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RESEARCH PAPER

Oestrogen confers cardioprotection by suppressing Ca^{2+}/calmodulin-dependent protein kinase II

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Background and purpose: Oestrogen confers cardioprotection by down-regulating the β_1-adrenoceptor and suppressing the expression and activity of protein kinase A. We hypothesized that oestrogen may also protect the heart by suppressing Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), another signalling messenger activated by the β_1-adrenoceptor, that enhances apoptosis.
Experimental approach: We first determined the expression of CaMKII in the heart from sham and ovariectomized rats with and without oestrogen replacement. We then determined the effects of CaMKII inhibition (KN93, 2.5 $\mu mol \cdot L^{-1}$) in the presence or absence of 10^{-7} $mol \cdot L^{-1}$ isoprenaline, a non-selective β-adrenoceptor agonist. We also determined the percentage apoptosis in myocytes from rats in each group with or without β-adrenoceptor stimulation.
Key results: Both CaMKIIδ and phosphorylated CaMKII were up-regulated in the hearts from ovariectomized rats, and they were restored to normal by oestrogen replacement. The infarct size and lactate dehydrogenase release were significantly greater after ovariectomy. Similarly, cardiac contractility, the amplitude of the electrically induced intracellular Ca^{2+} transient and the number of apoptotic cells were also greater in ovariectomized rats upon ischaemia/reperfusion in the presence or absence of isoprenaline. Most importantly, the responses to ischaemic insult in ovariectomized rats were reversed not only by oestrogen replacement, but by blockade of CaMKII with KN93.
Conclusions and implications: Oestrogen confers cardioprotection at least partly by suppressing CaMKIIδ. This effect of oestrogen on CaMKII is independent of the β-adrenoceptor and occurs in addition to down-regulation of the receptor.
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Keywords: oestrogen; CaMKII; ovariectomy; β-adrenoceptor; apoptosis

Abbreviations: 2-DOG, 2-deoxy-D-glucose; AC, adenylyl cyclase; AIP, autocamtide 2-related inhibitory peptide; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; $E[Ca^{2+}]_i$, electrically induced intracellular calcium transient; LDH, lactate dehydrogenase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end of diastolic pressure; MI/A, metabolic inhibition and anoxia; NCX, Na^+/Ca^{2+} exchanger; O+E, ovariectomy with oestrogen replacement; OVX, ovariectomy; phospho-CaMKII, phosphorylated CaMKII; PKA, protein kinase A; RyR, ryanodine receptor; TTC, 2,3,5-triphenyl-tetrazolium chloride; TUNEL, deoxynucleotidyl transferase-mediated dUTP nick-end labelling

Introduction

Before menopause, women are less vulnerable to coronary heart diseases than men (Wilson *et al.*, 1985; Colditz *et al.*, 1987). As women age, their risk of heart disease rises. These observations are taken as indications that oestrogen protects the heart against ischaemic insults, thus reducing coronary heart disease and mortality from cardiovascular disease (Stampfer *et al.*, 1991; Hayward *et al.*, 2000).

β-Adrenoceptor stimulation by the sympathetic nervous system plays an important role in contraction, heart rate and apoptosis in the heart (Communal *et al.*, 1999; Zaugg *et al.*, 2000). There are two β-adrenoceptor subtypes, namely, β_1 and β_2. The β_1-adrenoceptor subtype is the predominant subtype in the heart. The actions of β_1-adrenoceptor stimulation are mediated via two pathways. The primary pathway is the well-established $G\alpha_s$ protein/adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway, which enhances cardiac contractility and relaxation, as well as oxygen consumption (Dohlman *et al.*, 1991; Campbell and Strauss, 1995; Bers, 2002).

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Inhibition of PKA attenuates the increase in myocyte contraction mediated by short-term β_1-adrenoceptor stimulation (Kam *et al.*, 2005). The other signalling pathway activated by β_1-adrenoceptor stimulation is the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) pathway. Activation of CaMKII leads to apoptosis and enhanced activity of the L-type Ca^{2+} channel, ryanodine receptor (RyR), sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger (NCX) (Zhu *et al.*, 2003; Zhang *et al.*, 2005). It is known that the increase in myocyte contraction and Ca^{2+} transient during sustained β_1-adrenoceptor stimulation cannot be restored to normal by blocking the PKA pathway, but rather by blocking the CaMKII pathway (Wang *et al.*, 2004). Apoptosis has also been shown to occur independently of PKA activity (Zhu *et al.*, 2007). We therefore hypothesized that oestrogen may also suppress the CaMKII pathway, which is PKA-independent and plays an important role in the regulation of apoptosis.

To test this hypothesis, we first used Western blot to detect the expression of CaMKII in female rats after sham operation or ovariectomy with (O+E) and without (OVX) oestrogen replacement. In a preliminary study using a non-selective anti-CaMKII antibody, we observed two bands in the Western blot. One was stained more deeply and showed different expression in the hearts from the three groups of rats, while the other was lighter, suggesting lower expression, and showed no difference among the groups. Based on the fact that CaMKIIδ is the predominant CaMKII isoform (Braun and Schulman, 1995; Singer *et al.*, 1997; Zhang and Brown, 2004) and is known to induce apoptosis upon activation (Zhu *et al.*, 2003; 2007), we decided to investigate the expression of this isoform. We then determined the injury, contractile function and Ca^{2+} transient in responses to ischaemic insult, as well as the number of deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)-positive cells in the presence of isoprenaline, a non-selective β-adrenoceptor agonist, which stimulates both β-adrenoceptor subtypes, in isolated perfused hearts and isolated ventricular myocytes. The data showed that oestrogen suppresses CaMKII, thus protecting the heart against ischaemic insult. The novel finding is that oestrogen suppresses CaMKII independent of β-adrenoceptors. It also suppresses CaMKII via suppressing β-adrenoceptors in agreement with its effect of down-regulation of the receptor demonstrated previously (Thawornkaiwong *et al.*, 2003; Kam *et al.*, 2004). CaMKIIδ may be the isoform involved.

Methods

Experimental animals

All animal care and this study were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Adult female Sprague-Dawley rats weighing 190–210 g were randomly divided into two groups. One group served as the normal control (sham). The other group underwent bilateral ovariectomy (OVX) and was divided into two subgroups. One week after OVX, one subgroup (O+E) was implanted with subcutaneous 1.5 mg 60 day release oestrogen pellets (Innovative Research of America, Toledo, OH, USA), which maintained oestrogen concentration within the physiological range for 60 days, and the other subgroup was treated with vehicle. All surgical procedures were performed under anaesthesia with sodium pentobarbital (60 $mg \cdot kg^{-1}$, i.p., Abbott Laboratories, Chicago, IL, USA).

Serum oestrogen level

Blood samples were collected from the rats after decapitation, and serum oestrogen levels were measured by using the assay (ELISA) technique with an estradiol EIA kit (Cayman Chemical, MI, USA) according to the manufacturer's instructions.

Isolated heart preparation

Hearts were isolated 6 weeks after OVX and perfused with a Krebs-Henseleit (K-H) solution equilibrated with 95% O_2 and 5% CO_2 at a constant pressure of 80 cm H_2O and temperature (37°C). This solution contained (in $mmol \cdot L^{-1}$) 118 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$ and 11 glucose. A latex balloon connected to a transducer was inserted into the left ventricle and was filled with saline solution to adjust the end-diastolic pressure to 5–7 mmHg. The heart was allowed to stabilize for 15 min and then subjected to 45 min global ischaemia followed by 120 min reperfusion. A CaMKII inhibitor, KN93 (2.5 $\mu mol \cdot L^{-1}$, Sigma, St. Louis, MO, USA), or its inactive analogue, KN92 (2.5 $\mu mol \cdot L^{-1}$, Sigma, St. Louis, MO, USA), or a PKA inhibitor, KT5720 (2 $\mu mol \cdot L^{-1}$, Sigma, St. Louis, MO, USA) was added to the K-H solution 20 min before ischaemia and during the first 10 min of reperfusion. Activation of β-adrenoceptors was achieved by administration of 10^{-7} $mol \cdot L^{-1}$ isoprenaline (Sigma, St. Louis, MO, USA) for 10 min before ischaemia and during the first 10 min of reperfusion.

Left ventricular pressure recording

A latex balloon connected to a transducer was inserted into the left ventricle and was filled with saline solution to adjust the end-diastolic pressure to 5–7 mmHg. The left ventricular developed pressure (LVDP), left ventricular end of diastolic pressure (LVEDP), velocity of contraction and relaxation ($\pm dP/dt_{max}$) and heart rate were monitored continuously by a PowerLab/4SD analogue-to-digital converter (AD instruments, Castle Hill, Australia).

Isolation of ventricular myocytes

Ventricular myocytes were isolated from the hearts of sham, OVX and O+E rats, using the collagenase perfusion method described previously (Wu *et al.*, 1999; Wang *et al.*, 2001). Preliminary experiments showed that there was no statistically significant difference in the viability of freshly prepared and cultured myocytes from all the groups, which ensures that percentage survival is equivalent among individual preparations/plates. After isolation, they were allowed to stabilize for 30 min before experiments. Myocytes were plated at a density of 2×10^5 cells per well on laminin-coated (1 $\mu mol \cdot L^{-1}$, Sigma-Aldrich, St Louis, MO, USA) six-well plates. The minimal essential medium contained 1.25 $mmol \cdot L^{-1}$ Ca^{2+}, 5% fetal bovine serum and 1% penicillin-streptomycin. The designated reagents 17β-estradiol (10^{-9} $mol \cdot L^{-1}$, Sigma-Aldrich, St. Louis, MO, USA), the CaMKII inhibitors [autocamtide 2-related inhibitory peptide (AIP), KN93, or its

inactive analogue, KN92] were added 1 h prior to β-adrenoceptor stimulation with isoprenaline (10^{-7} mol·L^{-1}) in some subsets of experiments. All Western blot and apoptosis assays related to β-adrenoceptor stimulation were performed after isoprenaline treatment for 24 h.

Western blot analysis

Cultured myocytes were collected and homogenized. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951). Sixty micrograms of extracted protein from the left ventricular cardiomyocytes was diluted in loading buffer [130 mmol·L^{-1} Tris-HCl, pH 8.0, 20% (v v^{-1}) glycerol, 5% (w v^{-1}) SDS, 0.02% bromophenol blue, 2% DTT] and denatured for 5 min at 95°C. Proteins were loaded onto 10% sodium dodecylsulphate-polyvinylidene gel and then transferred to polyvinylidene difluoride membrane (0.2 μm pore size; Bio-Rad, Hercules, CA, USA) at 4°C in a transfer buffer containing 20% methanol with the Bio-Rad Trans-blot electrophoretic transfer system at 120 V for 1.5 h. After blocking in TBST (Tris-buffered saline, pH 7.4, 0.1% Tween-20) with 5% skimmed milk for 1 h at room temperature, the membrane was incubated with anti-CaMKIIδ (1:500, Santa Cruz Biotechnology, CA, USA) or anti-phosphorylated CaMKII (anti-phospho-CaMKII) (1:500, Chemicon International, CA, USA) overnight at 4°C. Monoclonal antibody against α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) was chosen as the internal control. After three 10 min washes with TBST, HRP-linked anti-mouse (1:7000, for anti-CaMKII primary antibody, Amersham Biosciences, NJ, USA) or anti-rabbit (1:7000, for anti-phospho-CaMKII primary antibody, Amersham Biosciences, NJ, USA) IgG was used as secondary antibody. The membrane was incubated with secondary antibody for 1 h at room temperature followed by three 10 min washes with TBST. Bands were detected by the chemiluminescence method (ECL Western blot detection kit; Amersham Biosciences, NJ, USA). The film was scanned, and the intensity of the bands was calculated with an image analysis system (Image J; National Institutes of Health). Membranes were stripped and reblotted with anti-α-tubulin antibody to ensure equal amount of protein loading. The data were normalized for the loading control.

Infarct size

At the end of reperfusion, the heart was frozen at –20°C for 1 h and then cut from apex to base into 2 mm slices. Then the slices were immersed in 1% 2,3,5-triphenyl-tetrazolium chloride in phosphate buffer (pH 7.4) for 10 min (Vila-Petroff *et al.*, 2007). Infarct areas were enhanced by immersing the slices in 10% formaldehyde overnight before measurement (Wang *et al.*, 2001). The infarct area was calculated by planimetry with image J software (National Institutes of Health) and expressed as a percentage of the total area of the heart.

Lactate dehydrogenase (LDH) determination

The effluent from isolated perfused hearts was collected for the first 1 min of reperfusion, and the LDH was assayed by an enzymatic method (Stanbio Laboratory, TX, USA).

Measurement of electrically induced intracellular calcium transient ($E[Ca^{2+}]_i$)

A spectrofluorometric method with Fura2-AM as the Ca^{2+} indicator was used to measure the $[Ca^{2+}]_i$ as previously described (Wu *et al.*, 1999). Ventricular myocytes from each group were isolated and allowed to stabilize for 30 min before experiments. The myocytes selected were rod-shaped and quiescent with clear striations. Some groups of myocytes were first subjected to 120 min pretreatment with 10^{-7} mol·L^{-1} isoprenaline. Ten minutes before isoprenaline incubation, 2.5 μmol·L^{-1} KN93, 2.5 μmol·L^{-1} KN92, or 10 μmol·L^{-1} AIP was added. After incubation and loading with 5 μmol·L^{-1} Fura2-AM for 40 min in the dark, the myocytes were added to a bathing chamber under the object lens of a fluorescence microscope. Myocytes contracted synchronously under the stimulation of a 0.2 Hz electrical field with 15 ms pulses at 60 V through two platinum wires in the chamber. After normal perfusion with K-H buffer under 95% O_2 and 5% CO_2, a single ventricular myocyte was selected and was subjected to 10 min of severe metabolic inhibition and anoxia (MI/A) in a glucose-free K-H solution containing 10 mmol·L^{-1} 2-deoxy-D-glucose (2-DOG) and 10 mmol·L^{-1} sodium dithionite. Reperfusion was mimicked by incubating the myocytes with normal K-H solution. Fluorescence signals obtained at 340 nm and 380 nm excitation wavelengths were recorded and stored in the computer for data processing and analysis.

Terminal TUNEL

After 24 h incubation, myocytes were collected and a TUNEL assay was performed with a cell death detection kit (Roche Diagnostics, Germany) according to the supplier's instructions. The percentage of TUNEL-positive cells was calculated from 20 randomly chosen fields in each culture dish containing approximately 800 myocytes.

Statistical analysis

Data are expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-test was used to determine differences among multiple groups. A value of $P < 0.05$ was considered statistically significant.

Materials

Water-soluble 17β-estradiol, KN92, AIP, KN93, KT5720, isoprenaline, type-1 collagenase, paraformaldehyde anti-α-tubulin antibody, 2,3,5-triphenyl-tetrazolium chloride and Fura2-AM were from Sigma-Aldrich. Specific anti-CaMKIIδ antibody was from Santa Cruz Biotechnology. Specific anti-phospho-CaMKII antibody was from Chemicon International. HRP-linked anti-mouse and anti-rabbit secondary antibodies and the ECL Western blot detection kit were from Amersham Biosciences. The 60 day release oestrogen pellets were from Innovative Research of America, and sodium pentobarbital was from Abbott Laboratories. The *in situ* cell death detection kit was from Roche Diagnostics. The LDH kit was from Stanbio Laboratory. The estradiol EIA kit was from Cayman Chemical. All drugs were dissolved in deionized water or K-H solution, except for KT5720, KN93 and Fura2-

AM, which were dissolved in DMSO. The final concentration of DMSO was ≤0.01% that itself had no effects on the hearts.

Results

Oestrogen level of experimental animals

The serum oestrogen concentration was significantly decreased at 6 weeks after OVX and was reversed by oestrogen replacement (Table 1) as in our previous studies (Kam *et al*., 2004; 2005; Kravtsov *et al*., 2007).

Expression of CaMKIIδ and phospho-CaMKII in hearts from sham, OVX and O+E rats

Both CaMKIIδ (Figure 1A) and phospho-CaMKII (Figure 1B) were up-regulated in myocytes from OVX rats. After 24 h incubation with 10^{-7} mol·L^{-1} isoprenaline, CaMKIIδ (Figure 1A) and phospho-CaMKII (Figure 1B) further increased in myocytes from both the sham control and OVX rats. All changes after OVX were restored to normal level after incubation with 10^{-9} mol·L^{-1} oestrogen for 24 h.

Effects of CaMKII inhibition on cardiac injury induced by ischaemia/reperfusion

Ovariectomy resulted in increases in infarct size (Figure 2) and LDH release (Figure 3) following ischaemia/reperfusion, and these effects were reversed by oestrogen replacement (Figure 2). Blockade of CaMKII with a selective inhibitor, 2.5 μmol·L^{-1} KN93, but not of PKA with its selective inhibitor, 2 μmol·L^{-1} KT5720, abolished the effects of OVX. And blockade of both CaMKII and PKA also abolished these effects (Figures 2 and 3). KN93 alone did not have any significant effect in control group. When the isolated perfused heart was subjected to ischaemia/reperfusion in the presence of 10^{-7} mol·L^{-1} isoprenaline, which mimics the sympathetic overreactivity during ischaemia *in vivo*, both the infarct size and LDH release were greater than the corresponding values in the absence of isoprenaline. Interestingly, blockade of either CaMKII or PKA or both attenuated/abolished the effects of OVX (Figures 2 and 3).

Effects of CaMKII inhibition on contractile recovery in hearts after ischaemia and reperfusion

The LVDP (Figure 4A) and average ± dP/dt_{max} (Figure 4C and D) evaluated at the end of reperfusion were significantly attenuated, while the LVEDP (Figure 4B) was elevated, in hearts from OVX rats. Oestrogen replacement abolished the effects of OVX (Figure 4). Blockade of CaMKII with 2.5 μmol·L^{-1} KN93 also abolished or attenuated the effects of OVX (Figure 4). KN93 alone had no significant effect in control group. In the presence of 10^{-7} mol·L^{-1} isoprenaline, the responses to the different treatments were the same. However, the LVDP (Figure 4A) and the average ±dP/dt_{max} (Figure 4C and D) were lower, while the LVEDP (Figure 4B) was higher, than the corresponding values in the absence of isoprenaline, indicating poorer contractile recovery in the presence of isoprenaline.

Table 1 Oestrogen levels in serum of experimental animals

	Serum oestrogen level (pg·mL^{-1})
Sham (n = 8)	57 ± 10.3
OVX (n = 8)	17 ± 1.4***
O+E (n = 7)	70 ± 11.7###

Levels were measured 6 weeks after OVX. Each value shows mean ± SEM. O+E, ovariectomy with oestrogen replacement; OVX, ovariectomy. ***P < 0.001 versus sham; ###P < 0.001 versus OVX.

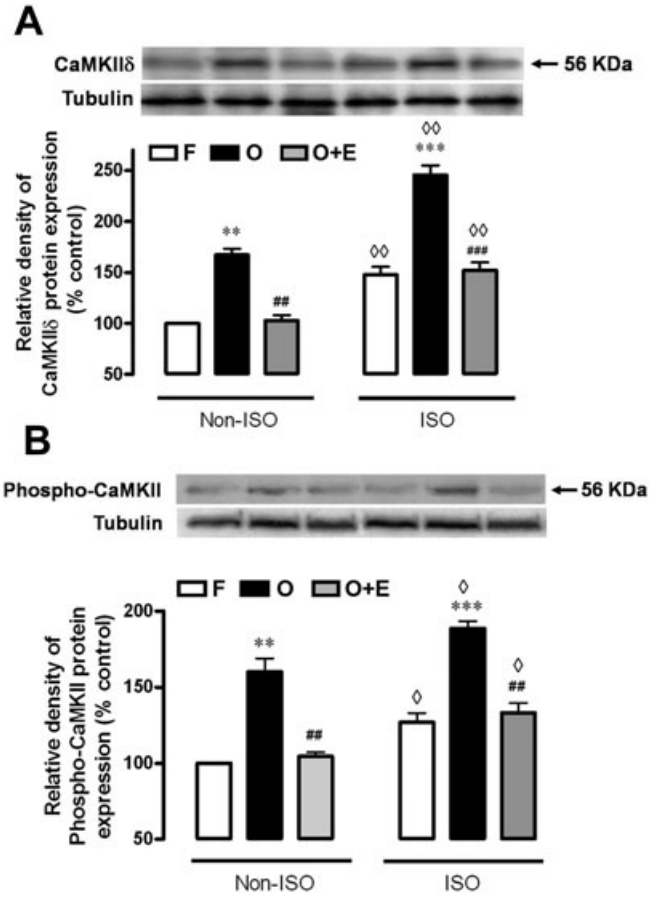

Figure 1 Expression of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)δ (A) and phosphorylated CaMKII (phospho-CaMKII) (B) in ventricular tissue from ovariectomized (OVX, O) and oestrogen-replaced (O+E) rats, assessed by Western blot. The bar graph shows the overall data from six experiments (isoprenaline, ISO). Data are expressed as mean ± SEM, **P < 0.01 versus control (F); ***P < 0.001 versus control; ##P < 0.01 versus OVX; ###P < 0.001 versus OVX; ◇P < 0.05 versus non-ISO treatment; ◇◇P < 0.01 versus non-ISO treatment.

Effects of CaMKII inhibition on resting intracellular Ca^{2+} ($[Ca^{2+}]_i$) and E$[Ca^{2+}]_i$ in the isolated ventricular myocyte after metabolic inhibition/anoxia and reperfusion

The resting $[Ca^{2+}]_i$ measured before MI/A was significantly higher in myocytes isolated from OVX rats both in the absence and in the presence of isoprenaline (Figure 5A). This effect was abolished or attenuated not only by oestrogen replacement, but also by blockade of CaMKII with KN93 or

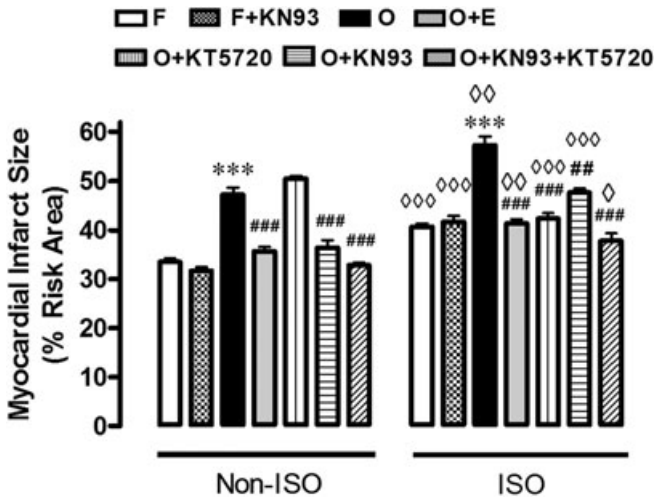

Figure 2 Cross-sections of TTC (2,3,5-triphenyl-tetrazolium chloride) staining in hearts from female rats (F), female rats with 2.5 μmol·L^{-1} KN93 (F + KN93), ovariectomy (OVX, O), OVX with oestrogen replacement (O+E), OVX with 2.5 μmol·L^{-1} KN93 (O + KN93), OVX with 2 μmol·L^{-1} KT5720 (O + KT5720) and OVX with both inhibitors (O + KN93 + KT5720). The bar graph shows the overall data from six experiments (isoprenaline, ISO). Data are expressed as mean ± SEM, ***P < 0.001 versus control (F); ##P < 0.01 versus OVX; ###P < 0.001 versus OVX; ◇P < 0.05 versus non-ISO treatment; ◇◇P < 0.01 versus non-ISO treatment; ◇◇◇P < 0.001 versus non-ISO treatment.

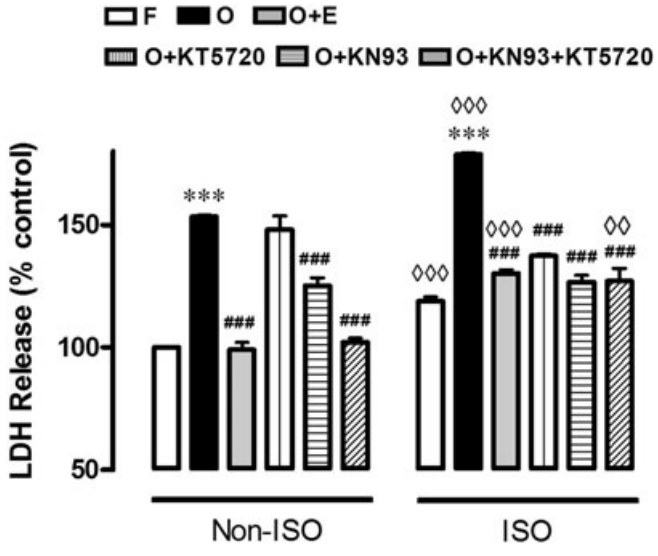

Figure 3 Lactate dehydrogenase release during the first 1 min of reperfusion from hearts of female rats (F), ovariectomy (OVX, O), OVX with oestrogen replacement (O+E), OVX with 2.5 μmol·L^{-1} KN93 (O + KN93), OVX with 2 μmol·L^{-1} KT5720 (O + KT5720) and OVX with both inhibitors (O + KN93 + KT5720). The bar graph depicts the overall results of six independent experiments in each group (isoprenaline, ISO). Data are expressed as mean ± SEM, ***P < 0.001 versus control (F); ###P < 0.001 versus OVX; ◇◇P < 0.01 versus non-ISO treatment; ◇◇◇P < 0.001 versus non-ISO treatment.

AIP. KN93 alone had no effect in normal rats. KN92, an inactive analogue of KN93, did not have a beneficial effect in OVX rats. All the values were greater than the corresponding values in the absence of isoprenaline.

Ovariectomy slightly, but significantly increased the amplitude of $[Ca^{2+}]_i$, which represents the amount of Ca^{2+} released from the sarcoplasmic reticulum (Figure 5B). It did not, however, affect either the time to peak (Figure 5C), which represents the speed of Ca^{2+} release, or the decay time, T_{50} (Figure 5D), which represents the rate of removal of Ca^{2+} from the cytosol. At the end of reperfusion, the amplitude (Figure 5B) was significantly reduced, while the time to peak (Figure 5C) and T_{50} (Figure 5D) were significantly greater, in myocytes from OVX rats. The effects of OVX were abolished both by oestrogen replacement and blockade of CaMKII with 2.5 μmol·L^{-1} KN93 or 10 μmol·L^{-1} AIP.

In the presence of 10^{-7} mol·L^{-1} isoprenaline, the amplitude (Figure 5A) was greater, while the time to peak (Figure 5C) and T_{50} (Figure 5D) were smaller, than the corresponding values in the absence of isoprenaline in myocytes from OVX rats, indicating that β-adrenoceptor stimulation enhances contractile function in the absence of the female sex hormone. At the end of reperfusion, the responses were also the same as those in the absence of isoprenaline. The amplitude values in response to the different treatments were lower in the presence of isoprenaline than in its absence, in keeping with the poor contractile responses observed in the isolated perfused heart (Figure 4). Again, the effects of OVX were abolished both by oestrogen replacement and blockade of CaMKII with 2.5 μmol·L^{-1} KN93 or 10 μmol·L^{-1} AIP. KN92 (2.5 μmol·L^{-1}) did not have any significant effect in OVX rats.

Effects of CaMKII inhibition on apoptosis

The number of apoptotic cells determined by the TUNEL assay was significantly increased in hearts from OVX rats. Incubation with 10^{-7} mol·L^{-1} isoprenaline for 24 h increased the ratio of apoptotic cells both in the sham and OVX groups and the number of these cells was significantly greater in the OVX group (Figure 6). The effect of OVX was attenuated/abolished not only by oestrogen replacement, but also by blockade of CaMKII with KN 93 or AIP. KN92 had no significant effect in OVX rats.

Discussion

There are two novel observations in the present study. First, both CaMKIIδ and phospho-CaMKII were up-regulated in the hearts from oestrogen-deficient rats, and these changes were reversed by oestrogen replacement. The second observation, which is more important, is that the deleterious effects of ischaemic insult and reperfusion on hearts from OVX rats, namely, poor contractile recovery and increased apoptosis, were attenuated or abolished not only by oestrogen replacement, but also by blockade of CaMKII with its inhibitor, KN93. These results are evidence that oestrogen confers cardioprotection by suppressing the CaMKII pathway. Expression studies suggested that the isoform involved may be CaMKIIδ. That blockade of CaMKII abolished the effect of OVX in the absence of 10^{-7} mol·L^{-1} isoprenaline indicates that oestrogen suppresses the kinase independently of β-adrenoceptor activation. It is known that oestrogen decreases the expression of β_1-adrenoceptors in OVX rats

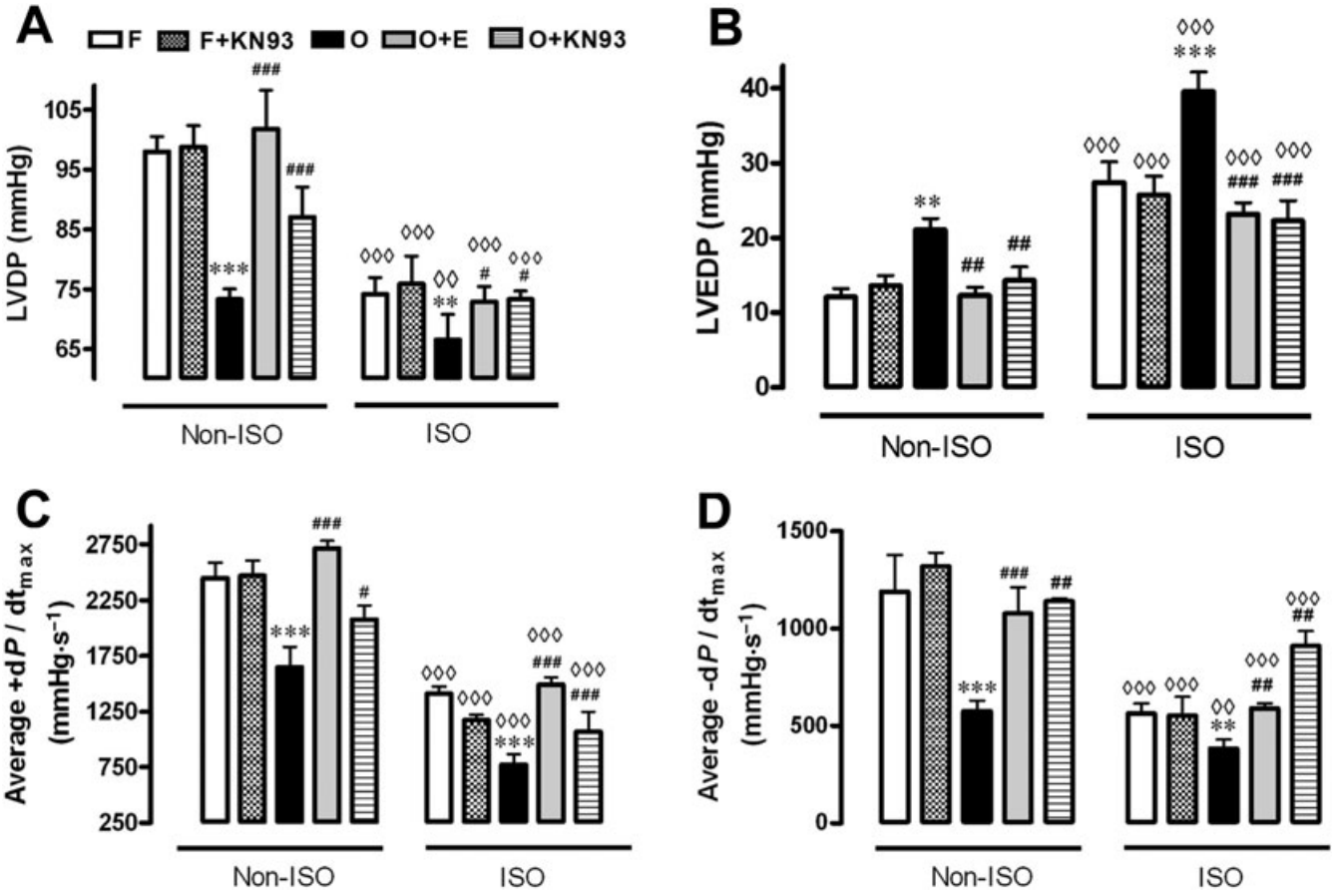

Figure 4 Effects of ischaemic insult in the presence or absence of 10^{-7} mol·L^{-1} isoprenaline (ISO) on cardiac contractile functions in isolated perfused hearts from female rats (F), female rats with 2.5 μmol·L^{-1} KN93 (F + KN93), OVX (O), OVX with oestrogen replacement (O+E) and OVX with 2.5 μmol·L^{-1} KN93 (O + KN93) rats. Values are expressed as mean ± SEM (n = 6 in each group), $^{**}P < 0.01$ versus control (F); $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$ versus OVX; $^{\#\#}P < 0.01$ versus OVX; $^{\#\#\#}P < 0.001$ versus OVX; $^{\diamond\diamond}P < 0.01$ versus non-ISO treatment; $^{\diamond\diamond\diamond}P < 0.001$ versus non-ISO treatment. +dP/dt_{max}, velocity of contraction; –dP/dt_{max}, velocity of relaxation; LVDP, left ventricular developed pressure; LVEDP, left ventricular end of diastolic pressure; OVX, ovariectomy.

(Thawornkaiwong *et al.*, 2003; Kam *et al.*, 2004). Our previous study also showed that oestrogen down-regulates β_1-adrenoceptors, thus conferring cardioprotection by diminishing their responses (Kam *et al.*, 2004). These observations suggest that oestrogen may suppress the CaMKII pathway via the β-adrenoceptor. In agreement with this, we showed in the present study that oestrogen also attenuated the effects of ischaemic insult in the presence of isoprenaline, which activates the β-adrenoceptor. So oestrogen may affect CaMKII both via the β-adrenoceptor and independently of the receptor.

Ca^{2+}/calmodulin-dependent protein kinase II is a functional serine/threonine protein kinase, which phosphorylates several important proteins in response to increasing intracellular Ca^{2+} concentration (Hook and Means, 2001). There are four different CaMKII isoforms (α, β, γ and δ). Only the γ and δ isoforms are present in the heart (Tobimatsu and Fujisawa, 1989). CaMKIIδ is the most abundant isoform in cardiomyocytes, while the expression of CaMKIIγ is low (Braun and Schulman, 1995; Singer *et al.*, 1997; Zhang and Brown, 2004). Previous studies have demonstrated that activation of CaMKIIδ induces myocyte apoptosis, which results in cardiac hypertrophy and heart failure (Zhu *et al.*, 2003; 2007). This is in agreement with the finding of the present study that the expression of CaMKIIδ was enhanced in hearts from OVX rats, which are more vulnerable to ischaemic insult. To date, 13 splice variants of the CaMKIIδ have been found, and only δ_B and δ_c are present at the protein level in mammalian myocytes. In this study, we were unable to determine which variant(s) was (were) up-regulated due to a lack of specific antibodies for the Western blots. However, CaMKIIδ_B activation results in hypertrophic gene expression (Ramirez *et al.*, 1997), while CaMKIIδ_c activation enhances phosphorylation of RyR2, leading to increased SR Ca^{2+} release and dysfunction of heart contractility. In addition, CaMKIIδ_c activation also triggers an apoptosis pathway in cardiomyocytes (Zhu *et al.*, 2007). So the C isoform of the CaMKIIδ is likely to be the isoform involved.

In the present study, we demonstrated that in hearts from OVX rats subjected to ischaemic insult and reperfusion both in the presence or absence of isoprenaline, the infarct size, LDH release, apoptosis and resting $[Ca^{2+}]_i$ (Ca^{2+} overload is a precipitating cause of injury) increased significantly, and contractile recovery was poor. These effects were reversed by oestrogen replacement. The data confirm the cardioprotective actions of the female sex hormone reported previously (Kam

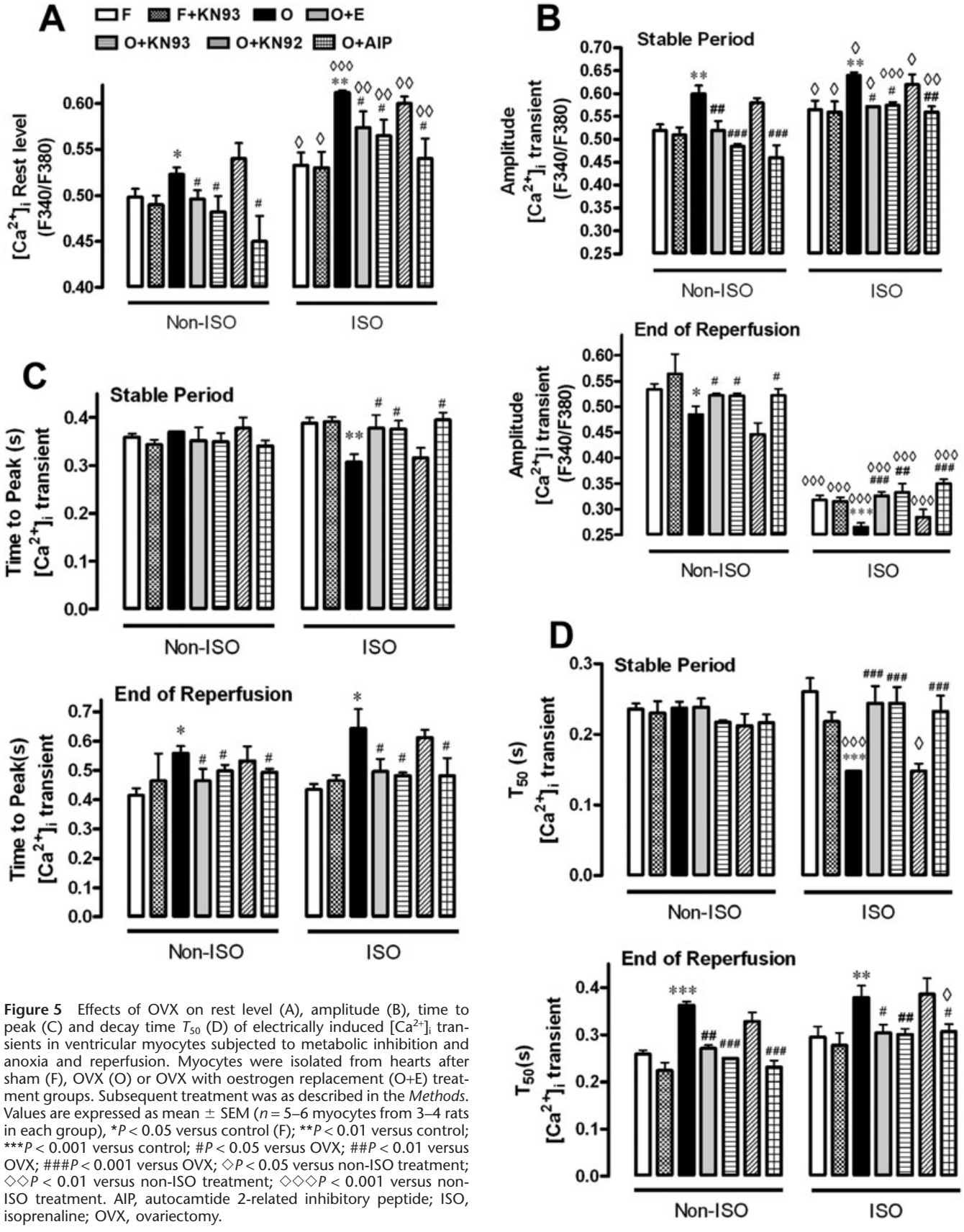

Figure 5 Effects of OVX on rest level (A), amplitude (B), time to peak (C) and decay time T_{50} (D) of electrically induced $[Ca^{2+}]_i$ transients in ventricular myocytes subjected to metabolic inhibition and anoxia and reperfusion. Myocytes were isolated from hearts after sham (F), OVX (O) or OVX with oestrogen replacement (O+E) treatment groups. Subsequent treatment was as described in the *Methods*. Values are expressed as mean ± SEM ($n = 5$–6 myocytes from 3–4 rats in each group), *$P < 0.05$ versus control (F); **$P < 0.01$ versus control; ***$P < 0.001$ versus control; #$P < 0.05$ versus OVX; ##$P < 0.01$ versus OVX; ###$P < 0.001$ versus OVX; ◇$P < 0.05$ versus non-ISO treatment; ◇◇$P < 0.01$ versus non-ISO treatment; ◇◇◇$P < 0.001$ versus non-ISO treatment. AIP, autocamtide 2-related inhibitory peptide; ISO, isoprenaline; OVX, ovariectomy.

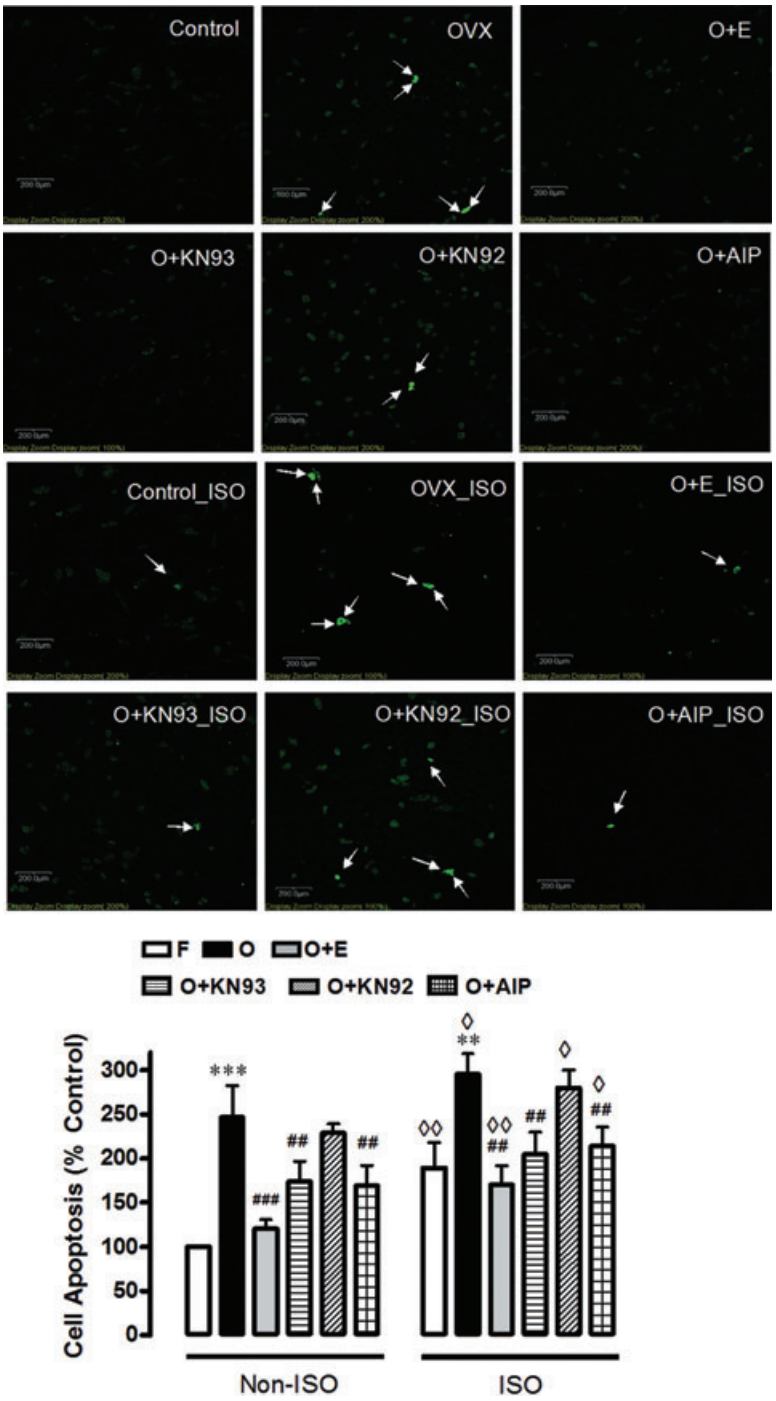

Figure 6 TUNEL-positive cells in control, OVX, O+E, O + KN93, O + KN92 and O + AIP rats after incubation with or without 10^{-7} mol·L^{-1} ISO. The fluorescent images show CaMKII-induced apoptosis, indicated by the increased number of TUNEL-positive cells (arrows). The bar graph below depicts the overall results for apoptosis after 24 h incubation. Data are expressed as mean ± SEM from 5–6 independent experiments from 5–6 hearts, $^{**}P < 0.01$ versus control; $^{***}P < 0.001$ versus control; $^{\#\#}P < 0.01$ versus OVX; $^{\#\#\#}P < 0.001$ versus OVX; $^{\diamond}P < 0.05$ versus non-ISO treatment; $^{\diamond\diamond}P < 0.01$ versus non-ISO treatment. AIP, autocamtide 2-related inhibitory peptide; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; ISO, isoprenaline; O+E, ovariectomy with oestrogen replacement; OVX, ovariectomy; TUNEL, deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

et al., 2004; 2005; Chu *et al.*, 2006; Nikolic *et al.*, 2007). We showed in a previous study that oestrogen down-regulates the β_1-adrenoceptor (Thawornkaiwong *et al.*, 2003; Kam *et al.*, 2004). We also showed in another study that PKA activity increased in the heart from OVX rats while oestrogen suppressed the PKA expression in the absence of β-adrenoceptor stimulation, indicating that oestrogen suppressed PKA independently of β_1-adrenoceptors (Kam *et al.*, 2005). In the present study we showed that oestrogen also suppressed CaMKII independently of β_1-adrenoceptors as well as via the

receptor. So oestrogen may act on the β-adrenoceptor as well on both CaMKII and PKA independently of the receptor, thus conferring cardioprotection. That down-regulation of the β_1-adrenoceptor is responsible for the cardioprotective effect was well established with the observation that oestrogen at physiological concentration for 24 h down-regulates the expression of the receptor and reduces LDH release. Activation by a β_1-adrenoceptor agonist, ICI182780, increased the LDH release, which was reversed by blockade of the β-adrenoceptors with propranolol (Kam *et al.*, 2004). The observations are evidence that β_1-adrenoceptors mediate the cardioprotective effect of oestrogen. In the present study, we showed that both the infarct size and the LDH release were increased in the heart from OVX rats and the effects were reversed not only by oestrogen replacement, but by blockade of CaMKII, indicating that CaMKII is also involved in cardioprotection of oestrogen. Interestingly, the effects of OVX were only partially reversed by inhibition of PKA in the presence, but not the absence, of β-adrenoceptor stimulation with isoprenaline. The observation suggests that in the absence of β_1-adrenoceptor stimulation, PKA may not play an important role in mediating the protective effect of oestrogen although the hormone was shown to suppress the expression of the kinase (Kam *et al.*, 2005). A recent study showed that oestrogen reduces apoptosis of cardiomyocytes *in vivo* and *in vitro* by activating the phospho-inositide-3 kinase/Akt signalling pathway (Patten *et al.*, 2004), which is a downstream pathway activated by β_2-adrenoceptor stimulation and functionally inhibits Gs/AC/cAMP/PKA mediated target protein phosphorylation (Xiao *et al.*, 2006). So oestrogen may also involve β_2-adrenoceptors and their signalling pathways, leading to reduced apoptosis and cardioprotection.

Our previous study showed that oestrogen suppresses the expression of PKA in the absence of β-adrenoceptor stimulation (Kam *et al.*, 2005). The present study also showed that the expression of CaMKII was suppressed by oestrogen in the absence of β-adrenoceptor stimulation. The observations suggest that oestrogen may exert its action via both messengers. However, we observed in the present study that the effects of OVX on injury were abolished by blockade of CaMKII, but not by blockade of PKA, in the absence of β-adrenoceptor stimulation. The observation means that when PKA activity is minimal, as in the absence of β-adrenoceptor stimulation, its interaction with oestrogen does not contribute significantly to the injury responses. One explanation may be that PKA is not apoptotic, in contrast to the apoptotic action of CaMKII.

Previous studies indicated that Ca^{2+} influx via L-type Ca^{2+} channel or NCX induces CaMKII activation (Vittone *et al.*, 2002; Vila-Petroff *et al.*, 2007). In addition, over-expression of CaMKII enhances cardiac necrosis and apoptosis by reducing LDH release and caspase-3 activity, and increasing Bcl-2/Bax ratio (Vila-Petroff *et al.*, 2007). It was shown that oestrogen can reduce the expression of L-type Ca^{2+} channels and the L-type Ca^{2+} current by as much as 50% (Kitazawa *et al.*, 1997; Ullrich *et al.*, 2007). Furthermore, a study showed that the ischaemia/reperfusion injury by over-expression of the NCX can be partially overcome by oestrogen (Cross *et al.*, 1998). These findings suggested that oestrogen deficiency may enhance Ca^{2+} influx through either L-type Ca^{2+} channels or the NCX, thus activating CaMKII. In this study we found that there was a higher expression of CaMKIIδ in oestrogen-deficient rats. We also showed an increase in basal Ca^{2+} in OVX rats, with or without isoprenaline incubation, supporting that an increase in cytosolic Ca^{2+} concentration is the leading cause for CaMKII activation.

It should be noted that oestrogen may also confer cardioprotection by mechanisms unrelated to the β-adrenoceptor and its signalling pathways. Oestrogen also protects the heart by up-regulating a series of beneficial genes including heart shock protein 70 (Nikolic *et al.*, 2007), by increasing the activity of mitochondrial respiratory complex IV (Hsieh *et al.*, 2006) and by other unknown mechanisms (Jovanović *et al.*, 2000).

Here, we found that in isolated perfused hearts from OVX rats subjected to ischaemic insult followed by reperfusion, LVEDP increased, while LVDP and average $+dP/dt$ and $-dP/dt$, reflecting decreases in velocity of contraction and relaxation respectively, decreased. These responses indicate impaired contractile recovery. We also demonstrated that in myocytes isolated from OVX rats subjected to ischaemic insult followed by reperfusion, the amplitude of the E[Ca^{2+}], which represents the amount of Ca^{2+} released from the sarcoplasmic reticulum, decreased while the time to peak, which represents the rate of Ca^{2+} release from the sarcoplasmic reticulum, and T_{50}, which represents the removal of Ca^{2+} from the cytosol, increased. These results suggest that slower and reduced Ca^{2+} release from the sarcoplasmic reticulum and slower removal of Ca^{2+} from the cytosol are most likely responsible for the decreased speed of contraction and relaxation of the heart respectively. It would be more convincing if both the contractile functions and the [Ca^{2+}] transient were measured simultaneously.

As expected, isoprenaline increased the amplitude of E[Ca^{2+}] in myocytes during the stable period, confirming that β-adrenoceptor stimulation increases cardiac contraction (Kam *et al.*, 2005; Yu *et al.*, 2008). On the other hand, in myocytes subjected to ischaemic insult followed by reperfusion, isoprenaline treatment during the insult led to a marked reduction in amplitude and marked increases in time to peak and T_{50}, indicating deleterious effects of β-adrenoceptor stimulation on contractile recovery. This is in agreement with the greater injury in response to β_1-adrenoceptor stimulation found in the present study, confirming the well-established deleterious role of β-adrenoceptor stimulation during ischaemia (Hu *et al.*, 2006; Li *et al.*, 2006; Otani *et al.*, 2006).

The L-type Ca^{2+} channel is reported to have higher expression and activity in oestrogen-deficient animals (Johnson *et al.*, 1997; Patterson *et al.*, 1998). In a previous study, we showed that blockade of PKA with its inhibitor, KT5720 at 2 $\mu mol \cdot L^{-1}$, abolishes the elevation in L-type Ca^{2+} channel activity in myocytes from OVX rats (Kam *et al.*, 2005). In contrast, we found in the present study that blockade of PKA with the same inhibitor at the same concentration failed to reverse the injury to the normal level induced by ischaemic insult and reperfusion in hearts from OVX rats. One likely explanation for this discrepancy is that the effect of blockade on L-type Ca^{2+} channels may not be sufficient to counteract the other effects of OVX on the heart, which cause injury. Further studies are warranted.

In the present study we administered a non-selective β-adrenoceptor agonist, isoprenaline, which activates both β_1-

and β_2-adrenoceptor subtypes. Activation of the former results in increased apoptosis, while activation of the latter reduced apoptosis (Communal *et al*., 1999). We found that isoprenaline increased apoptosis, indicating that the effects of activation of the former predominated over the latter.

There are two limitations in the study. The first is that, due to a lack of selective antagonists for CaMKIIδ, we were not able to employ a cause–effect approach by determining the effects of OVX after blockade of the isoform. The increased expression of the isoform provides evidence suggesting that this isoform may be involved. The second is that in the present study we chose the healthy cells according to two criteria, namely, rod-shape and striation. This is based on our previous experience. In a previous study we determined cardioprotection of preconditioning using rat ventricular myocytes. We looked at two parameters, namely, the shape of the myocyte and Trypan blue exclusion. We observed that rod-shaped cells are usually non-blue, while oval cells are blue, and that the patterns of changes in response to different treatments in these two parameters agree well with each other (Wu *et al*., 1999). However, the percentage of non-blue cells, that is, the healthy cells, is greater than that of rod-shaped cells. It would be better if more parameters such as contractile functions were to be used for choosing healthy cells.

In conclusion, the present study has provided evidence that oestrogen confers cardioprotection by suppressing CaMKII independently of as well as via β-adrenoceptors. CaMKIIδ is likely to be the isoform involved in this cardioprotection.

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Conflict of interest

The authors state no conflict of interest.

References

Bers DM (2002). Cardiac excitation-contraction coupling. *Nature* **415**: 198–205.

Braun AP, Schulman H (1995). The multifunctional calcium/calmodulin-dependent protein kinase-from form to function. *Annu Rev Physiol* **57**: 417–445.

Campbell DL, Strauss HC (1995). Regulation of calcium channels in the heart. *Adv Second Messenger Phosphoprotein Res* **30**: 25–88.

Chu SH, Goldspink P, Kowalski J, Beck J, Schwertz DW (2006). Effect of estrogen on calcium-handling proteins, beta-adrenergic receptors, and function in rat heart. *Life Sci* **79**: 1257–1267.

Colditz GA, Willett WC, Stampfer MJ, Rosner B, Speizer FE, Hennekens CH (1987). Menopause and the risk of coronary heart disease in women. *N Engl J Med* **316**: 1105–1110.

Communal C, Singh K, Sawyer DB, Colucci WS (1999). Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein. *Circulation* **100**: 2210–2212.

Cross HR, Lu L, Steenbergen C, Philipson KD, Murphy E (1998). Overexpression of the cardiac Na^+/Ca^{2+} exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. *Circ Res* **83**: 1215–1223.

Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ (1991). Model systems for the study of seven-transmembrane-segment receptors. *Annu Rev Biochem* **60**: 653–688.

Hayward CS, Kelly RP, Collins P (2000). The roles of gender, the menopause and hormone replacement on cardiovascular function. *Cardiovasc Res* **46**: 28–49.

Hook SS, Means AR (2001). Ca^{2+}/CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* **41**: 471–505.

Hsieh YC, Yu HP, Suzuki T, Choudhry MA, Schwacha MG, Bland KI *et al*. (2006). Upregulation of mitochondrial respiratory complex IV by estrogen receptor-beta is critical for inhibiting mitochondrial apoptotic signaling and restoring cardiac functions following trauma-hemorrhage. *J Mol Cell Cardiol* **41**: 511–521.

Hu A, Jiao X, Gao E, Koch WJ, Sharifi-Azad S, Grunwald Z *et al*. (2006). Chronic beta-adrenergic receptor stimulation induces cardiac apoptosis and aggravates myocardial ischaemia/reperfusion injury by provoking inducible nitric-oxide synthase-mediated nitrative stress. *J Pharmacol Exp Ther* **318**: 469–475.

Johnson BD, Zheng W, Korach KS, Scheuer T, Catterall WA, Rubanyi GM (1997). Increased expression of the cardiac L-type calcium channel in estrogen receptor-deficient mice. *J Gen Physiol* **110**: 135–140.

Jovanovic S, Jovanovic A, Shen WK, Terzic A (2000). Low concentrations of 17beta-estradiol protect single cardiac cells against metabolic stress-induced Ca^{2+} loading. *J Am Coll Cardiol* **36**: 948–952.

Kam KW, Qi JS, Chen M, Wong TM (2004). Estrogen reduces cardiac injury and expression of beta1-adrenoceptor upon ischaemic insult in the rat heart. *J Pharmacol Exp Ther* **309**: 8–15.

Kam KWL, Kravtsov GM, Liu J, Wong TM (2005). Increased PKA activity and its influence on isoprenaline-stimulated L-type Ca^{2+} channels in the heart from ovariectomized rats. *Br J Pharmacol* **144**: 972–981.

Kitazawa T, Hamada E, Kitazawa K, Gaznabi AK (1997). Non-genomic mechanism of 17 beta-oestradiol-induced inhibition of contraction in mammalian vascular smooth muscle. *J Physiol* **499**: 497–511.

Kravtsov GM, Kam KW, Liu J, Wu S, Wong TM (2007). Altered Ca^{2+} handling by ryanodine receptor and Na^+-Ca^{2+} exchange in the heart from ovariectomized rats: role of protein kinase A. *Am J Physiol Cell Physiol* **292**: C1625–C1635.

Li L, Zhang LK, Pang YZ, Pan CS, Qi YF, Chen L *et al*. (2006). Cardioprotective effects of ghrelin and des-octanoyl ghrelin on myocardial injury induced by isoproterenol in rats. *Acta Pharmacol Sin* **27**: 527–535.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Nikolic I, Liu D, Bell JA, Collins J, Steenbergen C, Murphy E (2007). Treatment with an estrogen receptor-beta-selective agonist is cardioprotective. *J Mol Cell Cardiol* **42**: 769–780.

Otani H, Matsuhisa S, Akita Y, Kyoi S, Enoki C, Tatsumi K *et al*. (2006). Role of mechanical stress in the form of cardiomyocyte death during the early phase of reperfusion. *Circ J* **70**: 1344–1355.

Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X *et al*. (2004). 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. *Circ Res* **95**: 692–699.

Patterson E, Ma L, Szabo B, Robinson CP, Thadani U (1998). Ovariectomy and estrogen-induced alterations in myocardial contractility in female rabbits: role of the L-type calcium channel. *J Pharmacol Exp Ther* **284**: 586–591.

Ramirez MT, Zhao X, Schulman H, Brown JH (1997). The nuclear δB isoform of Ca^{2+}/calmodulin-dependent protein kinase II regulates

atrial natriuretic factor gene expression in ventricular myocytes. *J Biol Chem* **272**: 31203–31208.

Singer HA, Benscoter HA, Schworer CM (1997). Novel Ca^{2+}/calmodulin-dependent protein kinase II gamma-subunit variants expressed in vascular smooth muscle, brain, and cardiomyocytes. *J Biol Chem* **272**: 9393–9400.

Stampfer MJ, Colditz GA, Willett WC, Manson JE, Rosner B, Speizer FE *et al.* (1991). Postmenopausal Estrogen Therapy and Cardiovascular-Disease – 10-Year Follow-up from the Nurses' Health Study. *N Engl J Med* **325**: 756–762.

Thawornkaiwong A, Preawnim S, Wattanapermpool J (2003). Upregulation of beta 1-adrenergic receptors in ovariectomized rat hearts. *Life Sci* **72**: 1813–1824.

Tobimatsu T, Fujisawa H (1989). Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. *J Biol Chem* **264**: 17907–17912.

Ullrich ND, Koschak A, MacLeod KT (2007). Oestrogen directly inhibits the cardiovascular L-type Ca^{2+} channel Cav1.2. *Biochem Biophys Res Commun* **361**: 522–527.

Vila-Petroff M, Salas MA, Said M, Valverde CA, Sapia L, Portiansky E *et al.* (2007). CaMKII inhibition protects against necrosis and apoptosis in irreversible ischaemia-reperfusion injury. *Cardiovasc Res* **73**: 689–698.

Vittone L, Mundiña-Weilenmann C, Said M, Ferrero P, Mattiazzi A (2002). Time course and mechanisms of phosphorylation of phospholamban residues in ischemia-reperfused rat hearts. Dissociation of phospholamban phosphorylation pathways. *J Mol Cell Cardiol* **34**: 39–50.

Wang GY, Wu S, Pei JM, Yu XC, Wong TM (2001). Kappa- but not delta-opioid receptors mediate effects of ischaemic preconditioning on both infarct and arrhythmia in rats. *Am J Physiol Heart Circ Physiol* **280**: H384–H391.

Wang W, Zhu W, Wang S, Yang D, Crow MT, Xiao RP *et al.* (2004). Sustained beta1-adrenergic stimulation modulates cardiac contractility by Ca^{2+}/calmodulin kinase signaling pathway. *Circ Res* **95**: 798–806.

Wilson PW, Garrison RJ, Castelli WP (1985). Postmenopausal estrogen use, cigarette smoking, and cardiovascular morbidity in women over 50. The Framingham Study. *N Engl J Med* **313**: 1038–1343.

Wu S, Li HY, Wong TM (1999). Cardioprotection of preconditioning by metabolic inhibition in the rat ventricular myocyte. Involvement of kappa-opioid receptor. *Circ Res* **84**: 1388–1395.

Xiao RP, Zhu W, Zheng M, Cao C, Zhang Y, Lakatto EG *et al.* (2006). Subtype-specific alpha1- and beta-adrenoceptor signaling in the heart. *Trends Pharmacol Sci* **6**: 330–337.

Yu QJ, Si R, Zhou N, Zhang HF, Guo WY, Wang HC *et al.* (2008). Insulin inhibits beta-adrenergic action in ischaemic/reperfused heart: a novel mechanism of insulin in cardioprotection. *Apoptosis* **13**: 305–317.

Zaugg M, Xu W, Lucchinetti E, Shafiq SA, Jamali NZ, Siddiqui MA (2000). Beta-adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes. *Circulation* **102**: 344–350.

Zhang R, Khoo MS, Wu Y, Yang Y, Grueter CE, Ni G *et al.* (2005). Calmodulin kinase II inhibition protects against structural heart disease. *Nat Med* **11**: 409–417.

Zhang T, Brown JH (2004). Role of Ca^{2+}/calmodulin-dependent protein kinase II in cardiac hypertrophy and heart failure. *Cardiovasc Res* **63**: 476–486.

Zhu WZ, Wang SQ, Chakir K, Yang D, Zhang T, Brown JH *et al.* (2003). Linkage of beta1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca^{2+}/calmodulin kinase II. *J Clin Invest* **111**: 617–625.

Zhu WZ, Woo AYH, Yang DM, Cheng HP, Crow MT, Xiao RP (2007). Activation of CaMKII delta C is a common intermediate of diverse death stimuli-induced heart muscle cell apoptosis. *J Biol Chem* **282**: 10833–10839.