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17- β Estradiol Attenuates Ovariectomy-Induced Changes in Cardiomyocyte Contractile Function via Activation of AMP-Activated Protein Kinase

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Abstract

Menopause increases the risk of cardiometabolic diseases in women. This circumstance is usually attributed to a deficiency in circulating estrogen levels although the underlying mechanism remains elusive. Given the pivotal role of AMP-activated protein kinase (AMPK) in the regulation of energy metabolism and cardiac function, this study was designed to examine the role of AMPK in estrogen deficiency and replacement-exerted cardiomyocyte responses. Adult female WT and AMPK kinase dead (KD) mice were subjected to bilateral ovariectomy (OVX) or sham operation. A cohort of ovariectomized mice received 17 β -estradiol (E2) (40 μ g/kg/day, i.p.) for 6 weeks. Mechanical and intracellular Ca²⁺ properties were evaluated including peak shortening (PS), time-to-PS (TPS), time-to-90%-relengthening (TR₉₀), and maximal velocity of shortening/relengthening ($\pm$ dL/dt). Levels of AMPK, Akt, JNK, ACC, SERCA, membrane Glut4, AS160 and PGC-1 α were assessed using Western blot. OVX significantly decreased PS, $\pm$ dL/dt and intracellular Ca²⁺ rise in response to electric stimulus, prolonged TR₉₀ and intracellular Ca²⁺ decay without affecting TPS and resting intracellular Ca²⁺, the effects of which were reconciled by E2 replacement. Western blot analysis depicted that OVX suppressed phosphorylation of Akt, AMPK and ACC although it promoted JNK phosphorylation, the effects of which were mitigated or significantly attenuated by E2 treatment in WT but not KD mice. Moreover, OVX procedure

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CONFLICT OF INTEREST

The authors declared no conflict of interest for this work.

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downregulated SERCA2a and membrane Glut4 while inhibiting AS160 phosphorylation without affecting PGC-1 α levels. *In vitro* study revealed that E2 corrected cardiomyocyte contractile dysfunction elicited by OVX in cardiomyocytes from WT but not the AMPK kinase dead mice. Taken together, these data suggest that E2 treatment ameliorates estrogen deficiency-induced changes in cardiac contractile function possibly through an AMPK-dependent mechanism.

Keywords

estrogen; estrogen replacement; AMPK; cardiomyocyte; contractile function

INTRODUCTION

Cardiovascular diseases remain the number one cause of morbidity and mortality for both men and women. It is estimated that over 40% of the US population will be afflicted with some forms of cardiovascular diseases by the year of 2030 (Heidenreich et al., 2011). Although females are often protected from cardiovascular events compared with their male counterparts, a drastic rise in cardiovascular incidence is seen following menopause in women (Ren and Kelley, 2009), a condition usually attributed to estrogen deficiency (Manolagas et al., 2013; Mauvais-Jarvis et al., 2013; Moolman, 2006). Although ample clinical and experimental evidence has validated that estrogen confers direct cardioprotective action (Manolagas et al., 2013; Mauvais-Jarvis et al., 2013; Moolman, 2006; Murphy, 2011), it still remain controversial with regards to the clinical value of hormonal replacement therapy (HRT) following menopause in women with preexisting cardiovascular anomalies (Gnatuk, 2002; Stevenson, 2009; Valdiviezo et al., 2013). It is perceived that HRT process initiated long after menopause may pose a greater risk for post-myocardial infarction and other cardiovascular events (Ren, 2006; Rossouw, 2007). It is thus imperative to identify the cellular mechanisms behind estrogen-offered cardiovascular protection to better guide the clinical application and risk management of HRT. Our earlier observations suggested that estrogen replacement ameliorates ovariectomy-induced changes in cardiomyocyte contractile function and intracellular Ca²⁺ homeostasis (Hintz et al., 2001; Ren et al., 2003), suggesting a role of intrinsic estrogen in the regulation of myocardial mechanical and protein integrity (Duan et al., 2004). Recent evidence depicted that 17 β -estradiol may elicit its beneficial cardioprotective action via an AMP-activated protein kinase (AMPK)/histone H3 acetylation-dependent mechanism (Bendale et al., 2013).

AMPK is a metabolic sensor governing energy balance (Hardie, 2008). AMPK can be turned on in response to a fall in the ATP/AMP ratio to facilitate energy supply by increasing glucose transport and utilization free fatty acids in a variety of cells including cardiomyocytes (Lee and Kim, 2013; Shirwany and Zou, 2010). Recent evidence has consolidated a role for AMPK in the pathogenesis and therapeutics of aging, type 2 diabetes, obesity, dyslipidemia, and cardiovascular diseases (Lee and Kim, 2013; Turdi et al., 2010; Xiao et al., 2011). Given the prevalence of these cardiometabolic comorbidities in postmenopausal women (Mauvais-Jarvis et al., 2013; Ren, 2006), it is pertinent to elucidate the precise interplay between estrogen and AMPK. Indeed, AMPK has been demonstrated to be activated by estrogen in multiple tissues (Oosthuysen and Bosch, 2012). A rise in the

androgen/estrogen ratio promotes visceral fat accumulation through AMPK inhibition and subsequent lipogenesis (McInnes et al., 2006). More evidence depicted that estrogen promotes the partitioning of free fatty acids toward oxidation rather than triglyceride storage through AMPK activation in adipocytes (D'Eon et al., 2005). Stimulation of estrogen receptor α (ER α) promotes insulin-stimulated glucose uptake into soleus and extensor digitorum longus muscles, attributing to activation of Akt and AMPK (Gorres et al., 2011). Along the same line, estradiol treatment was found to display a time- and concentration-dependent activation of AMPK and its downstream target acetyl coenzyme A carboxylase (ACC), a process deemed essential for estradiol-induced eNOS activation (Schulz et al., 2005). Further study revealed activation of AMPK in response to metabolites of estrogen/estradiol, such as 2-hydroxyestradiol (D'Eon et al., 2008). Nonetheless, little information is now available with regards to the role of AMPK in estrogen deficiency and estrogen replacement-elicited cardiac contractile responses. To this end, this study was designed to examine the role of AMPK in estrogen replacement-induced cardiomyocyte contractile responses, if any, in an experimental setting of estrogen deficiency. Activation of Akt and AMPK, as well as the downstream AMPK target ACC and the stress signal c-Jun N terminal kinase (JNK), was evaluated in myocardium. Given that AMPK and Akt are known to phosphorylate the Akt substrate of 160KD (AS160) to initiate glucose transport by Glut-4 translocation to the plasma membrane (Funai and Cartee, 2009), phosphorylation of AS160 and membrane Glut-4 levels were monitored in myocardium. To ascertain the permissive role of AMPK in estrogen replacement-offered cardioprotection, if any, the AMPK transgenic mice overexpress a dominant negative $\alpha 2$ subunit of AMPK (kinase dead, KD) driven by a muscle specific creatine kinase promoter to skeletal and cardiac muscles (Turdi et al., 2010; Turdi et al., 2011) were employed.

METHODS AND MATERIALS

Experimental animal and ovariectomy procedure

All animal procedures used were approved by the Institutional Animal Care and Use Committee at the University of Wyoming (Laramie, WY). In brief, 10 to 12-week-old nulliparous female C57/BL6 wild-type (WT) or AMPKKD mice were assigned to weight-paired sham-operated (sham) or ovariectomy (OVX) groups. For OVX, mice were anesthetized with intraperitoneal injection of ketamine/xylazine (1 ml/kg body weight) and were placed on a warm pad (37°C). The ovaries were exteriorized, ligated and removed via bilateral paralumbar incisions, which were then closed with sterile sutures. The sham procedure was consisted of anesthesia, visualization of ovaries through incisions into the abdominal cavity, and closure of the wound. One week following surgery, a subgroup of ovariectomized mice was assigned to the estrogen (E2) replacement group, receiving a daily intraperitoneal injection of 17 β -estradiol (40 μ g/kg, 100 μ l cottonseed oil) for six weeks. The sham group received vehicle only (Ren et al., 2003). At the time of sacrifice, adequacy of ovariectomy was determined by the absence of ovarian tissue and measurement of uterine weight in female mice. To discern the role of AMPK in ovariectomy-induced cardiac contractile responses, 10 to 12-week-old female WT and AMPKKD mice expressing a dominant negative AMPK $\alpha 2$ isoform under the control of the muscle-specific creatine kinase promoter (kindly provided by Professor Morris Birnbaum (University of

Pennsylvania, Philadelphia, PA) were used. The dominant negative AMPK α 2 subunit replaces functional α 1 and α 2 subunits in AMPK thus resulting in very low AMPK activity (Turdi et al., 2010; Turdi et al., 2011). All mice were housed in clear plastic cages in a temperature- and humidity-controlled environment with a 12/12-light/dark cycle and were allowed *ad libitum* to lab chow and water.

Murine cardiomyocyte isolation

Hearts were rapidly removed from anesthetized mice and mounted onto a temperature-controlled (37°C) Langendorff system. After perfusing with a modified Tyrode's solution (Ca²⁺ free) for 2 min, the heart was digested with a Ca²⁺-free Krebs-Henseleit buffer (KHB) containing liberase blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN) for 20 min. The modified Tyrode solution contained the following (in mM): NaCl 135, KCl 4.0, MgCl₂ 1.0, HEPES 10, NaH₂PO₄ 0.33, glucose 10 and butanedione monoxime 10 while the solution was gassed with 5% CO₂/95% O₂. The digested heart was removed from cannula and left ventricle was cut into small pieces in the modified Tyrode's solution. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca²⁺ was added incrementally to 1.20 mM over 30 min. A yield of ~60% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Cardiomyocytes with sarcolemmal blebs or spontaneous contraction were not chosen for mechanical study. Only rod-shaped cardiomyocytes with clear edges were selected for contractile and intracellular Ca²⁺ evaluation (Hintz et al., 2001). To assess the impact of estrogen on contractile function of cardiomyocytes from ovariectomized WT and AMPK KD mice, freshly isolated cardiomyocytes were treated with 17 β -estradiol (10 nM) for 2 hrs prior to the assessment of mechanical function (Liu et al., 2011).

Cardiomyocyte contractile function

Mechanical properties of murine cardiomyocytes were assessed by an IonOptix Myocam system (IonOptix Incorporation, Milton, MA). Cells were placed in a chamber mounted on the stage of an inverted microscope and superfused (at 30 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES at pH 7.4. The cells were field stimulated with a supra-threshold voltage at a frequency of 0.5 Hz, 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE). Cell shortening and relengthening were assessed using the following indices: peak shortening (PS) – indicative of peak ventricular contractility, time-to-90% PS (TPS) – indicative of systolic duration, time-to-90% relengthening (TR₉₀) – indicative of diastolic duration, maximal velocities of shortening (+ dL/dt) and relengthening (dL/dt) – indicatives of maximal velocities of ventricular pressure rise/fall (Doser et al., 2009; Turdi et al., 2011).

Intracellular Ca²⁺ transient measurement

Cardiomyocytes were loaded with fura-2/AM (0.5 μ M) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix). Cardiomyocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor $\times$ 40 oil objective. Cells were exposed to light emitted by a 75 W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract

at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 s then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca^{2+} levels were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths (360/380). Fluorescence decay time was measured as an indication of the intracellular Ca^{2+} clearing rate. A single exponential curve fit program was applied to calculate the intracellular Ca^{2+} decay (Doser et al., 2009).

Western blot analysis

Left ventricular tissues were homogenized and sonicated in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS) and a protease inhibitor cocktail (Turdi et al., 2010). Protein levels of ER α , ER β , total and phosphorylated Akt, AMPK and JNK, phosphorylated ACC (pACC), the mitochondrial biogenesis cofactor PGC-1 α , SERCA2a, phosphorylated AS160 and membrane Glut4 were examined using standard Western blot analysis. Membranes were probed with anti-ER α (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, sc-542), anti-ER β (1:1,000, Santa Cruz, sc-8974), anti-Akt (1:1,000, Cell Signaling Technology Inc., Beverly, MA, 9272), anti-pAkt (Thr⁴⁷³, 1:1,000, Cell Signaling, 4060), anti-AMPK (1:1,000, Cell Signaling, 2532), anti-pAMPK (Thr¹⁷², 1:1,000, Cell Signaling, 2531), anti-pACC (Ser⁷⁹, 1:1,000, Cell Signaling, 3661), anti-JNK (1:1,000, Cell Signaling, 9252), anti-pJNK (Thr¹⁸³/Tyr¹⁸⁵, 1:1,000, Cell Signaling, 9251), anti-Glut4 (1:1,000, Cell Signaling, 2299), anti-PGC-1 α (1:500, Cell Signaling, 2178), anti-pAS160 (1:1,000, Cell Signaling, 2447), anti-SERCA2a (1:1,000, Bethyl Laboratories Inc., Montgomery, TX, A010-25AP), anti-GAPDH (1:1,000, Cell Signaling, 2118), anti- α -Tubulin (1:1,000 Cell Signaling, 2125), and anti-Na⁺-K⁺ ATPase (1:1,000, Cell Signaling, 3010) antibodies. The membranes were then incubated with horseradish peroxidase (HRP)-coupled anti-rabbit (Cell Signaling) antibody. Immunoreactive bands were detected using Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, WI). The intensity of bands was measured with a scanning densitometer, and the intensity of immunoblot bands was normalized to that of GAPDH, α -tubulin or Na⁺-K⁺ ATPase (as a loading control for membrane protein).

Myocardial membrane protein extraction

Myocardial membrane protein was extracted using a membrane protein extraction kit (Biovision, Mountain View, CA). The membrane protein was subsequently used for Western blot analysis of Glut-4 (Turdi et al., 2011).

Statistics

Data are presented as Mean $\pm$ SEM. Statistical significance ($p < 0.05$) for each variable was estimated by one-way ANOVA followed by Newman-Keuls *post hoc* test.

RESULTS

General features of animals

At the time of sacrifice, body weights were comparable in mice from Sham, OVX and OVX with E2 replacement groups. Ovariectomy procedure significantly increased heart weights (absolute or normalized to body weight or tibial length), the effect of which was attenuated by the E2 replacement therapy. Neither ovariectomy nor E2 replacement significantly affected myocardial levels of estrogen receptors ER α or ER β . However, combined ovariectomy and E2 replacement slightly although significantly downregulated myocardial ER α but not ER β levels (Fig. 1).

Mechanical and intracellular Ca²⁺ properties of cardiomyocytes

The resting cell length was comparable among all groups. Ovariectomy overtly decreased peak shortening amplitude (PS) and maximal velocity of shortening/relengthening ($\pm$ dL/dt) as well as prolonged relengthening duration (TR₉₀) without affecting shortening duration (TPS) in cardiomyocyte, the effects of which were reconciled or significantly ameliorated by E2 replacement (Fig. 2). To explore the potential mechanism of action behind ovariectomy- and E2 replacement-induced cardiomyocyte contractile responses, the Fura-2AM fluorescent dye was employed to evaluate the intracellular Ca²⁺ handling in cardiomyocytes. Our data shown in Fig. 3 revealed that ovariectomy significantly suppressed electrically-stimulated rise in fura-2 fluorescence intensity (FFI) and intracellular Ca²⁺ clearance without affecting resting and peak FFI, the effects of which were mitigated or significantly attenuated by E2 treatment.

Effect of E2 treatment on cardiomyocyte contractile properties in WT and AMPKKD mice

To discern if AMPK plays a permissive role in ovariectomy- and E2-supplement-offered responses in cardiomyocyte contraction, cardiomyocytes from ovariectomized WT or AMPKKD mice were incubated with or without E2 *in vitro* prior to the assessment of mechanical function. As depicted in Fig. 4, cardiomyocyte contractile parameters were mostly comparable between WT and AMPKKD groups in the absence of ovariectomy. Ovariectomy procedure compromised cardiomyocyte mechanical function as manifested by decreased PS, $\pm$ dL/dt and prolonged TR₉₀ in a comparable manner in cardiomyocytes from WT and AMPKKD mice. Resting cell length and TPS were unaffected by the surgical procedure in both WT and AMPKKD mice. Consistent with our *in vivo* finding, acute E2 treatment effectively rescued ovariectomy-induced changes in PS, $\pm$ dL/dt and TR₉₀ without affecting resting cell length and TPS in WT group although such beneficial properties of E2 supplement were ablated by AMPK deficiency.

Phosphorylation of cell signaling molecules JNK, Akt, AMPK and ACC

Western blot analysis revealed that ovariectomy procedure overtly increased JNK phosphorylation while suppressing phosphorylation of Akt, AMPK and ACC in WT mice, the effects of which were negated or significantly attenuated by E2 replacement. Interestingly, the E2 replacement-offered beneficial effects on phosphorylation of JNK, Akt, AMPK and ACC were negated by AMPK deficiency (Fig. 5). To discern the effect of

ovariectomy and E2 replacement on mitochondrial biogenesis, cytosolic Ca^{2+} removal and glucose transport, levels of PGC-1 α , SERCA2a, phosphorylation of AS160 [a substrate of Akt/AMPK to initiate glucose transport by Glut-4 translocation to plasma membrane (Funai and Cartee, 2009)], and membrane fraction of Glut4 (using Na^{+} - K^{+} -ATPase as a loading control for membrane protein) were scrutinized. Our data presented in Fig. 6 revealed that ovariectomy significantly downregulated SERCA2a levels, AS160 phosphorylation and membrane Glut4 levels with little effect on PGC-1 α in WT mice, the effects of which were reconciled or significantly attenuated by E2 replacement. Consistent with its effects on phosphorylation of JNK, Akt, AMPK and ACC, the E2 replacement-offered favorite responses on SERCA2a, AS160 phosphorylation and membrane Glut4 levels were cancelled off by AMPK deficiency.

DISCUSSION

The salient findings from our study suggest that AMPK activation is permissive to estrogen replacement-induced protection against ovariectomy-induced cardiomyocyte contractile anomalies, phosphorylation of JNK, dephosphorylation Akt, AMPK, ACC and AS160, as well as downregulation of SERCA2a and membrane Glut4. Consistent with our previous reports (Hintz et al., 2001; Ren et al., 2003), our experimental evidence revealed compromised mechanical and intracellular Ca^{2+} properties in murine cardiomyocytes from ovariectomized mice including depressed PS, reduced $\pm \text{dL/dt}$, prolonged TR_{90} , decreased FFI and prolonged intracellular Ca^{2+} clearance. Estrogen replacement effectively rescued against ovariectomy-induced cardiomyocyte contractile and intracellular Ca^{2+} handling alterations. Our further Western blot analysis revealed elevated phosphorylation of stress signaling JNK as well as suppressed phosphorylation of Akt and AMPK (as well as that of the AMPK downstream target ACC) following ovariectomy, the effects of which were significantly ameliorated or negated by E2 replacement. Our results further suggested decreased SERCA2a levels, AS160 phosphorylation and Glut4 membrane fraction in the absence of altered mitochondrial biogenesis (PGC-1 α) following ovariectomy, the effects of which were reversed by estrogen replacement. Intriguingly, estrogen-induced cardioprotective responses against ovariectomy were negated by AMPK inhibition (using the unique kinase dead AMPK KD murine model), suggesting a permissive role of AMPK in ovariectomy- and estrogen replacement-induced cardiomyocyte mechanical responses.

Women exhibit a greater prevalence for cardiometabolic comorbidities after menopause, indicating an important role for estrogen in the progression of these pathological conditions (Gnatuk, 2002; Liu et al., 2011; Manolagas et al., 2013; Mauvais-Jarvis et al., 2013; Ren, 2006). Here we employed an established experimental model of estrogen deficiency verified by reduced serum estrogen levels and uterine weight (Ren et al., 2003), the effects of which may be rescued by estrogen replacement (Ren et al., 2003). In our hands, chronic estrogen therapy significantly attenuated ovariectomy-induced cardiac remodeling as evidenced by greater heart weights (absolute or normalized values). This observation is consistent with our previously published reports (Duan et al., 2003; Ren et al., 2003). It is known that transdermal 17 β -estradiol therapy effectively retards ventricular hypertrophy and improves cardiac performance in hypertensive women (Modena et al., 1999). Along the same line, high dose estradiol treatment resulted in less pronounced development of post-myocardial

infarction remodeling and improved left ventricular function (Beer et al., 2007). Our current results revealed that ovariectomy overtly suppressed cardiomyocyte contractile capacity (depressed PS, $\pm$ dL/dt and prolonged TR₉₀) and impaired intracellular Ca²⁺ handling (decreased FFI and prolonged intracellular Ca²⁺ clearance). These findings are consistent with our earlier observations (Duan et al., 2003; Ren et al., 2003). Gender difference in myocardial mechanical function has been recognized, as characterized by shorter contraction duration and faster tension development/decline associated with a comparable peak tension development in adult females (Ren and Kelley, 2009). It has been depicted that ovarian hormone-related disparity in myocardial contractile protein functions may be responsible for the mechanical differences (Ren and Kelley, 2009) although other intrinsic factors may also play a role. Estrogen receptors are present on a variety of cell types including cardiomyocytes. Data from our study did not favor a role for ER α and ER β receptors in the observed changes in cardiac contractile and intracellular Ca²⁺ properties. Although it is somewhat puzzling that combined ovariectomy and E2 replacement downregulated ER α but not ER β receptor expression, a direct downregulation of ER α may develop over chronic stimulation of the ligand. Several mechanisms may be postulated for ovariectomy or estrogen deficiency-induced changes in cardiomyocyte function. Ovariectomy was shown to alter L-type Ca²⁺ channel density (Kam et al., 2005), which may contribute to the compromised intracellular Ca²⁺ homeostasis found in our experimental setting. Ovarian hormones (e.g., estrogen, progesterone) alter myocardial contractile function such as myofilament Ca²⁺ sensitivity without significant change in the maximum force development (Wattanapernpool and Reiser, 1999). Intriguingly, our data revealed that estrogen replacement effectively rescued ovariectomy-induced alterations in cardiomyocyte contractile and intracellular Ca²⁺ handling properties, in conjunction with preserved SERCA2a levels. These data favor a seemingly role for intracellular Ca²⁺ handling in estrogen treatment-induced beneficial myocardial responses. The lack of a full recovery of relengthening duration (TR₉₀) and $-dL/dt$ may be related to contribution from other ovarian hormones upon ovariectomy or potential issues related to estrogen replacement dosage and/or duration. Meanwhile, other possible mechanisms may also contribute to the estrogen-offered protective responses including estrogen-induced antiapoptotic and antioxidant (Kim et al., 2006), antifibrotic (Stewart et al., 2006), antihypertrophic (van Eickels et al., 2001) and improved vasodilatory properties (Thompson et al., 2000) although this is beyond the scope of the current study.

Perhaps the most interesting piece of data from our study is that AMPK deficiency nullified estrogen-elicited cardiac mechanical benefit and signaling responses in ovariectomy, indicating a permissive role of AMPK in ovariectomy- and estrogen replacement-induced cardiac contractile responses and associated changes in cell signaling. AMPK is a heterotrimeric complex consisting of a catalytic isoform (α) and two regulatory isoforms (β and γ). There are two α -isoforms (α 1 and α 2) and two β -isoforms (β 1 and β 2) whereas three γ isoforms (γ 1, γ 2, and γ 3) have been identified (Hardie et al., 1998). AMPK α 2 is the predominant isoform in the heart (Stapleton et al., 1996). Ample of evidence has consolidated a role for AMPK in the homeostasis of myocardial function and energy metabolism in particular through preserved energy production (glucose and fatty acid oxidation) (Beauloye et al., 2011; Kim and Tian, 2011). Our data supported the presence of

impaired AMPK signaling in the heart following ovariectomy (reduced phosphorylation of AMPK and ACC). Interestingly, estrogen replacement restored AMPK signaling, favoring a likely role of AMPK in the maintenance of normal cardiomyocyte function. Hu and colleagues depicted that constitutively active AMPK α 2 is capable of upregulating estrogen-related receptor- α (ER α) and certain metabolic genes in cardiomyocytes, demonstrating a role for AMPK α 2 in the regulation of ER α in the heart (Hu et al., 2011). Consistent with our previous report (Ren et al., 2003), our data revealed suppressed Akt activation in the heart following ovariectomy, the effect of which was reversed by estrogen replacement therapy in WT but not AMPKKD group. Reduced Akt activation in ovariectomized mouse hearts and the ability of estrogen replacement to restore Akt activation coincides with ability of estrogen to turn on Akt signaling, which is believed to play a role in estrogen-regulated cardiac function (Ren and Kelley, 2009). The fact that estrogen replacement therapy reversed ovariectomy-induced elevation in JNK activation, in an AMPK-dependent manner, also favor a role for the pro-survival Akt and stress signaling under estrogen deficiency and supplementation.

Findings from our study revealed that estrogen replacement reconciled ovariectomy-induced dampened AS160 phosphorylation, a known Glut4 translocation mediator (Funai and Cartee, 2009). This is in line with the changes in membrane Glut4 following ovariectomy and estrogen replacement, suggesting a possible role for AS160-Glut4 in ovariectomy- and estrogen replacement-induced cardiomyocyte mechanical responses. AMPK is known to promote Glut-4 translocation through AS160 phosphorylation (Kramer et al., 2006). In fact, AS160 has been demonstrated to be a downstream target for both Akt (Sakamoto and Holman, 2008) and AMPK (Kramer et al., 2006). Reduced AS160 phosphorylation may be responsible for the impaired Glut-4 translocation in ovariectomized hearts. Our further finding revealed that AMPK deficiency negated estrogen replacement-induced AS160 phosphorylation and upregulation of membrane Glut4, supporting a role for AMPK-activated AS160-mediated glucose transport in the cardioprotective action of estrogen replacement.

In summary, our findings suggest that ovariectomy interrupts AMPK signaling *en route* to alterations in cardiomyocyte contractile and intracellular Ca²⁺ properties. Our results further revealed that AMPK serves as a permissive signaling molecule for estrogen replacement-offered beneficial effect in the maintenance of cardiac homeostasis. Although our present study has shed some light on the role of Akt, AMPK, AS160 and glucose transport in ovariectomy- and estrogen replacement-induced myocardial responses, the precise mechanism behind estrogen deficiency- and replacement-induced changes in cardiac contractile function and the overall prevalence of cardiovascular events still deserves in-depth investigation. As the controversy for the association between estrogen replacement therapy and overall cardiovascular risk continues to evolve in postmenopausal women, a better understanding for estrogen deficiency- and replacement-induced changes in cardiac geometry and function should help to understand not only menopause but also estrogen replacement therapy-associated cardiovascular events.

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Highlights

- We examined the role of AMPK in ovariectomy- and E2-induced cardiac responses;
- E2 replacement alleviates ovariectomy-induced changes in cardiac function;
- The beneficial effect of E2 replacement in ovariectomy was mediated by AMPK;

Fig 1.

Body and heart weights as well as cardiac expression of estrogen receptors ERα and ERβ in sham-operated (Sham) and ovariectomized (OVX) mice with or without daily E2 treatment (40 µg/kg body weight, i.p.) for 6 weeks. A: Body weight; B: Heart weight; C: Heart weight normalized to tibial length; D: Heart weight normalized to body weight; E: ERα expression; and F: ERβ expression. Inset: Representative gel blots depicting expression of ERα, ERβ and GAPDH (used as loading control) in murine hearts. Means ± SEM, n = 9 (panel A–D), 4 (panel E) and 7 (panel F) mice per group, *p < 0.05 vs. Sham group.

Fig 2. Cardiomyocyte contractile properties in sham-operated (Sham) and ovariectomized (OVX) mice with or without daily E2 treatment (40 μg/kg body weight, i.p.) for 6 weeks. **A:** Representative cell shortening traces in cardiomyocytes from Sham, OVX and OVX-E2 mice; **B:** Resting cell length; **C:** Peak shortening (normalized to resting cell length); **D:** Maximal velocity of shortening (+ dL/dt); **E:** Maximal velocity of relengthening (- dL/dt); **F:** Time-to-peak shortening (TPS); and **G:** Time-to-90% relengthening (TR₉₀). Means ± SEM, n = 50 cells from three mice per group, *p < 0.05 vs. Sham group, #p < 0.05 vs. OVX group.

Fig. 3. Intracellular Ca^{2+} transient properties in cardiomyocytes from sham-operated (Sham) and ovariectomized (OVX) mice with or without E2 treatment (40 $\mu\text{g}/\text{kg}$ body weight, i.p.) for 6 weeks. A: Representative Fura-2 traces in cardiomyocytes from Sham, OVX and OVX-E2 mice; B: Resting intracellular Ca^{2+} level; C: Electrically-stimulated rise in Fura-2 fluorescent intensity (FFI); D: Single exponential intracellular Ca^{2+} decay rate. Mean $\pm$ SEM, $n = 50$ cells from 3 mice per group, * $p < 0.05$ vs. Sham group, # $p < 0.05$ vs. OVX group.

Fig. 4.

Contractile properties in cardiomyocytes from ovariectomized wild type (WT) and AMPK kinase dead (AMPKDD) mice treated with or without E2 (10 nM) for 2 hrs. A: Resting cell length; B: Peak shortening (normalized to resting cell length); C: Maximal velocity of shortening (+ dL/dt); E: Maximal velocity of relengthening (dL/dt); E: Time-to-peak shortening (TPS); and F: Time-to-90% relengthening (TR₉₀). Mean $\pm$ SEM, n = 48 - 50 cells per group, *p < 0.05 vs. WT group, #p < 0.05 vs. WT-OVX group.

Fig. 5.

Myocardial levels of JNK, Akt, AMPK and ACC (total and phosphorylated forms) in sham-operated (Sham) and ovariectomized (OVX) WT or AMPK kinase dead (AMPKKD) mice with or without E2 treatment (40 µg/kg body weight, i.p.) for 6 weeks. A: pJNK-to-JNK ratio; B: pAkt-to-Akt ratio; C: pAMPK-to-AMPK ratio; and D: pACC level. Insets: Representative gel blots depicting levels of JNK, pJNK, Akt, pAkt, AMPK, pAMPK, pACC and GAPDH (used as loading control) in murine hearts. Vertical lines denote the non-continuous nature of immunoblot bands from the same gel. WT and AMPKKD groups were from independent Western blot runs. Mean ± SEM, n = 5–6 mice per group, *p < 0.05 vs. Sham group, #p < 0.05 vs. OVX group.

Fig. 6. Myocardial levels of PGC-1 α , SERCA2a, pAS160 and membrane Glut4 in sham-operated (Sham) and ovariectomized (OVX) WT or AMPK kinase dead (AMPKKD) mice with or without E2 treatment (40 μ g/kg body weight, i.p.) for 6 weeks. A: PGC-1 α expression; B: SERCA2a expression; C: pAS160 level; and D: Membrane Glut4 expression. Insets: Representative gel blots depicting levels of PGC-1 α , SERCA2a, pAS160, membrane Glut4 and GAPDH (used as loading control) in murine hearts. Na-K-ATPase was used as a loading control for membrane Glut4 protein. Vertical lines denote the non-continuous nature of immunoblot bands from the same gel. WT and AMPKKD groups were from independent Western blot runs. Mean $\pm$ SEM, $n = 5-6$ mice per group, * $p < 0.05$ vs. Sham group, # $p < 0.05$ vs. OVX group.