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RESEARCH PAPER

Oestrogen inhibits BMP4-induced BMP4 expression in cardiomyocytes: a potential mechanism of oestrogen-mediated protection against cardiac hypertrophy

Yu-Chun Wang*, Xiao-Lin Xiao*, Na Li, Di Yang, Yue Xing, Rong Huo, Ming-Yu Liu, Yan-Qiu Zhang and De-Li Dong

Department of Pharmacology (the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, Harbin, China

Correspondence
Professor De-Li Dong, Department of Pharmacology, Harbin Medical University, Baojian Road 157, Harbin 150086, Heilongjiang Province, China. E-mail: dongdeli@ems.hrbmu.edu.cn

*These authors contributed equally to this work.

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BACKGROUND AND PURPOSE

Oestrogen inhibits cardiac hypertrophy and bone morphogenetic protein-4 (BMP4) induces cardiac hypertrophy. Here we have studied the inhibition by oestrogen of BMP4 expression in cardiomyocytes.

EXPERIMENTAL APPROACH

Cultures of neonatal rat cardiomyocytes were used in *in vitro* experiments. Bilatαl ovariectomy (OVX) was carried out in female Kunming mice and cardiac hypertrophy was induced by transverse aortic constriction (TAC).

KEY RESULTS

Oestrogen inhibited BMP4-induced cardiomyocyte hypertrophy and BMP4 expression *in vitro*. The inhibition of BMP4-induced BMP4 protein expression by oestrogen was prevented by the inhibitor of oestrogen receptor-β, PHTPP, but not by the inhibitor of oestrogen receptor-α MPP. BMP4 induced smad1/5/8 activation, which was not affected by oestrogen in cardiomyocytes. BMP4 induced JNK but not ERK1/2 and p38 activation, and activated JNK was inhibited by oestrogen. Treatment with the p38 inhibitor SB203580 or the JNK inhibitor SP600125 inhibited BMP4-induced BMP4 expression in cardiomyocytes, but the ERK1/2 inhibitor U0126 increased BMP4-induced BMP4 expression, indicating that JNK, ERK1/2 and p38 MAPKs were all involved, although only JNK activation contributed to the inhibition of BMP4-induced BMP4 expression by oestrogen. TAC induced significant heart hypertrophy in OVX mice *in vivo* and oestrogen replacement inhibited TAC-induced heart hypertrophy in OVX mice. In parallel with the data of heart hypertrophy, oestrogen replacement significantly reduced the increased BMP4 protein expression in TAC-treated OVX mice.

CONCLUSIONS AND IMPLICATIONS

Oestrogen treatment inhibited BMP4-induced BMP4 expression in cardiomyocytes through stimulating oestrogen receptor-β and inhibiting JNK activation. Our results provide a novel mechanism underlying oestrogen-mediated protection against cardiac hypertrophy.

LINKED ARTICLES

This article is part of a themed section on Chinese Innovation in Cardiovascular Drug Discovery. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-23

Abbreviations

BMP4, bone morphogenetic protein-4; ER-α, oestrogen receptor-α; ER-β, oestrogen receptor-β; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride; OVX, ovariectomy; PHTPP, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol; TAC, transverse aortic constriction

Tables of Links

TARGETS
Nuclear hormone receptors[a]
ER-α, oestrogen receptor-α
ER-β, oestrogen receptor-β
Enzymes[b]
ERK1/2
Furin
Histone deacetylase
JNK
p38
Ion channels[c]
$Ca_v3.1$ channels
$K_v4.3$ channels

LIGANDS
ANP, atrial natriuretic peptide
BNP, brain natriuretic peptide
MPP
PHTPP
SB203580
SP600125
β-oestradiol

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Pathological cardiac hypertrophy progressively leads to heart failure. Oestrogen (β-oestradiol), the female hormone, has been reported to inhibit cardiac hypertrophy and apoptosis by different research groups (van Eickels *et al.*, 2001; Donaldson *et al.*, 2009; Liu *et al.*, 2011). Ovariectomized (OVX) female mice treated with oestrogen had less hypertrophy than OVX females treated with vehicle in pressure overload-induced cardiac hypertrophy model (van Eickels *et al.*, 2001; Donaldson *et al.*, 2009). Further mechanistic studies showed that oestrogen inhibited heart hypertrophy through activating oestrogen receptor-β (ER-β; Skavdahl *et al.*, 2005; Babiker *et al.*, 2006; Fliegner *et al.*, 2010), increasing degradation of calcineurin (Pedram *et al.*, 2008; Donaldson *et al.*, 2009) or regulating histone deacetylase proteins (Pedram *et al.*, 2013). Although numerous studies have been carried out to investigate the effects of oestrogen on pathological cardiac hypertrophy, the details of the mechanism are still under investigation.

Bone morphogenetic protein-4 (BMP4), a member of the BMP family, has been extensively studied in embryo, bone and cartilage development (Monsoro-Burq *et al.*, 1996). BMP4 is a mechanosensitive and pro-inflammatory gene and our earlier study found that BMP4 expression increased in pressure-overload and Ang II constant infusion-induced cardiac hypertrophy; in turn, BMP4 induced pathological cardiac hypertrophy (Sun *et al.*, 2013a). BMP4 is synthesized as an inactive precursor that requires intracellular proteolysis by members of the proprotein convertase family to become the activated mature form to induce cardiac hypertrophy (Guo and Dong, 2014). BMP4 was also involved in cardiac ion channel remodelling. We have recently found that BMP4 induced up-regulation of $Ca_v3.1$ channels in HL-1 atrial myocytes and contributed to the down-regulation of $K_v4.3$ channels in the left ventricle of hypertrophic heart (Sun *et al.*, 2013b; Hu *et al.*, 2014). It was very interesting that BMP4 up-regulated itself in cardiomyocytes (Sun *et al.*, 2013a; Lu *et al.*, 2014), which would magnify the harmful effects of BMP4. Therefore, blockade of BMP4 and its related signalling is of therapeutic significance (Guo and Dong, 2014). Moreover, BMP4 mediated oestrogen-regulated sensory axon plasticity in the adult female reproductive tract and oestrogen down-regulated BMP4 expression in vaginal smooth muscle (Bhattacherjee *et al.*, 2013), indicating that BMP4 is a target of oestrogen's actions. As BMP4 mediates pathological cardiac hypertrophy, if oestrogen inhibited BMP4 expression in the cardiomyocytes, it would be a novel mechanism by which oestrogen inhibits pathological cardiac hypertrophy. Therefore, the present study was designed to test this hypothesis.

Methods

Pressure overload-induced cardiac hypertrophy

All animal care and experimental procedures were approved by the IACUC of Harbin Medical University, China. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 60 animals were used in the experiments described here.

The pressure-overload heart hypertrophy model consisted of animals with transverse aortic constriction (TAC), as described previously (Dong *et al.*, 2010; Sun *et al.*, 2013a). Adult Kunming female mice (22–26 g body weight; SLAC laboratory Animal Co., Ltd., Shanghai, China) were anaesthetized and endotracheal intubation was performed. The chest was opened and the thoracic aorta was identified. A 7-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle, which was subsequently removed. The animals were ventilated artificially until they regained consciousness.

Bilateral OVX

The dorsal skin of female Kunming mice was shaved after being anaesthetized with pentobarbital sodium. Then, mice were fixed in the prone position and decontaminated with povidone-iodine. A single 1–2 cm dorsal midline longitudinal incision was made in the skin, which was centred by the intersection point of dorsal median line and the horizontal line 0.5 cm above the superior margin of the bilateral roots of the thigh. Skin was bluntly dissected from s.c. tissue leftward or rightward from the incision to find the white cellulite under muscular layer. Then, a 0.5 cm incision was made in muscular layer to exteriorize and remove the ovary. The tissues pulled out simultaneously were returned into the abdomen. After both ovaries were removed, the skin was closed with an interrupted suture pattern using 5-0 silk.

In vivo *experiment protocol*

After 1 week of acclimation, adult female Kunming mice (3 weeks of age) were randomly assigned to undergo either sham operation or OVX performed under 1% pentobarbital sodium anaesthesia. From 6 weeks of age to the end of the experiments, all the ovariectomized mice were given either β-oestradiol by s.c. injection (10 $\mu g \cdot kg \cdot day^{-1}$), which was dissolved in 5% alcohol and 95% peanut oil or the same dose of vehicle (Fujita *et al.*, 2001; Zhou *et al.*, 2011). Oestrogen at 10 $\mu g \cdot kg \cdot day^{-1}$ was equivalent to the physiological levels (Zhou *et al.*, 2011). At 8 weeks of age, all the animals were given the TAC procedure, except that a subset of sham OVX was performed with sham TAC. At 12 weeks of age, all the mice were killed after being anaesthetized and followed by isolation of the heart and uterus. The whole heart, the left ventricle and uterus were weighed, allowing the calculation of the tissue : body weight ratios.

Primary culture of neonatal rat cardiomyocytes

The isolation and culture of neonatal rat cardiomyocytes were carried out as described earlier (Dong *et al.*, 2010; Sun *et al.*, 2013a). The purity of cardiomyocytes was increased by supplementing culture media with 5-bromo-2′-deoxyuridine to prevent non-cardiomyocytes from developing. Culture medium was renewed after 48 h and cells were further cultured for 24 h. The cardiomyocytes were cultured in non-serum DMEM for 12 h before the experiments. For measurement of cardiomyocyte cell area, immunocytochemical methods were used. Monoclonal antibody against sarcomeric α-actinin (Sigma-Aldrich) was added at dilutions of 1:200. Nuclear staining was performed with 1.3 $\mu mol \cdot L^{-1}$ of bisbenzimide (Sigma-Aldrich). The cardiomyocyte surface was estimated using Image-Pro Plus version 5.0.1 software (Media Cybernetics, Rockville, MD, USA) and the relative surface area was expressed as arbitrary units (the number of pixels), for evaluating hypertrophy as described earlier (Sun *et al.*, 2013a).

H9c2 cell culture

H9c2 cells were kindly donated by Xu Gao of the Biochemistry Department of Harbin Medical University, China. They were routinely cultured as in other studies (Urata *et al.*, 2006) in DMEM with 10% FBS and antibiotics (100 $U \cdot mL^{-1}$ penicillin G and 100 $\mu g \cdot mL^{-1}$ streptomycin) in an incubator with humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged every 2 days and grown to 70% of confluence prior to exposure to the treatments. H9c2 cells were cultured in non-serum DMEM for 12 h before the experiments.

Western blot

Detailed information on Western blotting has been given in our earlier papers (Dong *et al.*, 2010; Sun *et al.*, 2013a). Western blot bands were quantified using Odyssey infrared imaging system (Li-Cor, Lincoln, NE, USA) and Odyssey v3.0 software (Li-Cor, Inc.).

Real-time PCR analysis

The real-time PCR analysis and the real-time PCR primer sequences for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and BMP4 have been described (Sun *et al.*, 2013a). Transcript quantities were compared by using the relative quantitive method, where the amount of detected mRNA normalized to the amount of endogenous control (GAPDH). The relative value to the control sample is given by $2^{-\Delta\Delta CT}$.

Data analysis

Data are presented as mean ± SEM. Significance was determined by using paired Student's *t*-test or one-way ANOVA, followed by Tukey's post test. $P < 0.05$ was considered significant.

Materials

Oestrogen (β-oestradiol, Cat. No. E8875), SB203580 (Cat. No. s8307) and SP600125 (Cat. No. S5567) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Furin antibody (Cat. No. sc-20801), PHTPP [4-(2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl)phenol] (Cat. No. sc-204191) and MPP [1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride] (sc-204098) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-BMP-4 antibodies (Cat. No. sc-12721) and anti-smad1/5/8 antibodies (Cat. No. sc-6031-R) were from Santa Cruz Biotechnology. Anti-phospho-p38 (Cat. No.

4631S), anti-p38 (Cat. No. 9212), anti-p-ERK1/2 (Cat. No. 4370S), anti-ERK1/2 (Cat. No. 4372S), anti-p-JNK (Cat. No. 4668s), anti-JNK (Cat. No. 9258P), anti-p-smad1/5/8 antibody (Cat. No. 9511) and U0126 (Cat. No. 9903) were from Cell Signaling Technology (Beverly, MA, USA). Recombinant human BMP-4 (Cat. No. 314-BP-050) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-sarcomeric α-actinin (ab1321) was from Abcam (Cambridge, UK). Anti-GAPDH (KC-5G4) was from Kangchen (Shanghai, China). Anti-β-actin (Cat. No. TA-09) was from Beijing Zhong Shan-Golden Bridge Biological Technology (Beijing, China).

Results

Oestrogen inhibits BMP4-induced cardiomyocyte hypertrophy

Our previous study showed that BMP4 induced cardiomyocyte hypertrophy *in vitro* (Sun *et al.*, 2013a). Here, we used this BMP4-induced cardiomyocyte hypertrophy model to test the effects of oestrogen on cultured cardiomyocytes. As shown in Figure 1A, oestrogen treatment significantly inhibited BMP4-induced increase of the cell area of cardiomyocytes. We further measured the effects of oestrogen on the mRNA expression of the hypertrophic markers, ANP and BNP. BMP4 treatment increased ANP and BNP mRNA expression in cardiomyocytes, but these increases were not inhibited by oestrogen treatment (Figure 1B). Oestrogen was reported to directly induce ANP and BNP expressions in cardiomyocytes, and oestrogen-mediated induction of ANP and BNP contributed to its antagonistic effects in cardiac hypertrophy and fibrosis (Babiker *et al.*, 2004; Pedram *et al.*, 2008). Therefore, it is possible that the oestrogen-mediated induction of ANP and BNP expressions balanced any possible oestrogen-induced inhibition of the BMP4-induced increase of ANP and BNP expression. Oestrogen alone did not affect the cell area of cultured cardiomyocytes (Figure 1C).

Oestrogen inhibits BMP4-induced BMP4 expression through ER-β

As oestrogen treatment inhibited BMP4-induced cardiomyocyte hypertrophy, next, we examined whether oestrogen treatment inhibited BMP4-induced BMP4 expression in cardiomyocytes. Oestrogen treatment inhibited BMP4-induced increase of BMP4 mRNA and protein expressions in cardiomyocytes (Figure 2A and B). Furin is one of the proprotein convertases and is responsible for the cleavage of pro-BMP4 to the activated mature form. We therefore assessed the effects of BMP4 and BMP4 + oestrogen on furin protein in cardiomyocytes and found no significant effect on expression of this enzyme (Figure 2B).

Two ERs, ER-α and ER-β, are known to mediate the effects of oestrogen. To identify the ERs mediating the inhibitory effect of oestrogen on cardiomyocyte hypertrophy, we used H9c2 cells because these cells are known to express ER-β but not ER-α (Urata *et al.*, 2006). In H9c2 cells, BMP4-induced BMP4 protein expression was inhibited by oestrogen treatment (Figure 2C). We further examined the effects of the ER-β inhibitor, PHTPP, and the ER-α inhibitor, MPP, on oestrogen-inhibited BMP4 expression. As shown in Figure 2D and E, in cultured cardiomyocytes, oestrogen treatment inhibited BMP4-induced BMP4 protein expression and the inhibition

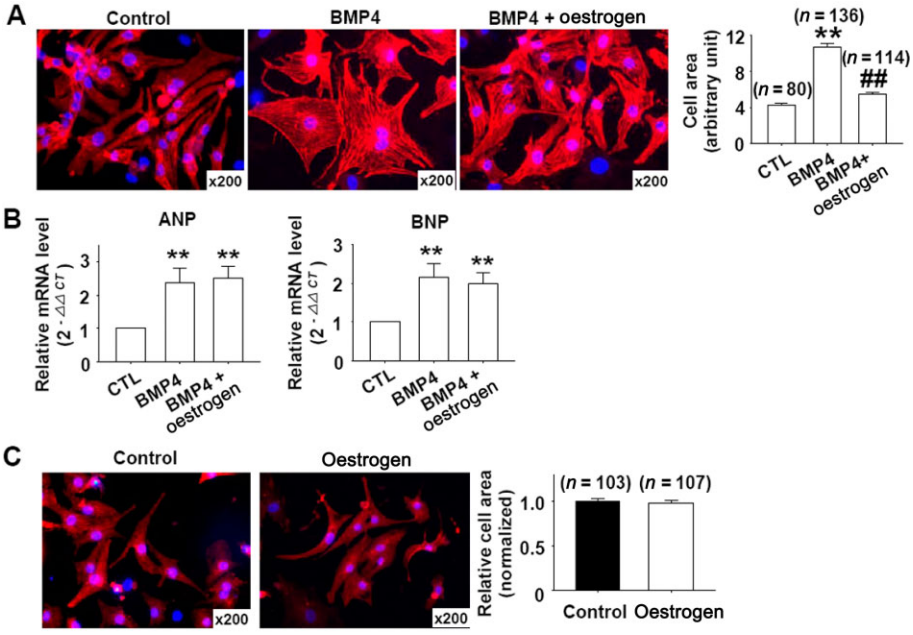

Figure 1

Oestrogen inhibits BMP4-induced cardiomyocyte hypertrophy. (A) Oestrogen (200 nM) inhibited BMP4 (50 ng·mL^{-1})-induced increase of the cell area of cardiomyocytes. **P < 0.01 versus control; ##P < 0.01 versus BMP4. (B) Oestrogen did not inhibit BMP4-induced increases of ANP and BNP mRNA expression. **P < 0.01 versus control. n = 10 per group. (C) Oestrogen (200 nM) alone did not affect the cell area of cardiomyocytes. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CTL, control.

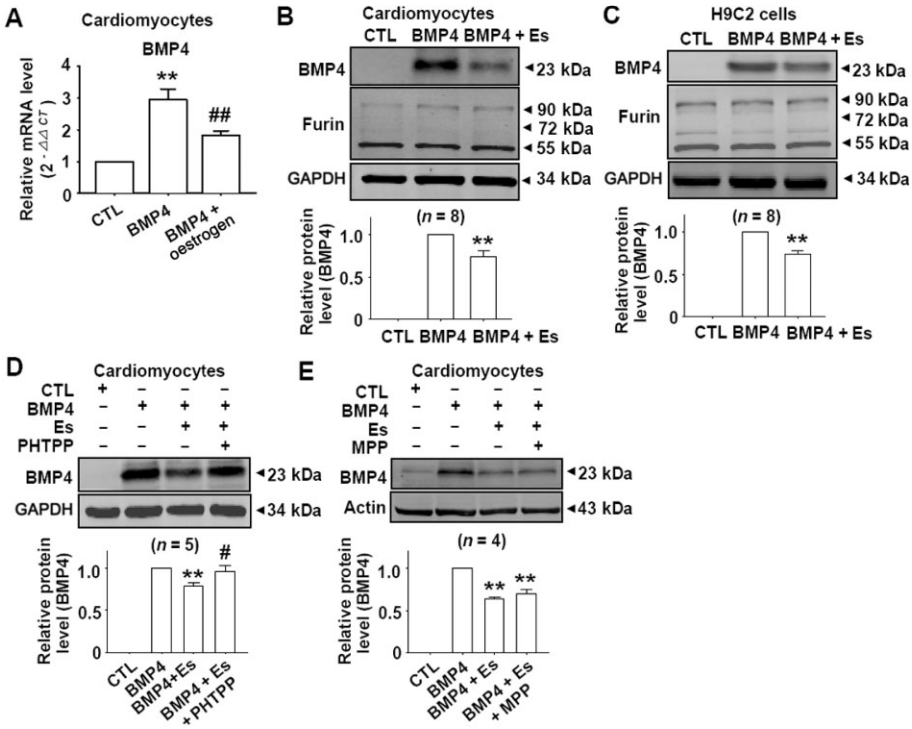

Figure 2

Oestrogen inhibits BMP4-induced BMP4 expression through ER-β. (A) Oestrogen (200 nM) treatment inhibited BMP4 (50 ng·mL^{-1})-induced increase of BMP4 mRNA expression in cardiomyocytes. $n = 16$. **$P < 0.01$ versus control; ## $P < 0.01$ versus BMP4 (50 ng·mL^{-1}). CTL, control. (B) Oestrogen (200 nM) treatment inhibited BMP4 (50 ng·mL^{-1})-induced BMP4 protein expression in cardiomyocytes. BMP4 and BMP4 + oestrogen showed no significant effect on furin protein expression in cardiomyocytes. **$P < 0.01$ versus BMP4. Es, oestrogen. (C) Oestrogen (200 nM) treatment inhibited BMP4 (50 ng·mL^{-1})-induced BMP4 protein expression in H9c2 cells. BMP4 and BMP4 + oestrogen showed no significant effect on furin protein expression in H9c2 cells. **$P < 0.01$ versus BMP4. Es, oestrogen. (D, E) Oestrogen treatment inhibited BMP4-induced BMP4 protein expression and the inhibition was prevented by the ER-β inhibitor PHTPP (100 nM) but not the ER-α inhibitor MPP (100 nM). **$P < 0.01$ versus BMP4; #$P < 0.05$ versus BMP4 + Es. Es, oestrogen.

was prevented by PHTPP but not by MPP. These results indicated that oestrogen inhibited BMP4-induced BMP4 expression through ER-β in cardiomyocytes.

Oestrogen does not affect BMP4-induced smad1/5/8 activation

The smad pathway is an important downstream signal of BMPs binding to their receptors and oestrogen inhibited p-smad 1 expression in dorsal root ganglion neurons (Bhattacherjee *et al.*, 2013). Therefore, we investigated whether the smad pathway was involved in oestrogen-induced inhibition of BMP4 expression. In order to observe the time-course of smad1/5/8 activation, we measured smad1/5/8 protein expression at three time points, 1, 6 and 48 h after BMP4 and oestrogen treatments. As shown in Figure 3A, smad1/5/8 was activated, but no BMP4 induction was observed 1 h after BMP4 treatment. As shown in Figure 3B and C, BMP4 activated smad1/5/8 at both 6 and 48 h after BMP4 treatment and oestrogen treatment showed no significant inhibition on smad1/5/8 activation. These data showed that the changes in smad1/5/8 activation and BMP4 expression were not parallel, indicating that inhibition of BMP4-induced BMP4 expression in cardiomyocytes by oestrogen did not involve smad1/5/8 activation.

Oestrogen inhibits BMP4-induced BMP4 expression through inhibiting JNK

BMP signalling includes smad and non-smad pathways (Miyazono *et al.*, 2005). As the classical smad pathway was not involved in BMP4-induced BMP4 expression in cardiomyocytes, we focused upon the non-smad pathway, which involves MAPK. BMP4 is known to activate JNK in cardiomyocytes (Pachori *et al.*, 2010; Sun *et al.*, 2013a) and oestrogen inhibited cardiac p38 and JNK MAPKs (van Eickels *et al.*, 2001; Satoh *et al.*, 2007). In the present experiments, we found that BMP4 induced activation of JNK but not of ERK1/2 or p38 and that oestrogen treatment inhibited BMP4-induced JNK activation (Figure 4 A-C). Oestrogen treatment alone did not affect JNK, ERK1/2 and p38 MAPKs.

In order to assess the relevance of JNK activation to BMP4-induced BMP4 expression in cardiomyocytes, we used selective kinase inhibitors on BMP4-induced BMP4 expression. As shown in Figure 5A, the p38 inhibitor SB203580 or the JNK inhibitor SP600125 significantly inhibited BMP4-induced BMP4 expression, but the ERK 1/2 inhibitor U0126 significantly increased BMP4-induced BMP4 expression. These results indicated that although JNK, ERK1/2 and p38 MAPKs were all involved in BMP4-induced BMP4 expression in cardiomyocytes, only activation of JNK was casually related to the

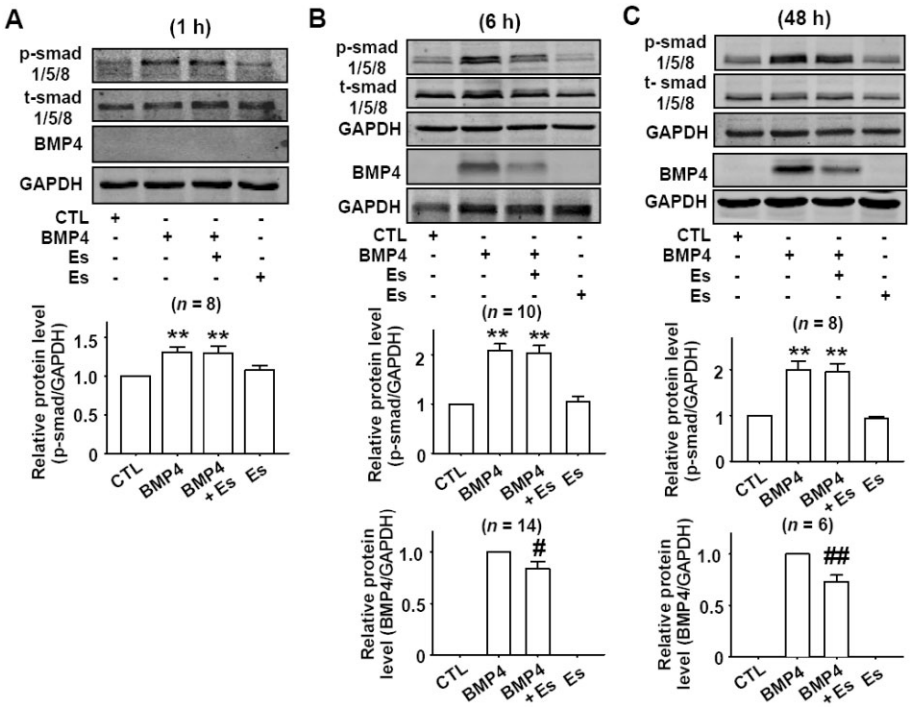

Figure 3

Oestrogen does not affect BMP4-induced smad1/5/8 activation. Phosphorylated and total smad1/5/8 protein expression were measured at 1, 6 and 48 h after BMP4 and oestrogen treatments. $^{**}P < 0.01$ versus CTL; $\#P < 0.05$, $\#\#P < 0.01$ versus BMP4. CTL, control; Es, oestrogen. The concentrations of BMP4 and oestrogen were 50 ng·mL^{-1} and 200 nM.

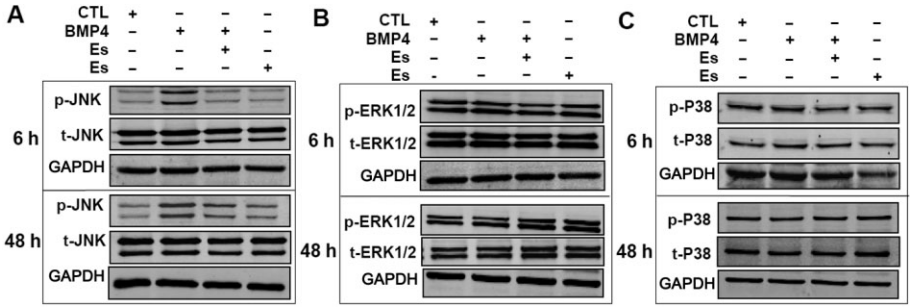

Figure 4

Oestrogen inhibits BMP4-induced BMP4 expression through inhibiting JNK. BMP4 (50 ng·mL^{-1}) induced JNK but not ERK1/2 and p38 activation. Oestrogen (200 nM) treatment inhibited BMP4-induced JNK activation. Oestrogen (200 nM) treatment alone did not affect JNK, ERK1/2 and p38 MAPKs. The Western blot represents five independent experiments. The concentrations of BMP4 and oestrogen were 50 ng·mL^{-1} and 200 nM. CTL, control; Es, oestrogen.

inhibition of BMP4-induced BMP4 expression by oestrogen (Figure 5B).

Oestrogen inhibits pressure overload-induced heart hypertrophy and BMP4 up-regulation in OVX mice in vivo

In order to elucidate whether oestrogen treatment inhibited BMP4 expression *in vivo*, we used the model with TAC in OVX mice and in OVX mice with oestrogen replacement. As shown in Figure 6A, TAC treatment significantly induced heart hypertrophy as demonstrated by the increased heart weight/body weight (HW/BW) and left ventricle weight/body weight (LVW/BW) ratios in control and OVX groups. Oestrogen replacement significantly inhibited TAC-induced heart hypertrophy in OVX mice. Uterus weight (UW) was measured to demonstrate the effectiveness of OVX and oestrogen replacement. As shown in Figure 6B, OVX decreased the UW/BW ratio and oestrogen replacement significantly increased the UW/BW ratio of OVX mice. We further com-

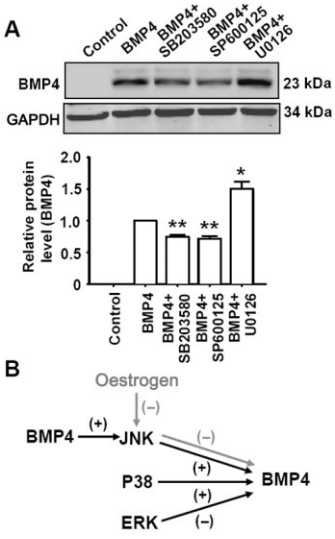

Figure 5

Effects of MAPKs on BMP4-induced BMP4 protein expressions in cardiomyocytes. (A)The p38 inhibitor SB203580 (10 μM) or the JNK inhibitor SP600125 (10 μM) inhibited BMP4-induced BMP4 protein expression, but the ERK1/2 inhibitor U0126 (20 μM) increased BMP4-induced BMP4 protein expression. *$P < 0.05$, **$P < 0.01$ versus control. $n = 5$ individual experiments. The concentration of BMP4 was 50 ng·mL^{-1}. (B) The diagram shows a mechanism for the inhibition, by oestrogen, of BMP4-induced BMP4 protein expression in cardiomyocytes. (+) stimulation; (–) inhibition.

pared the BMP4 protein expression in each group. As shown in Figure 6C, TAC increased BMP4 expression in control and OVX mice and there was a difference between TAC and OVX + TAC groups. Oestrogen replacement significantly reduced the increased BMP4 protein expression in OVX + TAC mice.

Discussion

Despite the numerous studies on the anti-hypertrophic effects of oestrogen, the mechanisms by which oestrogen inhibits cardiac hypertrophy are not fully understood. Here, we have demonstrated that oestrogen inhibited BMP4-induced BMP4 expression in cardiomyocytes. As we have previously found that BMP4 induced pathological heart hypertrophy and BMP4 up-regulated itself, the present results have allowed us to put forward a novel potential mechanism of oestrogen-mediated protection against cardiac hypertrophy.

Both ER-α and ER-β are expressed in the myocardium, but these receptors can regulate different genes (Murphy, 2011). In the cardiovascular system, ER-α is mainly responsible for activating endothelial NOS in adult endothelial cells, preventing vascular smooth muscle cell proliferation, and shows no effect on cardiac hypertrophy, whereas ER-β plays a crucial role in reducing pathological cardiac hypertrophy (Knowlton and Lee, 2012). There are a number of studies showing that oestrogen inhibits heart hypertrophy through ER-β and different mechanisms have been proposed. For example, Pedram *et al.* (2008) showed that oestrogen inhibited Ang II-induced cardiac hypertrophy in mice through ER-β and the mechanism involved inhibiting calcineurin activity (Pedram *et al.*, 2008), and modulating the histone deacetylase proteins (Pedram *et al.*, 2013). Similarly, other studies also showed that ER-β signalled heart hypertrophy by using ER-β knockout mice (Skavdahl *et al.*, 2005; Gurgen *et al.*, 2011). Recently, Kararigas *et al.*, (2011), using a public microarray dataset, showed that the lack of ER-β led to a significant increase of inflammatory pathways in pressure overload-induced hypertrophy .

In the present study, we used a range of experimental materials and pharmacological tools, including cultured neonatal cardiomyocytes, H9c2 cells, adult heart tissues, ER-α and ER-β selective blockers. The H9c2 cells, a cell line derived from embryonic rat heart tissues, expresses ER-β but not ER-α (Urata *et al.*, 2006). In cardiomyocytes from newborn and adult rats, there are also different outcomes of ER-α and ER-β expression (Jankowski *et al.*, 2001). However, our results showed that ER-β contributed to reducing BMP4-induced BMP4 expression in cardiomyocytes, which is consistent with the role of ER-β in reducing cardiac hypertrophy. Recently, several mechanisms have been proposed to explain the inhibitory effects of oestrogen on heart hypertrophy, such as increasing degradation of calcineurin (Pedram *et al.*, 2008; Donaldson *et al.*, 2009) or regulating histone deacetylase proteins (Pedram *et al.*, 2013). Inhibition of BMP4-induced BMP4 expression in cardiomyocytes by oestrogen is another possible mechanism.

In the present study, 200 nM oestrogen was used because our preliminary study showed that oestrogen at this concentration showed stable effects. Oestrogen concentrations from 10 nM to 50 μM have been used in the cardiovascular studies related to oestrogen (Nuedling *et al.*, 1999; Hamilton *et al.*, 2004; Satoh *et al.*, 2007; Cao *et al.*, 2011). CYP450 aromatase expressed in cardiomyocytes (Grohe *et al.*, 1998) and local oestrogen production potentially exerts important autocrine/paracrine influences on heart function and structural modelling (Bell *et al.*, 2013), indicating that local oestrogen level could be higher. Our *in vivo* experiments showed that oestrogen replacement reduced the increased BMP4 protein expression in OVX + TAC mice, supporting the probability that the *in vitro* dose of 200 nM oestrogen in the present work was of pharmacological significance.

In the present work, BMP4 protein measured by Western blot was endogenous. Although exogenous BMP4 was added in the experiments, this BMP4 was washed out before the cell proteins were collected. In our previous work (Sun *et al.*, 2013a), we have found that BMP4-induced increase of BMP4 mRNA and protein expression was inhibited by the endogenous BMP inhibitor, noggin, in cardiomyocytes. Together with the present data that oestrogen treatment inhibited BMP4-induced BMP4 mRNA and protein expression, we consider that BMP4 protein expression measured here was endogenous.

Oestrogen shows diverse effects on MAPKs. van Eickels *et al.* reported that oestrogen blocked the increased phosphorylation of p38 but did not affect ERK1/2 and JNK in TAC-treated mice (van Eickels *et al.*, 2001). Satoh *et al.* showed that oestrogen treatment inhibited the activation of cardiac JNK and p38 and reduced cardiac hypertrophy in $G\alpha_q$ transgenic (G_q) mice (Satoh *et al.*, 2007). Pedram *et al.* showed that oestrogen treatment inhibited Ang II-induced ERK activation

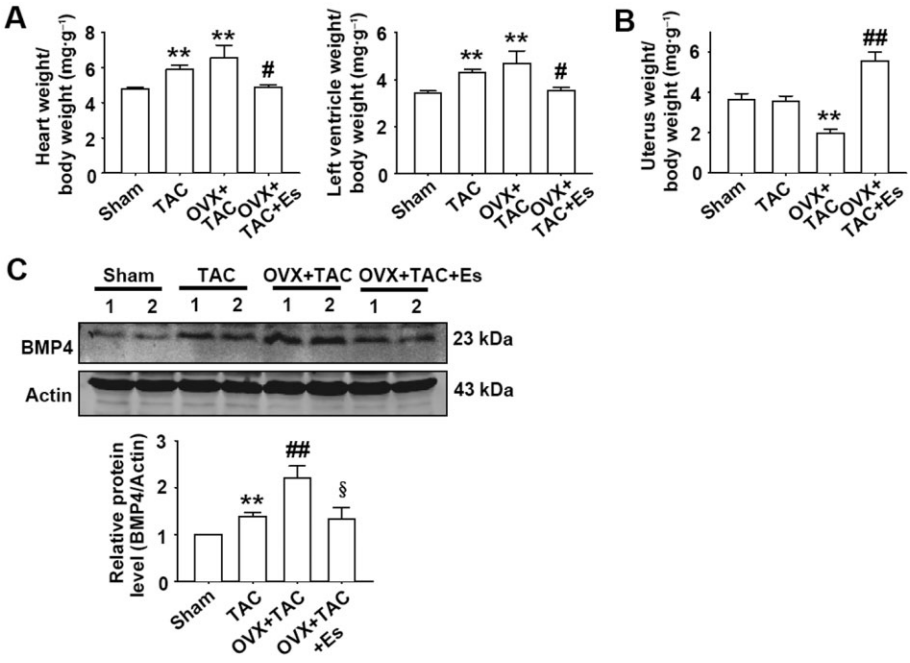

Figure 6

Oestrogen inhibits pressure overload-induced heart hypertrophy and BMP4 up-regulation in OVX mice *in vivo*. (A) TAC treatment induced heart hypertrophy as demonstrated by increased heart weight/body weight (HW/BW) and left ventricle weight/body weight (LVW/BW) ratios in control and OVX groups. **$P < 0.01$ versus sham; #$P < 0.05$ versus OVX + TAC. $n = 12, 12, 8, 8$ in sham, TAC, OVX + TAC, OVX + TAC + Es groups. Es, oestrogen. (B) OVX decreased the uterus weight/body weight ratio and oestrogen replacement increased the uterus weight/body weight ratio of OVX mice. **$P < 0.01$ versus sham; ## $P < 0.01$ versus OVX + TAC. $n = 12, 12, 5, 8$ in sham, TAC, OVX + TAC, OVX + TAC + Es groups. Es, oestrogen. (C) TAC induced increase of cardiac BMP4 expression in sham and OVX mice and oestrogen replacement reduced the increased cardiac BMP4 protein expression in OVX + TAC mice. Left ventricular tissues were used. $n = 5$ hearts per group. **$P < 0.01$ versus sham; ##$P < 0.01$ versus TAC; §$P < 0.05$ versus OVX + TAC. Es, oestrogen; OVX, ovariectomy; TAC, transverse aortic constriction.

and heart hypertrophy (Pedram *et al.*, 2008). In the present work, we found that oestrogen treatment inhibited BMP4-induced JNK activation and oestrogen treatment alone did not affect JNK, ERK1/2 and p38 MAPKs in cultured cardiomyocytes.

In summary, oestrogen inhibited BMP4-induced BMP4 expression in cardiomyocytes through stimulating ER-β and inhibiting JNK activation. As BMP4 mediates pathological cardiac hypertrophy, the present study proposes a novel mechanism by which oestrogen could inhibit pathological cardiac hypertrophy. Oestrogen is the predominant female hormone, affecting the development of several cardiovascular diseases in post-menopausal women (Khalil, 2013). Oestrogen replacement therapy has been shown to have protective effects against major cardiovascular diseases in post-menopausal women (Rockman *et al.*, 2012) and animal models (Bendale *et al.*, 2013). The present work provides further evidence supporting oestrogen replacement therapy for cardiovascular disease.

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Author contributions

Y-C. W., X-L. X., N. L., D. Y., Y. X., M-Y. L. and Y-Q. Z. performed the cell culture and Western blot experiments. R. H. performed real-time PCR. Y-C. W., X-L. X. and M-Y. L. performed the bilateral OVX and TAC operations. D-L. D. designed the project and wrote the article.

Conflict of interest

The authors declare no conflict of interest.

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