Loss of ischaemic preconditioning in ovariectomized rat hearts: possible involvement of impaired protein kinase C ε phosphorylation

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Aims The aims of this study were to determine whether chronic oestrogen withdrawal influences the development of ischaemic preconditioning (IPC) in female hearts, to investigate the mechanism whereby IPC is impaired, and to assess whether direct activation of protein kinase C (PKC) can mimic IPC in female hearts with chronic oestrogen depletion.

Methods and results We performed Sham-operation (Sham) or bilateral ovariectomy on 16-week-old Sprague–Dawley female rats. Ovariectomized rats were randomized to subcutaneous implantation of 17β-estradiol (OxE) or placebo (OxP) pellets. Four weeks later, isolated, perfused hearts were subjected to 30 min of ischaemia followed by 120 min of reperfusion with or without three cycles of 5 min ischaemia/5 min reperfusion. The cardioprotective effect of IPC was completely lost in the OxP group. Western immunoblots revealed that in the OxP group, IPC failed to translocate PKCε to the membranous fraction and that phosphorylation of PKCε (Ser^{729}) and phosphoinositide-dependent kinase (PDK) 1 (Ser^{241}) was impaired. Oestrogen replacement restored the IPC effect, the translocation and phosphorylation of PKCε, and the phosphorylation of PDK1. In the OxP group, pre-treatment with a PKCε selective activator peptide (Ψ–εRACK) mimicked the IPC effect. Pre-treatment with a phosphatidylinositol-3 kinase inhibitor before IPC abrogated the translocation and phosphorylation of PKCε in the Sham group.

Conclusions The cardioprotective effect of IPC is lost in female hearts with chronic oestrogen withdrawal and this is due, at least in part, to impaired translocation and phosphorylation of PKCε. Selective activation of PKCε-mediated signalling can fully restore the IPC effect in a manner analogous to oestrogen replacement.

1. Introduction

In most industrialized nations, coronary heart disease (CHD) is the leading cause of mortality in adult women. As in males, the incidence of CHD in females increases with age,[1] but it is significantly lower than that in males until approximately 70 years of age. Since the incidence of CHD in females changes dramatically with the menopausal status,[1] ovarian hormones are believed to protect female hearts from arteriosclerosis and CHD.

Some epidemiological studies have found that in-hospital mortality was higher in female patients with acute coronary syndrome (ACS) when compared with male patients.[2] A higher age at onset of ACS in female patients may contribute to higher in-hospital mortality. However, whether mortality after myocardial infarction is higher in females than in males remains controversial.

Clinical investigations of the effect of hormone replacement therapy (HRT), such as the Women's Health Initiative and the Heart and Oestrogen-Progestin Replacement Study

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II,[3,4] have failed to demonstrate the usefulness of HRT in preventing the development of CHD and in reducing the mortality in post-menopausal patients with CHD. Collectively, these clinical trials indicate that HRT should not be used for the purpose of protecting the cardiovascular system.[5] At least in part, the failure of these trials reflects the fact that our understanding of the cardiovascular effects of menopause and HRT is still limited.

The most powerful cardioprotective phenomenon identified to date is ischaemic preconditioning (IPC), an innate response that renders the heart relatively resistant to ischaemia (I)/reperfusion (R) injury.[6,7] Although the effect of chronic oestrogen deficiency on myocardial I/R injury (including myocardial infarction) has been actively investigated during the last decade,[8–12] little is known regarding whether chronic oestrogen deficiency affects the development of IPC. Accordingly, the aim of this study was to determine the influence of chronic oestrogen withdrawal on the development of IPC in female hearts and to investigate the mechanism(s) whereby the development of IPC is impaired in this setting. To accomplish these goals, we evaluated whether oestrogen replacement can restore the IPC effect in ovariectomized female rats. In addition, we investigated whether direct activation of protein kinase C (PKC) (which triggers the cardioprotective effect of IPC) can mimic the IPC effect in the absence of oestrogen replacement.

2. Methods

All procedures in the present study conformed to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996).

A total of 130 female Sprague–Dawley (SD) rats (16-week-old) were randomly divided into two groups. Sham-operation (Sham) or bilateral ovariectomy was performed as described previously.[10–12] Then, ovariectomized rats were randomized to receive either subcutaneous implantation of a 17β-estradiol pellet (60-day release pellet containing 500 µg, Innovative Research of America, Sarasota, FL, USA) (OxE group) or a placebo pellet (OxP group).[8,10,11] Successful ovariectomy was confirmed by measurements of serum 17β-estradiol levels and visible atrophy of the uterus when rats were sacrificed.

2.1 Langendorff perfusion of the hearts

Four weeks after the operation, the hearts were quickly excised under anaesthesia and perfused with modified Krebs–Henseleit buffer according to the Langendorff procedure. A plastic catheter with a latex balloon was inserted into the left ventricle (LV) through the left atrium, and the LV end-diastolic pressure was adjusted to 10 mmHg by filling the balloon with water, as described previously.[13,14] The hearts were paced at 5 Hz while the LV pressure was measured and pacing was turned off, and the balloon was deflated during global ischaemia and reperfusion.

A total of 16 rats from each group were assigned to two subgroups: IPC(+) and IPC(−). After a 10 min initial wash-out perfusion in a non-recirculating mode, the isolated rat hearts were perfused with Krebs–Heinseleit buffer in a recirculating mode. In the IPC(−) group, after initial recirculating perfusion for 40 min, the hearts were subjected to 30 min of global ischaemia, followed by 120 min of reperfusion. In the IPC(+) group, after 10 min of initial perfusion, IPC was induced by three cycles of 5 min ischaemia/ 5 min reperfusion, followed by 30 min of global ischaemia/120 min reperfusion. Infarct size (% of LV) was quantitated as described

previously.[15] The perfusate was collected during reperfusion, and total lactate dehydrogenase (LDH) and creatine kinase (CK) activity released into the perfusate was measured by standard enzymatic methods and expressed as IU/g wet weight of the ventricle.[13,14]

2.2 Measurement of serum parameters

Serum fasting glucose, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, and free fatty acid (FFA) levels were measured by standard enzymatic methods. Serum β-estradiol levels were measured by using commercially available ELISA kits.

2.3 Tissue sample preparation

Before the induction of sustained ischaemia, five hearts from each group were harvested quickly for western immunoblotting and measurement of PKC activity. For western immunoblotting of PKCδ and ε, cytosolic, nuclear, and membranous fractions were prepared as described previously.[16] For measurement of PKC activity and for western immunoblotting of phosphorylated PKCε and phosphorylated phosphoinositide-dependent kinase 1 (PDK1), cytosolic and membranous fractions were prepared as described previously.[15,17]

2.4 Measurement of protein kinase C activity

Total PKC activity in the cytosolic and membranous fractions was measured by using a PKC enzyme assay kit (Amersham Bioscience, Buckinghamshire, UK) and [γ-^{32}P] ATP, according to the manufacturer's instructions.

2.5 Western immunoblotting

Standard sodium dodecyl sulphate–polyacrylamide gel electrophoresis western immunoblotting techniques were used to assess the protein levels of PKCδ, PKCε, PDK1, the phosphorylated form of PKCε, and the phosphorylated form of PDK1, as described previously.[13,15,17] Initially, specific antibodies recognizing the phosphorylated form of PKCε or PDK1 were used; the membranes were then stripped and reprobed with standard PKCε or PDK1 antibodies recognizing both phosphorylated and non-phosphorylated forms in order to normalize the protein levels of the phosphorylated form. The total protein contents of PKCδ, PKCε, and PDK1 were expressed as a percentage of the corresponding value in the Sham IPC(−) group. Polyclonal antibodies against PKCδ, PKCε, and phosphorylated PKCε at the Ser^{729} residue (p-Ser^{729} PKCε) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and polyclonal antibodies against PDK1 and phosphorylated PDK1 at the Ser^{241} residue (p-Ser^{241} PDK1) from Cell Signalling Technology, Inc. (Beverly, MA, USA).

2.6 Effect of protein kinase C activators

Forty-two rats from the Sham and the Ox groups were assigned to three subgroups. After initial perfusion in a recirculating mode for 10 min, the perfusate was switched to the modified K–H buffer containing one of the following drugs: 1,2-dioctanoly *sn*-glycerol (DOG; Sigma Chemical, St Louis, MO, USA) (10 µM), ψ-εRACK (KAE1-1; Kai Pharmaceuticals, South San Francisco, CA, USA) (200 nM), or C1 (control peptide; Kai Pharmaceuticals) (200 nM) (*n* = 7, each). After 10 min, the perfusate was switched back to the normal buffer, and then the hearts were perfused in a non-recirculating mode for 10 min to wash out each drug. All the hearts were then subjected to 30 min of global ischaemia/120 min of reperfusion. The dose of DOG used in this study has previously been shown to attenuate myocardial I/R injury without changes in the heart rate or LV pressure in isolated perfused male rat hearts.[16,18] DOG was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was 0.02%. Inagaki *et al.*[19] have reported that pre-treatment with 500 nM of Ψ-εRACK attenuated myocardial I/R injury without affecting the heart rate or LV pressure in isolated perfused male

rat hearts. However, Ψ–εRACK at a concentration of 500 nM had a positive inotropic effect in our model (data not shown); therefore, we used 200 nM Ψ–εRACK. Immediately before use, Ψ–εRACK and C1 were directly dissolved in the Krebs–Henseleit buffer.

2.7 Effect of the phosphatidylinositol-3 (PI3)-kinase inhibitor

Five hearts in the Sham and OxP groups underwent initial perfusion in a recirculating mode for 10 min, following which the perfusate was switched to the modified Krebs–Henseleit buffer containing wortmannin (Wako Pure Chemical, Osaka, Japan) (100 nM).[18] After 10 min, the hearts were subjected to the IPC protocol (3×5 min ischaemia/5 min reperfusion) and immediately harvested for western immunoblotting of PKCε. Wortmannin was dissolved in DMSO and the final concentration of DMSO was 0.02%.

2.8 Statistical analyses

Data are reported as the mean $\pm$ SEM. For intragroup comparisons, haemodynamic variables were analysed by a two-way analysis of variance (ANOVA) (time and group), followed by a Scheffe's *post hoc* test. For intergroup comparisons, data were analysed by a one-way ANOVA, followed by a Scheffe's *post hoc* test. The P-value of less than 0.05 was defined as statistically significant. Statistical analyses were performed using Stat-View 5.0 software (SAS Institute, Cary, NC, USA) for Windows.

3. Results

Body weight and total ventricular weight increased significantly in the OxP group. However, the ratio of ventricular weight to body weight in the OxP group was lower than that in the Sham group (*Table 1*). Oestrogen replacement restored these values to the levels seen in the Sham group. Serum estradiol levels decreased significantly in the OxP group, but did not become zero (*Table 1*), because the adrenal glands also produce small amount of sex hormones. The average levels of serum estradiol in the OxE group were 2.5-fold higher than those in the Sham group but were still in the physiological range. Serum total cholesterol levels were higher and serum FFA levels were lower in the OxP group compared with the Sham group (*Table 1*). Oestrogen replacement restored serum FFA levels and increased serum HDL-cholesterol levels.

Left ventricular function at baseline did not differ among the three groups (*Table 2*). In the absence of IPC, infarct size did not differ among the three groups, although infarct size in the OxE group tended to be smaller than that in the OxP group ($0.1 < P < 0.05$) (*Figure 1A*). Ischaemic preconditioning significantly reduced infarct size and total CK and LDH release into the perfusate during reperfusion in the Sham and OxE groups (*Figure 1A, B and C*). However, in the OxP group, IPC failed to reduce infarct size and total CK and LDH release.

Pre-treatment with DOG, Ψ–εRACK, or C1 did not affect the LV function in any group (*Table 2*). Pre-treatment with Ψ–εRACK significantly reduced infarct size in both groups (*Figure 1A*). In contrast, a favourable effect of DOG on infarct size was observed only in the Sham group. Pre-treatment with C1 did not affect the infarct size in either group. In the Sham group, both DOG and Ψ–εRACK attenuated total CK and LDH release into the perfusate during reperfusion, whereas C1 had no effect (*Figure 1B and C*). In the OxP group, however, total CK and LDH release into the perfusate was reduced by Ψ–εRACK, not by the DOG.

There was no difference in the total PKC activity among groups (*Figure 2A*). In the absence of IPC, the ratio of PKC activity in the membranous fraction to total activity was lower in the OxP group compared with the Sham group. Ischaemic preconditioning increased the ratio of PKC activity in the membranous fraction to the total PKC activity in all groups, and there was no difference among groups (*Figure 2B*). The protein levels and the distribution of PKCδ and PKCε did not differ among the three groups without IPC (data not shown). Ischaemic preconditioning translocated PKCδ to the membranous fraction in all groups (*Figure 3A*). Ischaemic preconditioning translocated PKCε to the membranous fraction in the Sham and OxE groups, but not in the OxP group (*Figure 3B*).

Ischaemic preconditioning increased p-Ser^{729} PKCε in the cytosolic fraction in all groups, but the increase in the OxP group was significantly less than that in the Sham group (*Figure 4A*). In contrast, the levels of p-Ser^{729} PKCε in the membranous fraction did not differ between the preconditioned and non-preconditioned rats in each group. In the absence of IPC, the protein levels and the distribution of PDK1 did not differ among the three groups (data not

Table 1 Body weight, total ventricular weight, and serum parameters

	Sham	OxP	OxE
	($n = 16$)	($n = 16$)	($n = 16$)
Body weight (g)	305 ± 1	364 ± 1*	294 ± 3**
Total ventricular weight (g)	1.23 ± 0.01	1.36 ± 0.01*	1.17 ± 0.03**
Total ventricular weight/body weight (%)	0.41 ± 0.01	0.37 ± 0.01*	0.40 ± 0.03**
Serum parameters	($n = 8$)	($n = 8$)	($n = 8$)
Fasting blood sugar (mg/dL)	143 ± 15	147 ± 20	138 ± 16
Total cholesterol (mg/dL)	89 ± 7	102 ± 5*	98 ± 7
HDL-cholesterol (mg/dL)	26 ± 2	28 ± 1	30 ± 2*
Triglycerides (mg/dL)	10.3 ± 0.6	9.2 ± 0.9	9.4 ± 1.2
Free fatty acids (μEq/L)	604 ± 52	518 ± 61*	625 ± 56**
	($n = 16$)	($n = 16$)	($n = 16$)
Serum estradiol levels (pg/mL)	25.4 ± 1.4	6.0 ± 0.2*	63.0 ± 4.0*,**

Sham, Sham-operation group; OxP, bilateral ovariectomy plus placebo pellet implantation group; OxE, bilateral ovariectomy plus 17β-estradiol pellet implantation group; HDL, high-density lipoprotein; *$P < 0.05$ vs. the Sham group, **$P < 0.05$ vs. the OxP group.

Table 2 Cardiac parameters in each group

Parameter	Baseline					After treatment		
	LVSP (mmHg)	LVEDP (mmHg)	LVDP (mmHg)	+dP/dt	−dP/dt	LVSP (mmHg)	LVEDP (mmHg)	LVDP (mmHg)
Sham								
IPC(−) (n = 8)	91 ± 7	10.1 ± 0.1	80 ± 8	2480 ± 130	1510 ± 90			
IPC(+) (n = 8)	86 ± 3	10.1 ± 0.3	75 ± 3	2470 ± 110	1490 ± 80			
Sham IPC(−)								
+DOG (n = 7)	90 ± 7	10.3 ± 0.3	80 ± 7	2390 ± 120	1450 ± 80	85 ± 6	10.5 ± 0.3	74 ± 6 (93 ± 4)
+C1 (n = 7)	91 ± 8	10.5 ± 0.3	81 ± 8	2480 ± 140	1500 ± 90	95 ± 5	10.5 ± 0.4	84 ± 5 (104 ± 4)
+ψ-εRACK (n = 7)	89 ± 5	10.3 ± 0.3	79 ± 5	2420 ± 110	1480 ± 70	93 ± 5	10.6 ± 0.6	82 ± 5 (105 ± 5)
OxP								
IPC(−) (n = 8)	86 ± 6	10.3 ± 0.3	76 ± 6	2350 ± 120	1440 ± 80			
IPC(+) (n = 8)	87 ± 3	10.5 ± 0.1	77 ± 3	2410 ± 100	1480 ± 70			
OxP IPC(−)								
+DOG (n = 7)	85 ± 4	10.3 ± 0.4	75 ± 4	2390 ± 120	1450 ± 90	82 ± 4	10.6 ± 0.4	71 ± 4 (96 ± 3)
+C1 (n = 7)	86 ± 4	10.5 ± 0.2	75 ± 4	2420 ± 110	1480 ± 80	86 ± 5	10.6 ± 0.2	75 ± 5 (100 ± 4)
+ψ-εRACK (n = 7)	90 ± 3	10.5 ± 0.3	79 ± 3	2380 ± 90	1440 ± 70	91 ± 5	10.5 ± 0.3	81 ± 5 (103 ± 8)
OxE								
IPC(−) (n = 8)	92 ± 7	9.9 ± 0.1	82 ± 6	2510 ± 110	1610 ± 90			
IPC(+) (n = 8)	94 ± 6	10.0 ± 0.3	84 ± 7	2530 ± 100	1620 ± 90			

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; +dP/dt, peak positive dP/dt; −dP/dt, peak negative dP/dt; data in parentheses, percentage change of corresponding value at baseline.

shown); furthermore, without IPC, the ratio of p-Se[241] PDK1 to total PDK1 was higher in the OxE group when compared with the Sham group (*Figure 4B*). Ischaemic preconditioning increased p-Se[241] PDK1 in the cytosolic fraction in the Sham and OxE groups, but not in the OxP group.

In the Sham group, treatment with wortmannin prior to IPC abrogated PKCε translocation to the membranous fraction following IPC (*Figure 5A*). Treatment with wortmannin prior to IPC also abrogated the increase in p-Ser[729] PKCε in the cytosolic fraction following IPC in the Sham group (*Figure 5B*). No further changes in the distribution or phosphorylation of PKCε were observed in the OxP group treated with wortmannin (data not shown).

4. Discussion

This study provides several major new findings. Our data demonstrate that, in female hearts with chronic oestrogen withdrawal, (i) the cardioprotective effects of IPC are abrogated; (ii) IPC fails to translocate PKCε to the membranous fraction; (iii) the IPC-induced phosphorylation of PKCε at the Ser[729] residue is impaired; (iv) oestrogen replacement restores the IPC effect, as well as the translocation and phosphorylation of PKCε; and (v) pre-treatment with a selective activator of PKCε-mediated signalling, but not with a nonselective PKC activator, also rescues the phenotype and successfully mimics the salubrious effects of IPC. In addition, our data demonstrate that in the female heart, PI3-kinase-mediated signalling is located upstream of the activation of PKCε during the development of IPC. Our findings imply that the mechanism responsible for the loss of IPC in ovariectomized rats is at the level of PKCε or proximal to it, and that the downstream signalling pathways involved in the IPC are intact and can be effectively mobilized.

Although in the present study, chronic oestrogen withdrawal did not affect myocardial I/R injury in the non-preconditioned state; the results of previous studies of this issue have been controversial.[8–12] Likewise, the data regarding whether chronic oestrogen depletion interferes with the development of IPC have not been entirely consistent.[20–22] The apparent discrepancy in the previous reports might be due, at least in part, to different experimental models (*in vivo* vs. *ex vivo*), different I/R or IPC protocols, different parameters used to assess myocardial damage, and, possibly, species differences. Song *et al.*[22] demonstrated that chronic oestrogen withdrawal by ovariectomy increased infarct size and exacerbated the recovery of the LV function after I/R in isolated female mouse hearts. They further indicated that gonadectomy impaired the development of IPC in both male and female mice. Peng *et al.* reported that ovariectomy did not affect the recovery of the LV function after I/R in isolated perfused rat hearts; however, the effect of IPC on the recovery of the LV function was less in ovariectomized rats than in Sham-operated rats.[20] Sbarouni *et al.*[21] demonstrated that in an *in vivo* rabbit model, ovariectomy did not affect infarct size either in the absence or in the presence of IPC. However, ovariectomized rabbits showed considerable variability in infarct size and the effect of IPC on infarct size tended to be attenuated when compared with intact rabbits (infarct size: intact rabbits, 0.144 ± 0.021 cm^3 vs. ovariectomized rabbits, 0.249 ± 0.028 cm^3; $n = 6$ each), although the difference was not statistically significant.[21] Together, these reports support the concept that the IPC effect is, in some way, impaired in female hearts with chronic oestrogen withdrawal; however, they do not provide mechanistic insights into how chronic oestrogen withdrawal impairs the IPC effect.
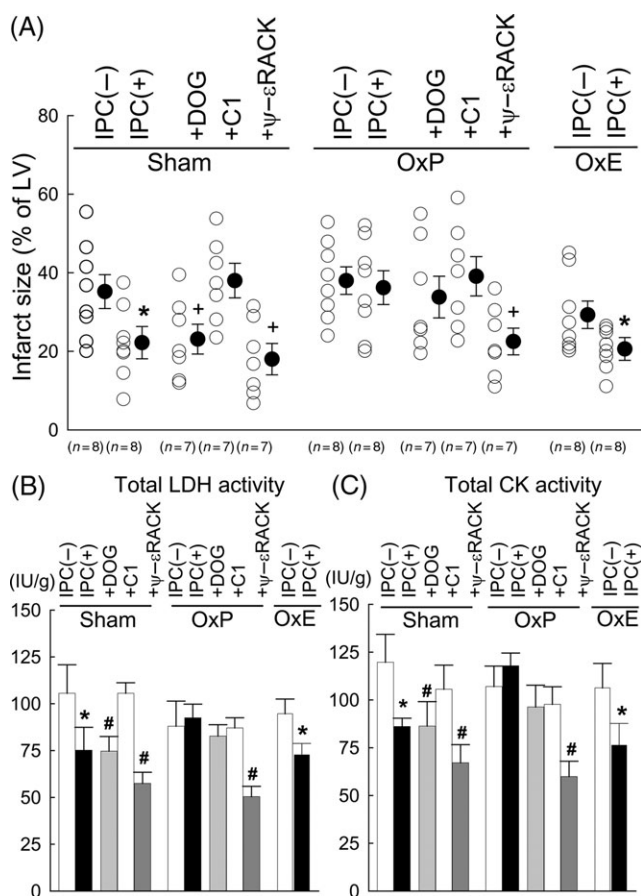
Figure 1 The effect of ischaemic preconditioning, direct protein kinase C activation by 1,2-dioctanoyl sn-glycerol, ψ-εRACK (KAE1-1), or control peptide (C1) on infarct size in each group. (A) Infarct size. (B) Total lactate dehydrogenase activity released into the perfusate during reperfusion. (C) Total creatine kinase activity released into the perfusate during reperfusion. Data are represented as the mean ± SEM. *$P < 0.05$ vs. the corresponding IPC(−) group, +$P < 0.05$ vs. the corresponding +C1 group.
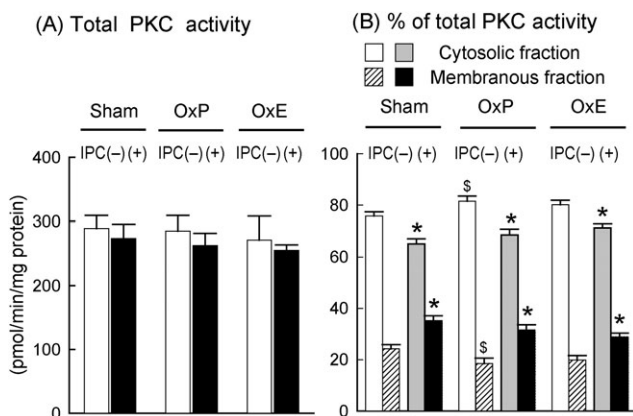
Figure 2 Protein kinase C activity. (A) Total protein kinase C activity. (B) Percentage of protein kinase C activity in the membranous or cytosolic fraction as a fraction of the total protein kinase C activity. Data are represented as the mean ± SEM. *$P < 0.05$ vs. the corresponding IPC(−) group, $P < 0.05$ vs. the corresponding Sham group.

In the absence of IPC, infarct size in ovariectomized rats implanted with 17β-estradiol pellets tended to be smaller than that in ovariectomized rats implanted with placebo pellets (Figure 1). This finding may support the previous

reports that exogenous 17β-estradiol administration, in itself, exerts the cardioprotective effect.[8,10] However, the evidence that chronic oestrogen replacement interferes with myocardial ischaemic/reperfusion injury has not been entirely consistent. As expected, oestrogen replacement successfully restored the IPC effect in the ovariectomized rat heart (Figure 1).

Protein kinase C plays a key role in the IPC signalling cascade in cardiomyocytes.[6,7,23,24] Among the various isoforms of PKC, PKCα, δ, and ε have been reported to be translocated to the membranous fraction by IPC in young male rat hearts.[25] We previously demonstrated that the IPC effect is lost in middle-aged male rat hearts.[14] Additionally, Tani et al.[16] have demonstrated that IPC failed to translocate both PKCδ and ε to the membranous fraction in middle-aged male rat hearts. Accordingly, we first investigated the intracellular distribution of PKCδ and ε before and after IPC. In contrast to middle-aged male hearts, IPC translocated PKCδ to the membranous fraction but failed to translocate PKCε to the membranous fraction in female hearts with chronic oestrogen withdrawal.

We do not have direct evidence that, in the preconditioned myocardium, PKC and PDK1 translocated instead of pre-existing PKC and PDK1 being degraded in one location and synthesized and accumulated in another location. However, the rapidity of the changes in the subcellular distribution of PKC and PDK1 after IPC (observed within 30 min) makes it unlikely that they reflected de novo synthesis of those proteins. Mounting evidence also supports the concept that PKC and other proteins translocate in preconditioned myocardium.[7,17,23] Thus, the most plausible interpretation of our observations is that a relatively small amount of PKC and PDK1 translocate after IPC, but this small change is essential for the development of the IPC effect.

Several possibilities might explain the impairment in PKCε translocation in the OxP group. We examined the phosphorylated state of PKCε before and after IPC because recent investigations demonstrated that phosphorylation of the three residues located in the kinase domain is essential for activation and translocation of novel PKC isozymes in various cell types.[26–29] The phosphorylation of the Thr^{566} residue located in the activation loop leads to the autophosphorylation of the Thr^{710} residue at the turn motif.[26,28] The Ser^{729} residue in the hydrophobic motif at the C-terminus may then be autophosphorylated[26,28] or it may be phosphorylated by an autologous kinase controlled by mTOR,[30] thus making PKCε ready for translocation. Accordingly, the impaired p-Ser^{729} PKCε observed in our study (Figure 4A) suggests a decrease in the capacity of PKCε for translocation to the membranous fraction. In fact, Xu et al.[31] have demonstrated that phosphorylation of the Ser^{729} residue is required for the determination of the intracellular localization of PKCε and for its interaction with target proteins in 3T3 fibroblasts.

In cardiomyocytes, the exact mechanism whereby the three residues in the kinase domain of PKCε are phosphorylated remains unsolved. Rybin et al.[32] reported that in neonatal cardiomyocytes, the Ser^{729} residue of PKCε is phosphorylated by PKCδ and, in turn, the Thr^{505} residue of PKCδ is phosphorylated by PKCε. Whether this concept can be applied to adult female hearts is unclear because of the differences in the expression levels of novel PKC isoforms
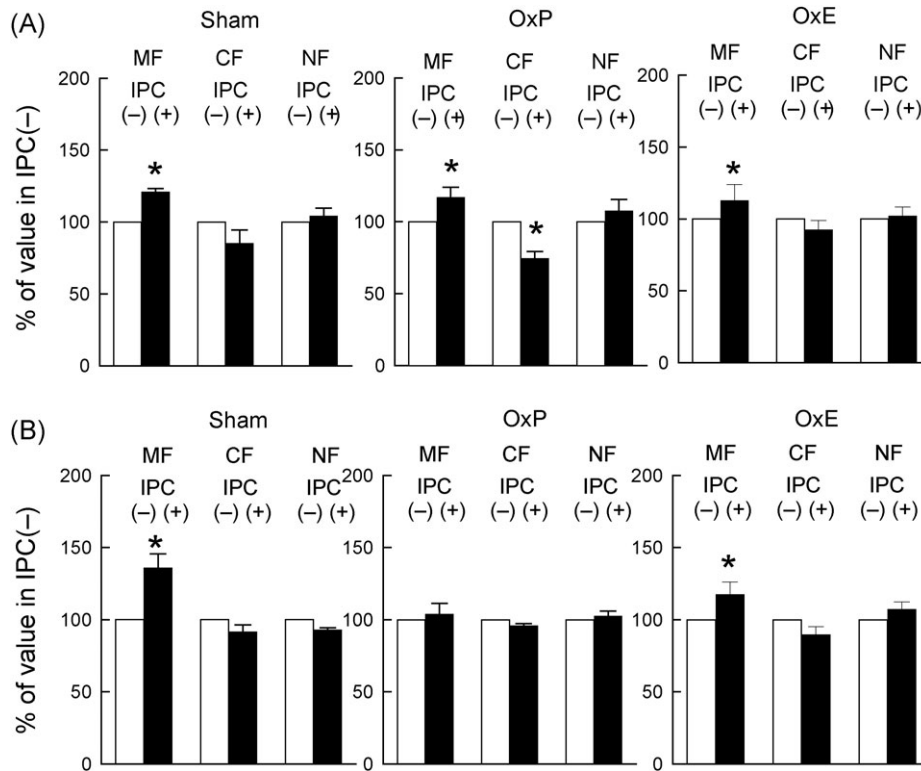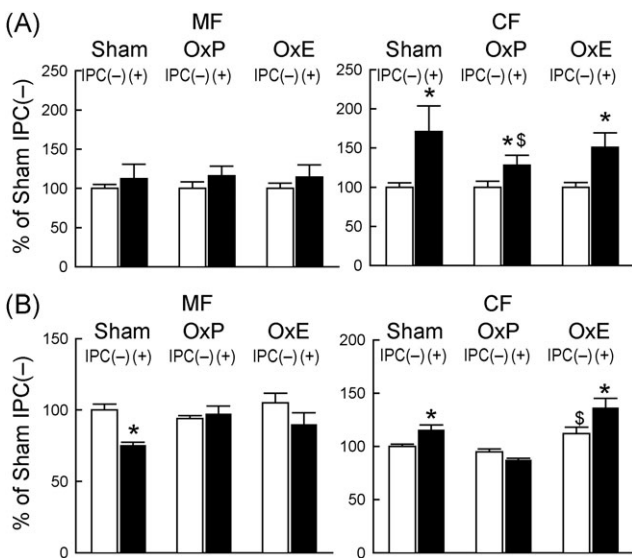
Figure 3 Western immunoblotting for protein kinase C δ and ε. (*A*) Densitometric analysis of protein kinase C δ in the membranous (MF), cytosolic (CF), and nuclear fractions (NF). (*B*) Densitometric analysis of protein kinase C ε in MF, CF, and NF. The densitometric measurements of protein kinase C are normalized to the total protein detected and expressed as a percentage of the average values measured in the Sham IPC(−) group. Data are represented as the mean ± SEM. *$P < 0.05$ vs. the corresponding IPC(−) group, $^{\$}P < 0.05$ vs. the corresponding Sham group.

Figure 4 Western immunoblotting for phosphorylated forms of protein kinase C ε and phosphoinositide-dependent kinase 1. (*A*) Densitometric analysis of phosphorylated protein kinase C ε at the Ser^{729} residue in the membranous (MF) and the cytosolic fractions (CF). (*B*) Densitometric analysis of phosphorylated phosphoinositide-dependent kinase 1 at the Ser^{241} residue in MF and CF. The densitometric measurements of phosphorylated proteins are normalized to the total protein detected and expressed as a percentage of the average values measured in the Sham IPC(−) group. Data are represented as the mean ± SEM. *$P < 0.05$ vs. the corresponding IPC(−) group, $^{\$}P < 0.05$ vs. the corresponding Sham group.
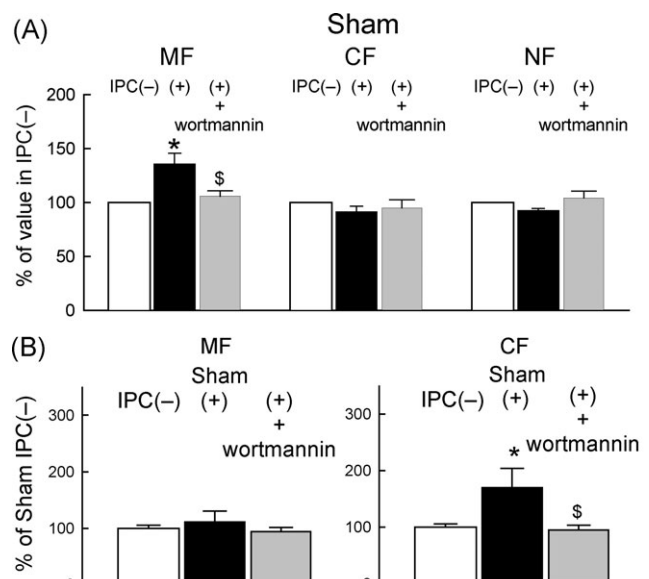

Figure 5 Effect of the PI3-kinase inhibitor (wortmannin) on the translocation and the phosphorylation of protein kinase C ε after IPC in the Sham group. (*A*) Densitometric analysis of protein kinase C ε in MF, CF, and NF. (*B*) Densitometric analysis of p-Ser^{729} protein kinase C ε in MF and CF. Data are represented as the mean ± SEM. *$P < 0.05$ vs. the corresponding IPC(−) group, $^{\$}P < 0.05$ vs. the corresponding Sham group.

between immature and mature cardiomyocytes.[33] Although most data have been obtained in 3T3 fibroblasts rather than in cardiomyocytes, increasing evidence demonstrates

that PDK1 is the upstream kinase that directly phosphorylates the active loop of PKCε.[27–29] PDK1 is bound to and activated by lipid metabolites that are produced by PI3-kinase. PDK1 phosphorylated at the Ser^{241} residue (an

active form of PDK1) translocates to the cytosolic fraction to mediate further signalling pathways.[28,34] Tong *et al.*[18] demonstrated that IPC induced phosphorylation of Akt/PKB and translocation of PKCε and, in male rat hearts, a PI3-kinase inhibitor blocked these effects of IPC. They also found that a PI3-kinase inhibitor abrogated the cardioprotective effect of IPC but did not completely eliminate the effect of direct PKC activation by DOG. They concluded that in male hearts, IPC activates PI3-kinase upstream of PKCε. In the present study, IPC failed to translocate the p-Ser[241] PDK1 to the cytosolic fraction in female hearts with chronic oestrogen withdrawal (*Figure 4B*). In addition, administration of a PI3-kinase inhibitor prior to IPC abrogated the translocation of PKCε to the membranous fraction and attenuated the p-Ser[729] PKCε in intact female hearts in analogy with the effects observed in female hearts with chronic oestrogen withdrawal (*Figure 5*). Our finding that the effect of non-selective PKC activation by DOG was insufficient in female hearts with chronic oestrogen withdrawal (*Table 2* and *Figure 2*) would not contradict the findings of Tong *et al.*,[18] if PI3-kinase-mediated signalling is impaired in the ovariectomized rat heart. Mounting evidence indicates that activation of oestrogen receptors directly modulates PI3-kinase/PDK/AKt signalling in cardiomyocytes.[35,36] Therefore, it is plausible that chronic oestrogen withdrawal may cause dysfunction of PKCε via impaired PI3-kinase/PDK/Akt signalling. In the absence of IPC, the ratio of p-Se[241] PDK1 to total PDK1 in the cytosolic fractions in the OxE group was higher than that in the Sham group (*Figure 4B*), suggesting that oestrogen replacement affects PI3-kinase/PDK/Akt signalling. Activation of PI3-kinase/PDK/Akt signalling might be responsible for the attenuation of myocardial I/R injury in the OxE group without IPC. As expected, oestrogen replacement completely restored the translocation and phosphorylation of PKCε after IPC in the ovariectomized rat heart (*Figures 3B* and *4A*).

We did not evaluate the phosphorylated state of PKCε at the Thr[566] residue, which is directly phosphorylated by PDK1,[26,28,29] because a specific antibody is not available. Thus, there is still no direct evidence that impaired PI3-kinase/PDK/Akt signalling in female hearts with chronic oestrogen depletion contributes to the loss of PKCε translocation. The Thr[505] residue located in the activation loop of PKCδ is also phosphorylated by PDK1.[26,28,29] However, in the present study, translocation of PKCδ to the membranous fraction was preserved in female hearts with chronic oestrogen depletion. Recent investigations suggest that unlike PKCε, phosphorylation of PKCδ appears to be dispensable.[29] The difference between the activation mechanisms of PKCδ and PKCε might explain the discrepancy in the subcellular trafficking of PKC after IPC in the present study. Further investigations are required to establish the interaction between PDK1 and PKCε in cardiomyocytes.

In summary, we have demonstrated that the cardioprotective effect of IPC is lost in female hearts with chronic oestrogen depletion and that this phenomenon is caused, at least in part, by impaired phosphorylation of PKCε at the Ser[729] residue and loss of PKCε translocation following IPC. We have also demonstrated that not only oestrogen replacement but also selective activation of PKCε-mediated signalling can fully restore a protective phenotype analogous to that conferred by IPC. The latter finding indicates that the cellular defect caused by oestrogen withdrawal is at the

level of PKCε or proximal to it, and that the distal cardioprotective machinery is intact and can be effectively recruited by appropriate interventions. Our results suggest that direct activation of PKCε-mediated signalling is a potential strategy for restoring protection against ischaemia in female hearts with chronic oestrogen depletion that might be more efficacious than HRT.

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