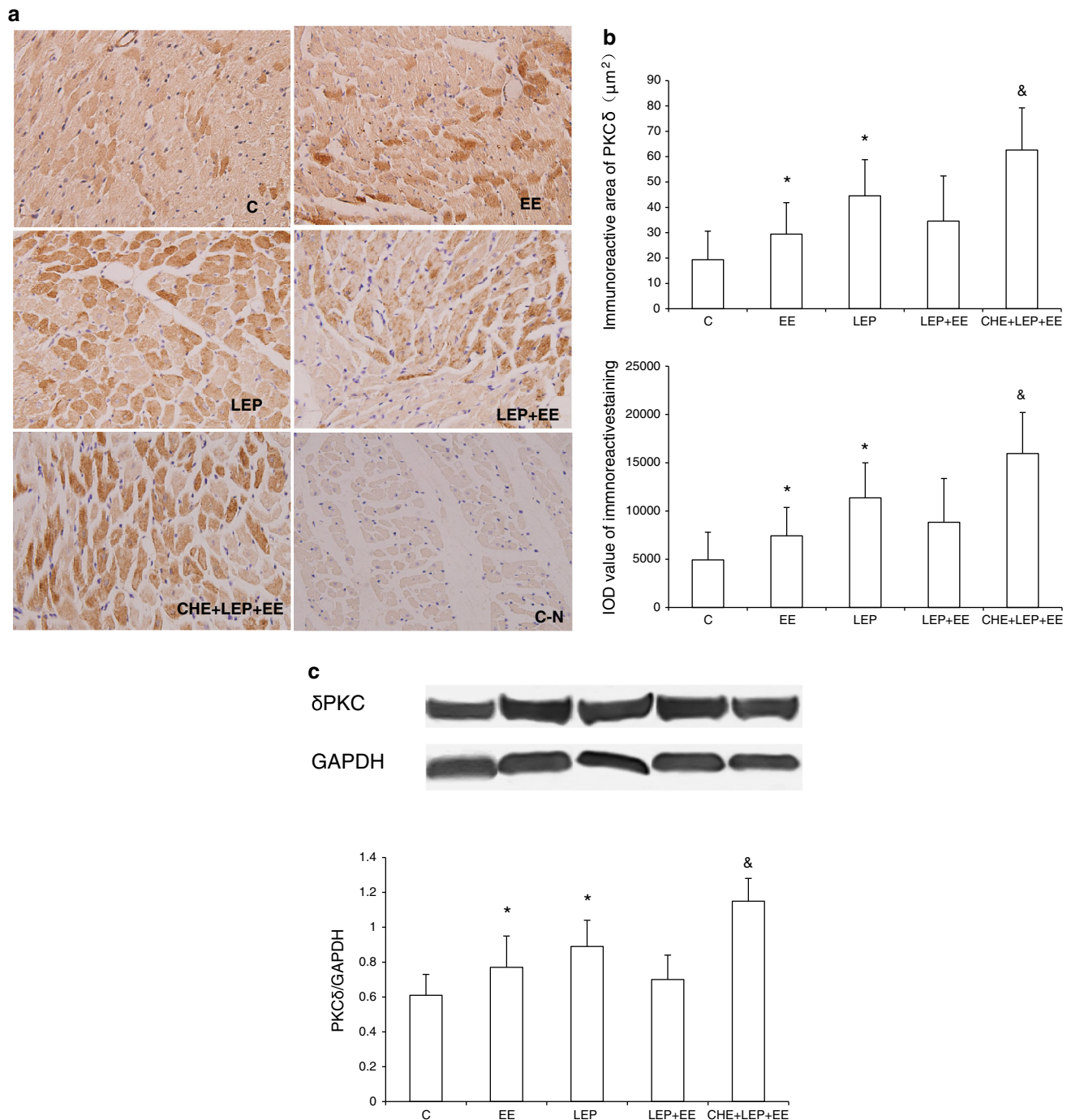
Group	<i>n</i>	MDA (nmol/ml)
C	20	$5.17 \pm 1.12$
EE	15	$7.04 \pm 1.96^*$
LEP	20	$5.33 \pm 0.93$
LEP + EE	20	$5.44 \pm 0.95^\#$
CHE + LEP + EE	20	$5.90 \pm 0.76$

Values are mean  $\pm$  SD, significant differences ( $P < 0.05$ ) are indicated as follows: from group C (\*), from group EE (#)

Serum MDA levels (Table 2) in group EE were significantly higher than those in group C ( $P < 0.05$ ). Compared with group EE, serum MDA levels in group LEP + EE decreased significantly ( $P < 0.05$ ). Serum MDA levels in group CHE + LEP + EE were higher than those in group LEP + EE; however, this reduction was not statistically significant ( $P = \text{NS}$ ).

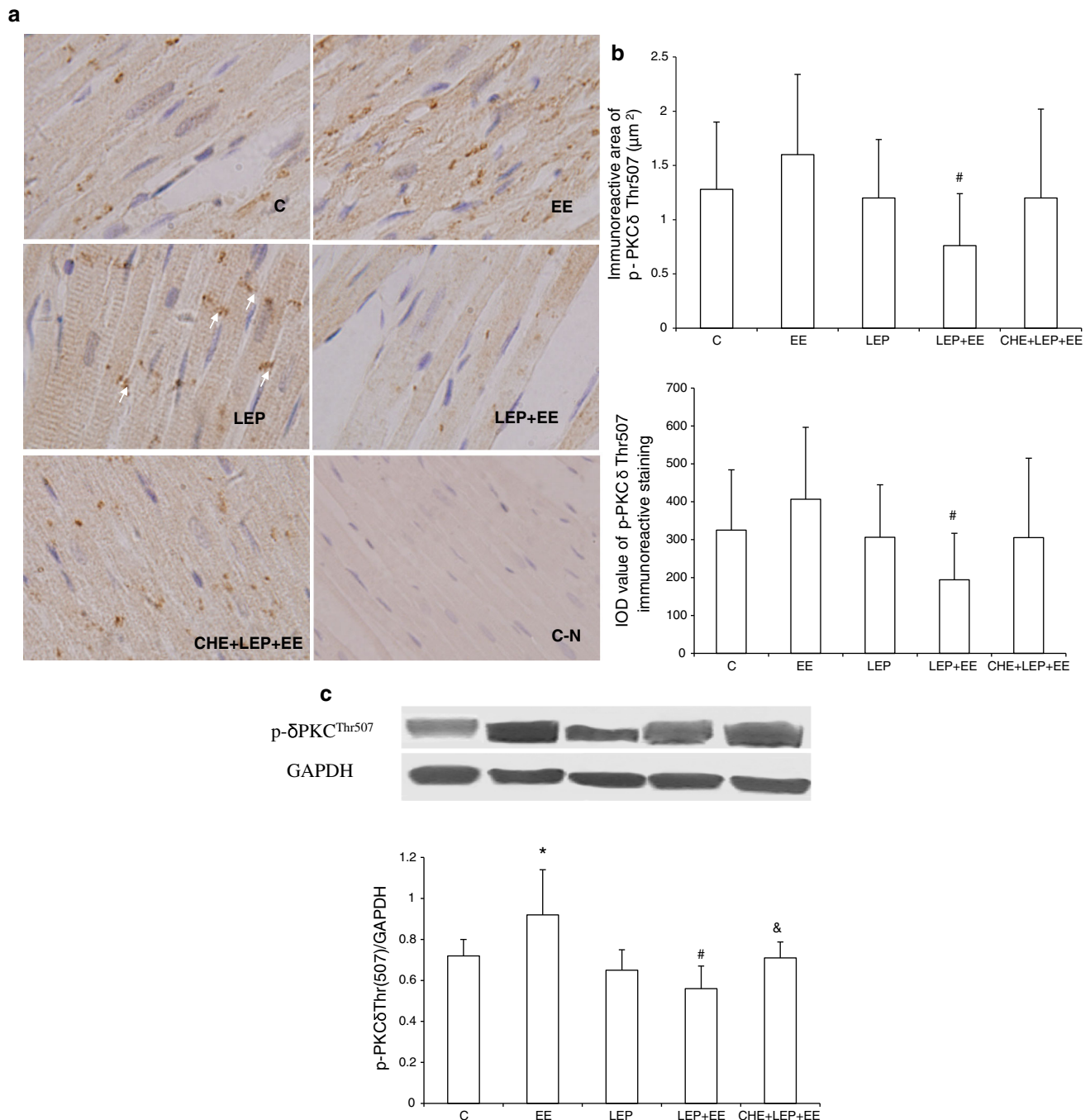
### Expression of PKC $\delta$ protein in the late cardiac effect of EP

Immunoreactivity for PKC $\delta$  was detected in cardiomyocytes from the left ventricular anterior free wall at the level of the near apex (Fig. 2a). PKC $\delta$  demonstrated a diffuse cytoplasmic staining in cardiomyocytes. Positive basal staining was noted in a diffuse cytoplasmic pattern in group C. Stronger staining has been observed in group EE, group LE, and group LEP + EE. Contrary to our hypothesis, immunoreactivity was elevated after CHE pretreatment in group CHE + LEP + EE. The semiquantitative analysis (Fig. 2b) showed that the positive area and IOD of the immunostaining in group EE and group LEP were significantly higher than those in group C ( $29.44 \pm 12.40$  and  $44.55 \pm 14.26$  vs.  $19.33 \pm 11.28$   $\mu\text{m}^2$ ,  $P < 0.05$ ;  $7,419.66 \pm 2,941.17$  and  $11,361.38 \pm 3,637.49$  vs.  $4,929.62 \pm 2,876.56$ ,  $P < 0.05$ ). Compared with group LEP + EE, the two values in group CHE + LEP + EE significantly increased ( $62.59 \pm 16.65$  vs.  $34.60 \pm 17.81$



**Fig. 2** Alteration of PKC $\delta$  expression in the late cardiac effect of EP. **a** Immunohistochemistry staining of PKC $\delta$  in cardiomyocytes. PKC $\delta$  demonstrated diffuse cytoplasmic staining in cardiomyocytes in all the groups (original magnification,  $\times 400$ ). C–N demonstrated the negative result of none treated with PKC $\delta$  primary antibody in control group. **b** Semi-quantitative analysis of PKC $\delta$  immunostaining. The positive area and IOD of the immunostaining in group EE and group LEP were significantly higher than those in group C ( $P < 0.05$ ). Compared with group LEP + EE, the two values in group CHE + LEP + EE

significantly increased ( $P < 0.05$ ). **c** Myocardial PKC $\delta$  levels determined by western blotting. Consistent with the results of immunostaining, total PKC $\delta$  levels in group EE and group LEP were also significantly higher than those in group C ( $P < 0.05$ ). Compared with group LEP + EE, PKC $\delta$  levels in group CHE + LEP + EE significantly increased ( $P < 0.05$ ). Values are mean  $\pm$  SD, significant differences ( $P < 0.05$ ) are indicated as follows: from group C (\*), from group LEP + EE (&)



**Fig. 3** Alteration of p-PKC $\delta^{\text{Thr507}}$  expression in the late cardiac effect of EP. **a** Immunohistochemistry staining of PKC $\delta$  in cardiomyocytes. p-PKC $\delta^{\text{Thr507}}$  demonstrated a granular, scattered cytoplasmic staining pattern in group C and group EE while the pattern changed in group LEP such that p-PKC $\delta^{\text{Thr507}}$  was preferentially localized to intercalated disks (arrow), but not in group LEP + EE and group CHE + LEP + EE. C–N demonstrated the negative result of none treated with p-PKC $\delta^{\text{Thr507}}$  primary antibody in the control group, original magnification,  $\times 1,000$ . **b** Semiquantitative analysis of p-PKC $\delta^{\text{Thr507}}$  immunostaining. The positive area and IOD of the immunostaining in group LEP + EE were significantly lower than those in group EE ( $P < 0.05$ ). Compared with group LEP + EE, the

two values in group CHE + LEP + EE were increased but not significantly ( $P < 0.05$ ). **c** Myocardial p-PKC $\delta^{\text{Thr507}}$  levels determined by western blotting. Consistent with the results of immunostaining, total p-PKC $\delta^{\text{Thr507}}$  levels in group EE were significantly higher than those in group C ( $P < 0.05$ ). However, compared with group EE, total p-PKC $\delta^{\text{Thr507}}$  levels in group LEP + EE were significantly decreased ( $P < 0.05$ ). Compared with group LEP + EE, p-PKC $\delta^{\text{Thr507}}$  levels in group CHE + LEP + EE were significantly increased. Values are mean  $\pm$  SD, significant differences ( $P < 0.05$ ) are indicated as follows: from group C (\*), from group EE (#), and from group LEP + EE (&)

$\mu\text{m}^2$ ,  $15,961.34 \pm 4,244.87$  vs.  $8,823.95 \pm 4,541.68$ ,  $P < 0.05$ ). No significant differences were detected between group LEP + EE and group EE ( $34.60 \pm 17.81$  vs.  $29.44 \pm 12.40 \mu\text{m}^2$ ,  $8,823.95 \pm 4,541.68$  vs.  $7,419.66 \pm 2,941.17$ ,  $P = \text{NS}$ ).

Figure 2c shows total PKC $\delta$  levels in cardiomyocytes determined by western blotting. Total PKC $\delta$  levels in group EE ( $n = 9$ ) and group LEP ( $n = 9$ ) were significantly higher than those in group C ( $n = 9$ ) ( $0.77 \pm 0.18$  and  $0.89 \pm 0.15$  vs.  $0.61 \pm 0.12$ ,  $P < 0.05$ ). Compared with group LEP + EE, PKC $\delta$  levels in group CHE + LEP + EE ( $n = 9$ ) significantly increased ( $1.15 \pm 0.13$  vs.  $0.70 \pm 0.14$ ,  $P < 0.05$ ). No differences were found between group LEP + EE ( $n = 9$ ) and group EE ( $0.70 \pm 0.14$  vs.  $0.77 \pm 0.18$ ,  $P = \text{NS}$ ).

#### Expression of p-PKC $\delta^{\text{Thr}507}$ protein in the late cardiac effect of EP

The immunostaining of p-PKC $\delta^{\text{Thr}507}$  (Fig. 3a) demonstrated a granular, scattered cytoplasmic staining pattern in group C, and the intensity of staining was stronger in group EE. However, the pattern changed in group LEP such that p-PKC $\delta^{\text{Thr}507}$  was preferentially localized to intercalated disks (arrow), but not in group LEP + EE and group CHE + LEP + EE. The staining was almost completely lost in group LEP + EE but restored in group CHE + LEP + EE. The semiquantitative analysis (Fig. 3b) showed that the positive area and IOD in group EE were higher than those in group C, but not significantly ( $1.60 \pm 0.74$  vs.  $1.28 \pm 0.62 \mu\text{m}^2$ ,  $406.89 \pm 189.82$  vs.  $325.12 \pm 158.97$ ,  $P = \text{NS}$ ). Compared with group EE, the two values in group LEP + EE were significantly decreased ( $0.76 \pm 0.48 \mu\text{m}^2$  vs.  $1.60 \pm 0.74$ ,  $P < 0.05$ ;  $194.30 \pm 122.58$  vs.  $406.89 \pm 189.82$ ,  $P < 0.05$ ). Compared with group LEP + EE, the two values in group CHE + LEP + EE were increased but not significantly ( $1.20 \pm 0.82$  vs.  $0.76 \pm 0.48 \mu\text{m}^2$ ,  $305.33 \pm 209.70$  vs.  $194.30 \pm 122.58$ ,  $P = \text{NS}$ ). No differences were found between group LEP and group C ( $1.20 \pm 0.54$  vs.  $1.28 \pm 0.62 \mu\text{m}^2$ ,  $306.29 \pm 138.51$  vs.  $325.12 \pm 158.97$ ,  $P = \text{NS}$ ). The p-PKC $\delta^{\text{Thr}507}$  semiquantitative analysis in LEP of intercalated disks showed that the positive area and IOD were  $0.19 \pm 0.09 \mu\text{m}^2$ ,  $26.40 \pm 12.14$ . The area analysis in LEP of p-PKC $\delta^{\text{Thr}507}$  localized to intercalated disks for total p-PKC $\delta^{\text{Thr}507}$  was 15.8 %.

Figure 3c shows that total p-PKC $\delta^{\text{Thr}507}$  levels determined by western blot in group EE ( $n = 9$ ) were significantly higher than those in group C ( $n = 9$ ) ( $0.92 \pm 0.22$  vs.  $0.72 \pm 0.08$ ,  $P < 0.05$ ). Compared with group EE, total p-PKC $\delta^{\text{Thr}507}$  levels in group LEP + EE ( $n = 9$ ) were significantly decreased ( $0.56 \pm 0.11$  vs.  $0.92 \pm 0.22$ ,  $P < 0.05$ ). Compared with group LEP + EE, p-PKC $\delta^{\text{Thr}507}$  levels in group CHE + LEP + EE ( $n = 9$ )

were significantly increased ( $0.71 \pm 0.08$  vs.  $0.56 \pm 0.11$ ,  $P < 0.05$ ). No differences were found between group LEP ( $n = 9$ ) and group C ( $0.65 \pm 0.10$  vs.  $0.72 \pm 0.08$ ,  $P = \text{NS}$ ).

#### The activated ratio of PKC $\delta$

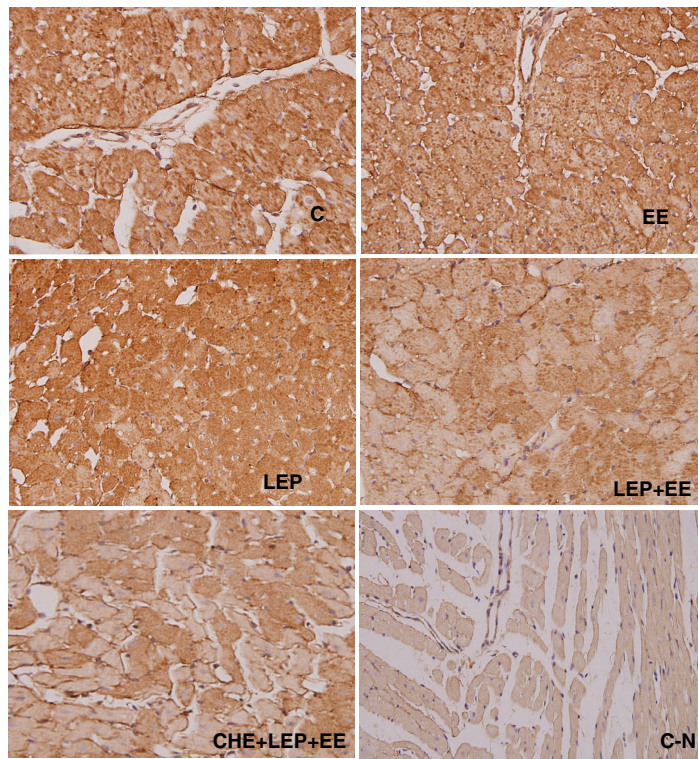
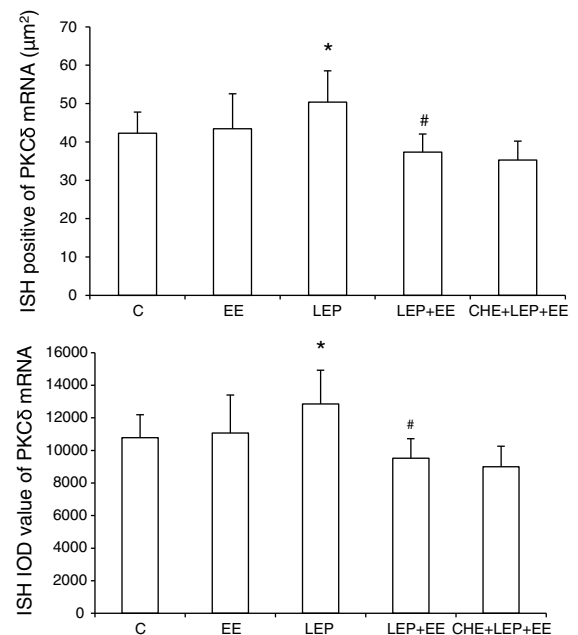
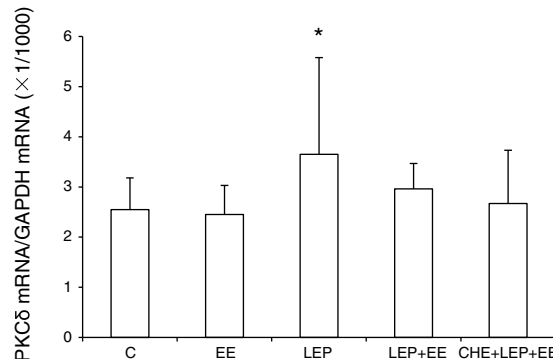
The activated ratio of PKC $\delta$  was evaluated by phosphorylated PKC $\delta^{\text{Thr}507}$  to total PKC $\delta$  (data from western blot) while the activated ratio of PKC $\delta$  in group C was 24 %, group EE 24 %, group LEP 13 %, group LEP + EE 16 %, and group CHE + LEP + EE 12 %.

#### Expression of PKC $\delta$ mRNA in the late cardiac effect of EP

Distribution of PKC $\delta$  mRNA by ISH was detected in cardiomyocytes from the left ventricular anterior free wall at the level of the near apex (Fig. 4a). The positive signal of PKC $\delta$  mRNA demonstrated a granular, diffuse cytoplasmic distribution pattern in cardiomyocytes. No difference was found in the intensity of positive signal between group C and group EE while a markedly enhanced signal was observed in group LEP. The signal in group LEP + EE was diminished compared with group EE. The intensity of the reaction was downregulated by CHE pretreatment in group CHE + LEP + EE. The semiquantitative analysis (Fig. 4b) showed that the area and IOD of the positive signal of PKC $\delta$  mRNA in group LEP were significantly higher than those in group C ( $50.39 \pm 8.16$  vs.  $42.28 \pm 5.53 \mu\text{m}^2$ ,  $P < 0.05$ ;  $12,848.98 \pm 2,080.83$  vs.  $10,780.88 \pm 1,411.32$ ,  $P < 0.05$ ). Compared with group EE, the two values in group LEP + EE significantly decreased ( $37.34 \pm 4.70$  vs.  $43.42 \pm 9.14 \mu\text{m}^2$ ,  $9,520.80 \pm 1,199.10$  vs.  $11,071.33 \pm 2,330.23$ ,  $P < 0.05$ ). No significant differences were detected between group C and group EE ( $42.28 \pm 5.53$  vs.  $43.42 \pm 9.14 \mu\text{m}^2$ ,  $10,780.88 \pm 1,411.32$  vs.  $11,071.33 \pm 2,330.23$ ,  $P = \text{NS}$ ), and group LEP + EE and group CHE + LEP + EE ( $37.34 \pm 4.70$  vs.  $35.29 \pm 4.93 \mu\text{m}^2$ ,  $9,520.80 \pm 1,199.10$  vs.  $8,997.90 \pm 1,258.05$ ,  $P = \text{NS}$ ).

Figure 4c shows PKC $\delta$  mRNA levels in cardiomyocytes determined by quantitative real-time PCR. PKC $\delta$  mRNA levels in group LEP ( $n = 8$ ) were significantly higher than those in group C ( $n = 8$ ) ( $3.65 \pm 1.93$  vs.  $2.55 \pm 0.63 \times 10^{-3}$ ,  $P < 0.05$ ). No differences were found between group EE ( $n = 9$ ) and group C ( $2.45 \pm 0.58$  vs.  $2.55 \pm 0.63 \times 10^{-3}$ ,  $P = \text{NS}$ ), group LEP + EE ( $n = 9$ ) and group EE ( $2.96 \pm 0.51$  vs.  $2.45 \pm 0.58 \times 10^{-3}$ ,  $P = \text{NS}$ ), group CHE + LEP + EE ( $n = 8$ ) and group LEP + EE ( $2.67 \pm 1.06$  vs.  $2.96 \pm 0.51 \times 10^{-3}$ ,  $P = \text{NS}$ ).



**a****b****c**

**Fig. 4** Alteration of PKCδ mRNA expression in the late cardiac effect of EP. **a** Distribution of PKCδ mRNA in cardiomyocytes by in situ hybridization. The positive signal of PKCδ mRNA demonstrated a granular, diffuse cytoplasmic distribution pattern in cardiomyocytes. The intensity of the hybridization signal was markedly strong in group LEP. C–N, the negative result of none treated with PKCδ mRNA probes in the control group. **b** Semiquantitative analysis

of the positive signal of PKCδ mRNA. The area and IOD of the positive signal of PKCδ mRNA in group LEP were significantly higher than those in group C. The **c** PKCδ mRNA levels determined by quantitative real-time PCR. PKCδ mRNA levels in group LEP were significantly higher than those in group C ( $P < 0.05$ ). Values are mean  $\pm$  SD, significant differences ( $P < 0.05$ ) are indicated as follows: from group C (\*), from group EE (#)

## Discussion

The key findings of this study can be summarized as follows: 1) EP significantly increased the running distance to exhaustion of rats, and alleviated the exhaustive exercise-induced injury in cardiomyocytes, but pretreatment with PKC inhibitor CHE did not abolish the late phase cardioprotection of EP. 2) After 24 h of EP, PKCδ was found to be markedly upregulated in left ventricular myocardium of

rats. During the late phase cardioprotection of EP, p-PKCδ<sup>Thr507</sup> was translocated to intercalated disks. Even though PKCδ was markedly upregulated during exhaustive exercise, but p-PKCδ<sup>Thr507</sup> was mainly distributed in the cytoplasm. CHE injection before EP did not suppress the activation or translocation of PKCδ.

To our surprise, EP in the late phase markedly enhanced the exercise performance, which was usually improved by weeks or months of training. Several investigators have

demonstrated that EP in the late phase significantly decreased infarct size induced by ischemia/reperfusion in animals, Domenech et al., had dogs take treadmill training after 1 h coronary artery ligation and 4.5 h reperfusion. They found that exercise prior to a coronary occlusion induced early and late preconditioning of the infarct size, the early effect was mediated through mitochondrial ATP-sensitive potassium channels [8]. Yamashita et al., used a rat model to determine whether exercise can reduce ischemic injury to the heart and elucidate a mechanism for the cardioprotective effect of exercise. Results showed that exercise significantly reduced the magnitude of a myocardial infarction in a biphasic manner. The time course for cardioprotection resembled that of the change in manganese superoxide dismutase (Mn-SOD) activity [30]. Then Yamashita et al., had rats exercise on a treadmill for 30 min at 23–27 m/min, then 24 h later, hearts were Langendorff perfused and subjected to 35 min left main coronary artery occlusion followed by 120 min reperfusion. They found that infarct size was reduced to 17.3 from 48.4. To examine the potential signalling pathway, and determine protein kinase C activation during exercise appears to be an important signal mediator of this protective response. Melling et al., found that that selected isoforms of PKC played an important role in exercise-mediated protection of the myocardium during ischemia–reperfusion injury [12]. Jimenez et al., investigated acute (swimming) exercise, and found it was sufficient such that sustained beneficial effects against cardiac functional decline were observed after high-dose isoproterenol administration [31]. In our previous study, EP in the late phase markedly attenuated the isoproterenol-induced myocardial injury in rats [22]. Studies also revealed that the underlying mechanisms might be involved in the increased manganese superoxide dismutase (Mn-SOD) activity and heat shock protein 70 (HSP70) expression in the late phase of EP [13, 30].

In this experiment, serum cTnI was chosen for evaluation of myocardial injury. The high serum cTnI levels may suggest membrane disruption of mechanical stress or irreversible myocyte degeneration during exercise [32]. In addition, serum MDA was chosen for evaluation of oxidative damage. The high serum MDA levels may suggest a myocardial injury occurred [33]. HBFP staining techniques were used in the present experiment to prove early myocardial ischaemia, and myocardial ischemic fiber staining produced a vivid crimson color [34]. Our results showed that the even though prolonged exercise finally resulted in more severe ischemia/hypoxia (HBFP staining), myocardial damage was still markedly attenuated (serum cTnI and MDA level). These results were similar with many investigations such that EP could attenuate myocardial injury. Quindry et al. [35] reported that the mitochondrial or sarcolemmal ATP-sensitive potassium channel mediates

exercise-induced cardioprotection against post-IR cell death and apoptosis in rats. Lee et al. [36] reported that three to five consecutive days of exercise training promotes cardioprotection, at least in part, by increasing mitochondrial antioxidants, preventing mitochondrial release of reactive oxygen species, and protecting cardiac mitochondria against IR-induced oxidative damage and functional impairment. In addition, several observations found that HBFP staining was not a useful tool in the recognition of myocardial necrosis [37]. Therefore, EP could alleviate the exhaustive exercise-induced oxidative damage and myocardium injury, but prolonged exercise could aggravate ischemia/hypoxia of myocardial in rats.

We also found that PKC $\delta$  was significantly increased both at the protein and the mRNA levels in the late phase of EP. The changes of PKC $\delta$  mRNA expression may be sufficient to account for the elevation of PKC $\delta$  protein, although other factors operating on the protein level may also be involved [38]. Studies showed that a high level of PKC $\delta$  might be involved in cardioprotection against ischemic damage, such as enhanced ability to cardiac ischemic tolerance [39], increased NCX-mediated Ca<sup>2+</sup> influx [40], and improved the recovery of left ventricular developed pressure and mechanical function [41]. For example, Kudo et al. [42] observed a significant protection against myocardial infarction 24 h after treatment with the selective adenosine A<sub>1</sub> receptor agonist 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA) in rats, PKC $\delta$  was upregulated 24 h after treatment with CCPA, and inhibition of PKC $\delta$  abolished the cardioprotective effect of CCPA. Neckár et al. [43] demonstrated that adaptation to chronic intermittent hypoxia (CIH) led to a marked upregulation of PKC $\delta$  and CIH-induced cardioprotection against ischemic insults in rats is partially mediated by PKC $\delta$ . It has been believed that the distinctive activation mechanism of PKC $\delta$  was involved in the coordination of phosphorylation and translocation events [44]. The beneficial role of PKC $\delta$  associated with its translocation to mitochondria and/or sarcolemma was confirmed in a variety of experiments on ischemic and pharmacological preconditioning [40, 45]. Melling et al. [14] showed that membrane levels of PKC $\delta$  in rat myocardium were significantly elevated in the late phase of EP. In our study, the activated ratio of PKC $\delta$  (data from western blot) in group C was 24 %, group EE 24 %, group LEP 13 %, group LEP + EE 16 %, and group CHE + LEP + EE 12 %. These results could not explain whether activated PKC $\delta$  had a key role in EP-induced cardioprotection. But our novel finding is that p-PKC $\delta^{\text{Thr507}}$  was preferentially localized to intercalated disks in the late phase of EP. The p-PKC $\delta^{\text{Thr507}}$  localized to intercalated disks in the LEP positive area to total p-PKC $\delta^{\text{Thr507}}$  was 15.8 %. These results demonstrated that components of intercalated disks such as connexin43

( $C \times 43$ ) may represent a molecular target of PKC $\delta$  in EP induced cardioprotection [46]. However, there still exist several controversial opinions, which reported that the activated PKC $\delta$  at reperfusion is potential harmful [47–49]. Our results showed that, compared with exhaustive exercise alone, exhaustive exercise pretreated with EP led to a markedly downregulation of p-PKC $\delta^{\text{Thr507}}$ , suggesting that after prolonged exercise, the activated PKC $\delta$  at reperfusion was downregulated in order to stabilize the intracellular environment and maintain cell survival in the myocardium. Taken together, EP promoted PKC $\delta$  localized to intercalated disks. A high level of PKC $\delta$  may enhance the ability of the myocardium to a subsequent exercise-induced myocardial injury, resulting in a marked increase in exercise capacity of rats. Nevertheless, after prolonged exercise, whether activated PKC $\delta$  at reperfusion has a key role in cardioprotection is still an outstanding question.

In addition, we found that the increase at protein level was enhanced by CHE treatment, but the response was not apparent at the mRNA level. This was consistent with the results from a study by Carson where the research showed that significant decreases were found in myocardial PKC $\epsilon$  mRNA levels immediately and 24 h after one bout of acute exercise [15]. This and the assessments of PKC $\delta$  protein expression, which showed protein PKC $\delta$  to be highly expressed by CHE treatment, showed that PKC $\delta$  mRNA undergoes downregulation upon sustained stimulation. The molecular mechanisms underlying the downregulation of PKC are still unclear, but studies have shown that chronic activation of PKC eventually results in the complete dephosphorylation and degradation of the enzyme by an ubiquitin/proteasome-dependent mechanism, which we here refer to as downregulation [50, 51].

It was observed that administration of CHE suppressed the membrane translocation of myocardial PKC $\delta$  in the late phase of EP and attenuated the exercise mediated reduction of myocardial infarct size during I/R injury [14]. However, our study showed that CHE treatment before EP neither prevented the translocation of PKC $\delta$  nor accelerated the exhaustive exercise-induced ischemia/hypoxia and damage in cardiomyocytes. On the contrary, it promoted the expression of PKC $\delta$  and provided a strong resistance to the myocardial injury. These results were consistent with our previous study that CHE treatment before EP did not attenuate the protective effect of EP against ISO-induced myocardial injury, and unexpectedly provided a more prominent cardioprotective effect [22]. In fact, the influence of CHE on the cardiac effect varied depending on the dosage and the experimental model [52–54], and an independent role of CHE on Na $^{+}$ -K $^{+}$ -ATPase activity was also reported [55]. Thus, its influence on cardioprotection should be interpreted with caution with respect to the role of PKC.

## Conclusions

The present work demonstrated that EP enhanced the exercise capacity of rats, and attenuated exhaustive exercise-induced myocardial injury in the late phases of cardioprotection. A significant increase of PKC $\delta$  in the late phase of EP, both at the protein level and the mRNA level in the left ventricular myocardium, was accompanied with p-PKC $\delta^{\text{Thr507}}$  translocation to intercalated disks. Compared with exhaustive exercise alone, exhaustive exercise pretreated with EP led to a markedly downregulation of p-PKC $\delta^{\text{Thr507}}$ . These results suggested that a high level of PKC $\delta$  might be involved in cardioprotection against ischemic damage, but whether activated PKC $\delta$  at reperfusion took a key role in cardioprotection was still an outstanding question. CHE injection during exercise might not be an appropriate means of demonstrating the role of PKC $\delta$ .

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**Conflict of interest** The authors declare no conflict of interest.

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