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Research Article

Estrogen Differentially Affects Expression of Calcium Handling Genes in Female and Male Adult Cardiomyocytes

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Premenopausal women have a lower risk of developing heart disease compared with postmenopausal women and age-matched men. However, the debate about whether estrogen is cardioprotective is ongoing due to conflicting results from basic science and clinical trials as well as signaling pathways that make interpretation of effects difficult. Calcium handling in the contracting cells of the heart, the cardiomyocytes, is one of the most important pathways involved in heart function. We sought to determine if calcium-handling genes are regulated by estrogen in a sexually dimorphic manner to explain differences in heart health between men and women. Cardiomyocytes were isolated from the hearts of healthy male and female rats and were treated with different doses of estrogen (300pM, 10nM). Expression of a set of calcium handling genes was then measured to determine the effect of estrogen. Our results demonstrate that estrogen differentially regulates calcium-handling genes in female and male cells, an effect that is also dose-dependent. To our knowledge, this is the first study to examine expression of this comprehensive set of calcium-handling genes in response to estrogen and to consider its effects separately on cardiomyocytes isolated from males and females.

Keywords: Estrogen, ARVM, NRVM, calcium handling, genetic expression

Introduction

Worldwide, cardiovascular disease has become the leading cause of death for both women and men[1]. However, there are important differences in the incidence of heart disease between women and men[2]. For example, mortality rates among premenopausal women with chronic heart failure are significantly lower than age-matched men[2, 3]. Although younger women generally experience better heart health, the incidence of heart disease increases significantly in postmenopausal women[4]. Additionally, older women with some forms of heart disease have different symptoms and worse prognoses than men[5]. Therefore, younger women appear to be protected from heart disease, but when hormone levels dramatically drop at menopause, their cardiovascular outcomes decline. Therefore, we tested the hypothesis that estrogen contributes to normal cardiac function through regulation of genes that mediate contractility.

The effects of estrogen are mainly mediated by binding to estrogen receptors (ERs)α and -β resulting in their translocation to the nucleus; however, there are non-receptor-mediated effects as well. Both ERα and ERβ mediate sex-specific cardioprotective effects of estrogen in the cardiovascular system. ERα is required to protect the myocardium from acute ischemia-reperfusion injury in females and ERβ systemic knockout mice with myocardial infarction and pressure overload have increased cardiac pathology in females but not males[6, 7]. Other studies have also demonstrated markers of heart failure and mortality increase in both male and female ERβ systemic knockout mice following myocardial infarction, suggesting that ERβ attenuates the development of chronic heart failure in this setting.[8] To further define the role of ERβ in the heart and limit the complicated interpretation of a systemic knockout, we studied an ERβ cardiac-specific knockout mice in which only male mice show cardiac hypertrophy (data not shown). Other unpublished studies in our lab demonstrate functional cardiac pathology in ERα cardiac-specific knockout female but not male mice. The mechanism by which ERs and estrogen signaling affect cardiac function remains to be fully understood.

The effects of estrogen are complex because of the numerous signaling pathways it modulates. Binding of estrogen to ERs leads to receptor dimerization and recruitment of coregulators. The estrogen-ER complex is then translocated to the estrogen response elements (ERE) on DNA to alter gene expressions. However, some genes regulated by ER do not have an ERE. In this situation, transcription factors are required to activate and inactivate gene expression, such as AP1, which controls numerous cellular process including differentiation, proliferation and apoptosis, and Sp1, which is involved in early development of organism. Even without estrogen, ER can influence EREs via phosphorylation by growth factor and other plasma membrane estrogen receptors to modulate gene transcription[9].

Research on breast cancer cells indicates that estrogen regulates many cellular processes, including cell division, actin dynamics and expression of ion channels[9]. Although not directly implicated in this research, we hypothesized that regulation of ion channels involved in calcium handling in the cardiac myocytes may also be regulated by estrogen. This is of particular interest because cyclical maintenance of appropriate intracellular calcium concentrations is required for normal cardiac muscle contraction and relaxation.

The cardiac myocyte is a specialized muscle cell composed of bundles of myofibrils. The basic contractile units of the myocytes, sarcomeres, are primarily comprised of myosin and actin, which interact both chemically and physically to change cell length and thus cause contraction. When the cell is depolarized by inward calcium current, other plasma membrane voltage-gated calcium channels open and intracellular stores of calcium are released from the sarcoplasmic reticulum (SR). Calcium binds to the myofilament protein troponin, which then switches on the

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interaction between actin and myosin, causing contraction[10]. To facilitate this process, intracellular calcium concentrations must be precisely regulated. Therefore, the genes involved in calcium handling have key roles in appropriate heart function.

To address the mechanism by which estrogen modulates cardiac function, expression of components of the excitation-contraction coupling process were measured using quantitative PCR. Several channels are involved in maintaining intercellular calcium concentrations in the physiological range (Figure 1)[11]. The sarcolemmal L-type voltage dependent calcium channels Cav1.1, also known as dihydropyridine receptor (DHPR) or CACNA1S, Cav1.2 and sodium-calcium exchanger (NCX) are channels that allow extracellular calcium to enter the cardiomyocyte. The ryanodine receptor 2 (RyR2) localized in the SR membrane also plays an important role in increasing calcium concentrations upon excitation of the cell. To reduce calcium concentrations in the cardiomyocyte after contraction, the plasma membrane calcium-ATPase (PMCA) transports calcium out of the cells in exchange for protons. Among those genes, none has the ERE sequence (5' GGTCAnnnTGACC 3') in mouse or human[12]; these genes may be regulated indirectly by estrogen signaling.

To determine whether estrogen regulates expression of these important channels, cardiomyocytes were isolated from adult and neonatal rat hearts and treated with vehicle or different concentrations of estrogen (10nM or 300pM). Quantitative PCR was used to detect the expression levels of these calcium handling genes to determine the effects of estrogen. Our data show that estrogen regulates expression of these genes differently in female and male adult rat ventricular myocytes (ARVMs) in a highly dose-dependent manner. Importantly, our data also demonstrate that the commonly used neonatal cardiac cell model may not be appropriate for studies of sex hormones in the heart, especially those that may be sexually dimorphic.

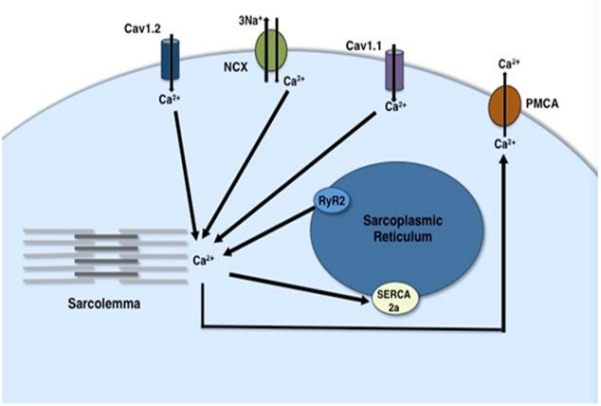

Figure 1: The relationship between key components in the excitation-contraction coupling process. Calcium concentrations in the cardiomyocyte are regulated by several ion channels in the cardiomyocyte. Cav1.1 and Cav1.2, voltage-gated L-type calcium channels, as well as NCX move calcium into the cell from the extracellular space. Calcium also enters the cytoplasm from the sarcoplasmic reticulum. Following contraction, calcium is sequestered into the SR by SERCA2a and is returned to the extracellular space via PMCA.

Experimental Procedures

Adult Rat Ventricular Myocyte Isolation

All animal procedures were performed with the approval by the University of Colorado at Boulder Institutional Animal Care and Use Committee. ARVMs were isolated as previously described with minor modification as follows[13, 14]. Briefly, male or female 6-8-week-old rats that were approximately 350g were injected with 250U heparin (Sagent, Schaumberg, IL). After ten minutes, the rat was injected intraperitoneally with 35mg/kg sodium pentobarbital solution (Vortech, Dearborn, MI). When the rat was completely and deeply anesthetized, as determined by firm toe pinch, the thoracic cavity was exposed by cutting the peritoneal muscle and ribs on both sides. The heart was excised including about 5mm of aorta required for cannulation. The heart was then transferred into 0.9% NaCl solution to manually pump the blood from the heart. Forceps were used to cannulate the aorta while immersed in 0.9% NaCl solution to prevent the introduction of bubbles and the aorta was secured using silk suture. Isotonic buffer heated to 37°C was used to perfuse the heart for five minutes and 1.455mg/ml collagenase type II solution was then used to digest the heart for 20-30 minutes. The heart was removed, cut into small pieces in the collagenase solution, incubated for two minutes, and dissociated using glass Pasteur pipets bent at 90°. Cells were transferred to a 15mL tube and centrifuged at 100 rpm for 3 minutes to pellet the cells. Supernatant containing dead cells and non-myocytes was discarded. 10ml Media 199 (Invitrogen, Carlsbad, CA) was added; cells were resuspended and centrifuged as above. Cell concentration was determined and the pellet was resuspended at 10,000-20,000 cells/mL in Springhorn Media (Media 199 with 0.2% bovine serum albumin, 1.6mmol/L L-carnitine, 4.4mmol/L creatine monohydrate, 5mmol/L L-taurine, 4µmol/L L-glutamine, 5µmol/L sodium pyruvate, 10mmol/L 2,3-butanedione monoxime, 17.4nmol/L insulin) and plated on 60mm plastic cell culture plates coated with 10µg/mL laminin (Invitrogen, Carlsbad, CA). Plates were incubated at 37°C with 5% CO_2 for 3-5 hours. Cells were then washed with Springhorn media to remove dead cells and treated with vehicle (ethanol), 300pM estradiol (E2), or 10nM E2 for 36h. The dose of 300pM was chosen because it represents a physiologic level of estrogen exposure. Adult female rats have serum estrogen between 20pg/mL and 40pg/mL (73.5pM to 147pM) during cycle. During pregnancy, serum estrogen peaks at 140pg/mL (514.5pM)[15]. The 10nM dose was chosen because this higher dose is commonly used in other studies that examine the effects of estrogen in cardiomyocytes, regardless of sex[16, 17]. Two to three plates were used per treatment and three separate isolations were performed per sex.

Neonatal Rat Ventricular Myocyte (NRVM) Isolation

NRVMs were isolated as previously described with modification of cell culture conditions as follows[19]. Briefly, cells were isolated, plated at 100,000 cells/mL on 60mm plastic cell culture plates and incubated for 24 hours at 1% CO_2 in growth media containing calf serum. Cells were then washed and transferred to serum-free media. Vehicle (ethanol), 300pM E2, or 10nM E2 were applied to the media and plates were incubated for 48 hours. Cells isolated in each preparation are a pooled population of cells isolated from 50-

75 neonatal rats, and pooled cells are distributed among the members of the laboratory for use. 300,000 cells were plates per 60mm plate thus approximately 14-16 neonatal per experiment. Three plates were used per treatment and two isolations were performed.

RNA Isolation

300µl TRI Reagent (Molecular Research Center, Cincinnati, OH) was added to two to three plates per treatment. Cells were scraped into a single 1.5mL tubes so each tube contained lysates from the same treatment. RNA was then isolated according to manufacturer protocol.

cDNA synthesis

RNA concentration was determined using a Nanodrop (Thermo Scientific, Waltham, MA). The appropriate volume of RNA was used to obtain 500ng, 1µg or 2µg in a 20µL reaction. cDNA was synthesized using, Superscript II™ reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers according to manufacturer protocol.

The RNA concentrations obtained from ARVMs were in the range of 70ng/µl to 160ng/µl and A260/A280 ratios indicating the degree of protein contamination were in an acceptable range (above 1.75). RNA concentrations from NRVMs were between 200ng/µl and 678ng/µl. A260/A280 ratios were above 1.9. Primer-BLAST (National Library of Medicine) was used to verify that the designed primers amplified the intended product *in silico*. A single melt curve of each quantitative PCR product with a peak at the expected melt temperature suggested that the reaction amplified a single product. The R-squared values for standard curves using each primer set were above 0.99, indicating the technical replicates were reliable. The slope of the standard curves for each primer set produced using 10-fold dilutions fro[20]m 40ng to 0.004ng per reaction and a non-template control were all less than -3.0, indicating that the products were amplified efficiently (-3.3 is approximately equal to 100% efficiency).

Primer Design

Ensembl Genome Browser was used to identify the cDNA sequence of each gene and select sequence that included least two exons[21]. Primer3 was then used to select different sets of primers that spanned an intron to eliminate possible amplification of genomic DNA[22]. The primer length was set as 18-22bp, primer melting temperature as 52-58°C, and GC content as 40-60%. Primer sequences are shown in Table 1.

Quantitative PCR

Expression of each gene was quantified by measuring SYBR® Green (Invitrogen, Carlsbad, CA) incorporated into PCR products using an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA). 4ng of cDNA were used per well with 6µL of SYBR® Green master mix and appropriate primer pairs. Each sample was loaded in triplicate and standard curves were produced for each gene examined. Expression of genes of interest was normalized to expression of 18S ribosomal RNA for each sample and changes in expression were determined by comparing to untreaed cells. 18S was chosen as a reference gene because it varied by less than 2.3% and 4.8% in male or female samples, respectively. Data were reported relative to vehicle-treated cells for each gene of interest. The thermocycler program used is shown in Figure 2.

Data presentation

All data are shown in bar graphs produced using Prism 4 for Macintosh as mean ± standard error from the mean (S.E.M.), calculated from three biological replicates per experiment. Expression from individual biological replicates was calculated using the algorithm in the Bio-Rad CFX Manager software, which normalizes expression according to the primer efficiency determined in the standard curve for the primer set. Statistically significant differences between groups were determined using unpaired Student's t-test; significance was defined as $p < 0.5$.

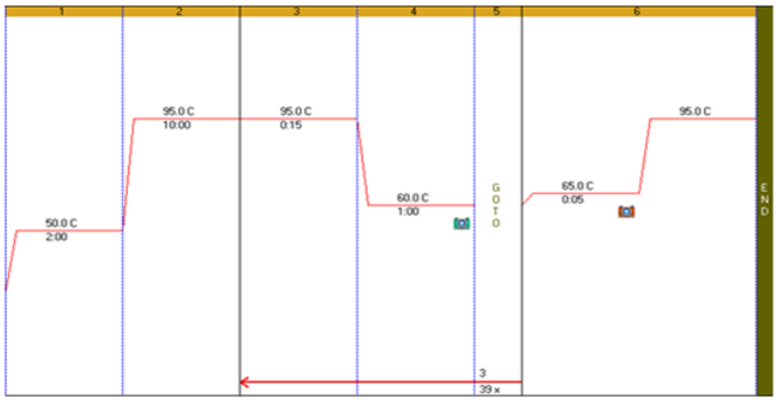

Figure 2: Quantitative PCR program.

Table 1: DNA sequences of oligonucleotide primers for Cav1.1, Cav1.2, NCX, PMCA and RyR2. Primers for 18S ribosomal RNA were used as a reference.

Gene	Forward Primer	Reverse Primer	Size (bp)
Cav1.1	5’ GCCTACGGACTTCTCTTCCA 3’	5’ GCTCCTTTCCCTCCTAGAGC 3’	149
Cav1.2	5’ GCCAGAGGATGACAACAACA 3’	5’ GATGGAATAGGAAGCCGTAGG 3’	119
NCX	5' CGTCTGGTGGAGATGAGTGA 3'	5' TGGTGTGTTCGCCTAGAATG 3'	139
PMCA	5’ TGGCAGCATGTATATCTTTCGT 3’	5’ GGCTACCAAGATAAGCAGAATTACA 3’	101
RyR2	5’ ACGGATGATGAAGTGGTCCT 3’,	5’ GATCAGGGGGTACATTCTGC 3’	139
18S rRNA	5’ GCCGCTAGAGGTGAAATTCTTG 3'	5’ CTTTCGCTCTGGTCCGTCTT 3’	66

PCR products were amplified by repeated denaturation (95°C) and elongation (60°C). The amount of fluorescence, an indication of the amount of PCR product, was measured after each elongation step (blue camera icon). At the end of the program, a melt curve was obtained by increasing the temperature by 2°C for 5 seconds and measuring the fluorescence after each increase (orange camera icon). The peak temperature was determined to be the melt temperature where 50% of the product was denatured. Image obtained from Bio-Rad CFX Manager software.

Results

ARVM isolation yielded healthy rod-shaped cardiomyocytes

Healthy rod-shaped cardiomyocytes were identified visually using a light microscope and a 20X objective. The percentage of myocytes increased significantly after washing, with the majority of cells appearing to be rod-shaped cardiomyocytes after the wash. All cardiomyocytes were adhered tightly to the surface of the plates. Some of them were spontaneously contracting, indicating that they were in healthy condition.

Quantitative PCR revealed different patterns of expression of calcium handling genes in female and male ARVMs.

Female and male cardiomyocytes treated with different doses of estrogen showed different genes expression patterns. Female ARVMs treated with 300pM E2 had down-regulated genes compared to vehicle-treated control as follow: Cav1.1 (0.956 ± 0.058), Cav1.2 (0.601 ± 0.060), NCX (0.542 ± 0.130), PMCA (0.802 ± 0.003), RyR2 (0.841 ± 0.037). Male ARVMs treated with 300pM E2 showed similar down-regulated gene expression, except for NCX, which was undetectable in these experiments [Cav1.1 (0.978 ± 0.048), Cav1.2 (0.604 ± 0.030), PMCA (0.539 ± 0.03), RyR2 (0.703 ± 0.040)] (Figure 3A). Expression of only one gene (PMCA) was statistically different between male and female cells treated with 300pM E2. NCX expression is not reported for male ARVMs because we were unable to obtain reproducible amplification data. Amplification was only observed when 40ng of cDNA were used but the technical replicates were inconsistent. We conclude that NCX expression was insufficient to be detected using our methods.

When cells were exposed to a higher dose of estrogen (10nM), male and female ARVMs had dramatically different gene expression changes. This supraphysiologic dose is commonly used to model cellular responses to E2[6, 16]. Calcium handling genes were still down-regulated in female ARVMs [Cav1.1 (0.619 ± 0.101), Cav1.2 (0.580 ± 0.070), NCX (0.574 ± 0.018), PMCA (0.613 ± 0.038), RyR2 (0.803 ± 0.074)]. Cav1.1, PMCA, and RyR2 had a dose-dependent decrease in expression compared to female ARVMs treated with 300pM E2. However, the male ARVMs exhibited the opposite response with statistically significant ($p < 0.01$) up-regulation of all calcium handling genes except for RyR, which was increased but was not determined to be significantly different from expression in female cells (Figure 3B). Male ARVMs treated with 10nM E2 showed similar down-regulated gene expression, except for NCX, which was undetectable in these experiments [Cav1.1 (1.33 ± 0.102), Cav1.2 (0.953 ± 0..020), PMCA (1.159 ± 0.101), RyR2 (0.985 ± 0.030)] (Figure 3A).

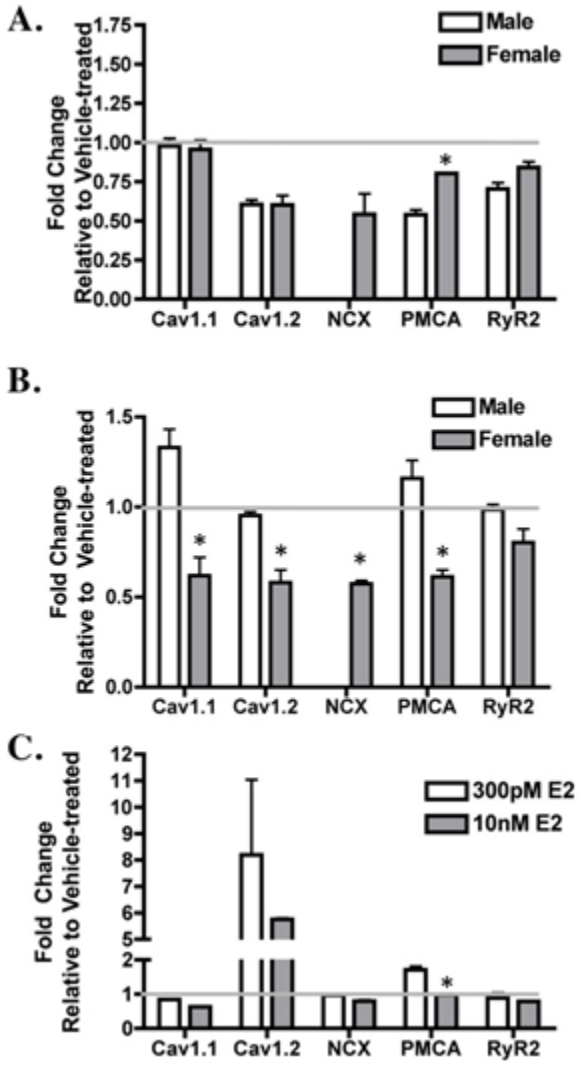

Figure 3: **(A)** Expression of calcium handling genes in male and female ARVMs treated with 300pM E2. Open bars represent male ARVMs and gray bars represent female ARVMs. * $p < 0.01$ **(B)** Expression of calcium handling genes in male and female ARVMs treated with 10nM E2. Open bars represent male ARVMs and gray bars represent female ARVMs. * $p < 0.01$ **(C)** NRVMs treated with 300pM E2 (open bars) and 10nM E2 (gray bars). Gray line in each graph indicates no change in expression relative to vehicle-treated. * $p < 0.01$ Error bars indicate S.E.M.

Neonatal rat ventricular myocytes (NRVMs) were also isolated and treated to determine whether calcium handling gene expression is also modulated by estrogen in these cells. NRVMs are a commonly used cell model to study cardiac responses to many hormone, drug and pathologic conditions[23-25]. Although convenient because of ease of isolation and longer-term maintenance in culture[19], these cells represent a mix of cells from male and female hearts and may not be as

responsive as adult cells to sex hormones. Significant differences were found for NRVMs treated with 300pM E2 and 10nM E2 compared to ARVMs isolated from rats of either sex (Figure 3C). A dose-dependent decrease in expression of all genes tested was observed in NRVMs treated with 10nM E2 compared to 300pM E2 (Cav1.1 -26.2%, Cav1.2 -%, NCX -28.7%, PMCA -62.5%, RyR2 -21.3%). With the exception of Cav1.2, all genes were down-regulated with both doses of E2; only the reduction of RyR2 was significant. The response of NRVMs did not accurately mimic the response of male or female ARVMs.

Discussion

Estrogen regulates calcium handling genes.

Evaluation of ARVMs showed that calcium handling gene expression is modulated by estrogen. With both physiologic (300pM) and supraphysiologic (10nM) estrogen (see methods), expression of genes responsible for encoding channels that transport calcium in male and female adult cardiomyocytes is altered. NCX was below detection in male ARVMs, which was probably because the expression level was too low in male cardiomyocytes. Other groups have demonstrated that NCX is expressed in female and male rat heart ventricular tissues by using western blots. They also proved that NCX and RyR had higher mRNA levels in female rat heart ventricular tissue compared to males, which is consistent with our studies[26]. In addition, NRVMs treated with estrogen also showed different calcium handling gene expression compared to vehicle-treated. These data demonstrate that estrogen regulates expression of calcium handling genes in cardiomyocytes and the effect is dose-dependent. To our knowledge, these are the first studies to examine this combined set of calcium handling genes to determine expression changes with exposure of estrogen in both female and male cardiomyoctyes. Importantly, we also show different responses between male and female adult cell and between adult and neonatal cells.

Physiologic and supraphysiologic doses of estrogen regulate calcium handling genes in males and females differently.

Other studies have already shown that ERα knockout mice had increased L-type calcium channels[27]. In our studies, calcium handling genes were down-regulated fin both female and male ARVMs treated with 300pM E2, in agreement with this study. With this physiological dose of estrogen, female and male showed similar responses. However, when the estrogen dose was increased to 10nM, a dose that is normally used for estrogen-related research, expression of calcium handling genes still decreased for female cardiomyocytes in a dose-dependent manner, but increased dramatically for male cardiomyocytes. These data therefore suggest that male cardiac myocytes may be more sensitive to this supraphysiologic estrogen dose. Our preliminary data (not shown) suggest that there are significant differences in ER expression in male and female ARVMs, which could account for this increased sensitivity. Hence, researchers should be aware of this effect when studies are performed in adult cardiomyocytes, especially in males because higher doses of estrogen may causes abnormal regulation of calcium handling genes resulting in incorrect interpretation of results.

NRVMs may not be appropriate for studying sex-specific responses to estrogen.

NRVMs are a common model for studying sex hormones because of the ease of preparation and health in culture[19]. However, our data from NRVMs suggest that it might not be a reliable model to study the effect of sex hormones on calcium handling genes. Because male and female neonatal rats are not easily sexed visually, ventricles from multiple hearts are typically pooled, so NRVMs preparations represent a mix cells from both males and females[28]. The male-specific SYR gene can be measured using PCR to distinguish between male and female neonates[29], however this technique is rarely used in studies using cultured NRVMs, even in studies involving sex hormones[23, 30, 31]. Additionally, NRVMs are isolated from a neonatal environment rather than an adult one, so the effect of sex hormones may be different. In our study, data from NRVMs did not reflect the gene expression portfolio of either male or female ARVMs. Therefore, researchers should be aware of this issue in NRVMs if the effects of sex hormones are to be studied in isolated cardiomyocytes.

Estrogen supplementation might be harmful to cardiac health in males.

The debate of whether estrogen supplementation should be provided to males for cardiac protection has been active for many years. Despite the suggestion that estrogen may be protective in females, these studies have been recently questioned and many studies have shown that estrogen might be harmful to males, especially in the context of heart disease[32, 33]. For example, estrogen supplementation in men can cause negative cardiac effects, leading to increased death rates after myocardial infarction[34, 35]. In light of our data on physiologic doses of estrogen in male and female ARVMs, we hypothesized that exposure to higher doses of estrogen would down-regulate calcium handling genes. However, male but not female ARVMs treated with 10nM estrogen had the opposite gene expression pattern. Dramatic increases in expression of calcium handling genes that move calcium into the sarcoplasm could be responsible for pathology. Calcium overload in cardiomyocytes is believed to cause pathology in cardiac myocytes, indicating that the dramatic up-regulation of calcium handling genes in male ARVMs treated with high dose of estrogen might be harmful to the cardiac health of males. Interestingly, however, estrogen has been shown to have cardioprotective effects on female cardiac myocytes because of a decrease of intercellular calcium but not on male cardiac myocytes[36]. Our studies agree with this; isolated female cardiomyocytes to estrogen down-regulated calcium handling genes. Thus, the dose of estrogen can have dramatic effects on the health of the heart and males and females should be considered differently in this determination.

Conclusions

Much remains to be understood about the effects of estrogen in males and females in terms of cardiac health. The data presented here comprehensively examine the effects of estrogen on calcium handling in isolated adult cardiac myocytes, an important model that excludes effects of other cells in the heart or of pathology in the cardiovascular system that can indirectly affect cardiac function and allows

consideration of male and female cells separately. We show that estrogen affects male and female ARVMs differently and raise important questions about its effects on calcium handling in the heart. Additionally, our experiments make a direct comparison between adult and neonatal cells and suggest that ARVMs are a superior model for the study of sex hormones in cardiomyocytes.

Limitations and future directions

The effect of estrogen is complicated because of involvement of numerous signaling pathways and genes. Even when considering only calcium handling pathways, it proves to be a complex story. Estrogen can protect the heart cells by reducing the intercellular calcium in females, but it also can cause pathology by increasing calcium. The key point here is female and male cells have different responses to varying doses of estrogen. Male cardiomyocytes appear to be more sensitive to estrogen dose, which would make identification of the correct dose for men difficult. This issue may also exist for choosing the correct dose for post-menopausal women. From our studies, it is not appropriate to make the conclusion that high dose estrogen caused pathological changes on male cardiomyocytes however, our data suggest that more studies are required to examine appropriate doses in males and females if estrogen supplementation is to be considered to protect the heart.

While many of the ARVMs were still contracting when they were first isolated, this spontaneous activity did not continue in cell culture. This suggests that many of the normal calcium handling processes have been made inactive in these cells. Despite this, our studies still suggested a dramatic effect on calcium handling genes regulation by estrogen. In the future, the contractile function of male and female ARVMs treated with different dose estrogen should be measured to make interpretation of these data more clear.

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