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Expression, Distribution and Regulation of Sex Steroid Hormone Receptors in Mouse Heart

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Key Words

Cardiac • Ovariectomy • Subcellular distribution • Protein fraction

Abstract

The effects of sex hormones on the heart are dependent on the presence and distribution of sex steroid hormone receptors (SSHR) in cardiac tissue. This study used subcellular fractionation, Western blot analysis and densitometry to characterize the subcellular distribution and abundance of estrogen receptor (ER) α, ERβ and androgen receptor (AR) in atrial and ventricular tissue from male and female mice. The results showed that in both atrial and ventricular tissue ERα was primarily found in the sarcolemma, whereas ERβ and AR were predominantly located in the nucleus and cytosol. Interestingly, ERα expression was greater in the ventricles compared to the atria, whereas ERβ and AR expression were similar in both heart chambers. Furthermore, the distribution and abundance of SSHR in the atria and ventricles did not differ between sexes. This study also showed that a reduction in hormone levels (as a result of ovariectomy) resulted in a significant increase in the abundance of ERα in the ventricular sarcolemmal fraction. Overall, the results suggest ERα, ERβ and AR distribution and expression are not sex dependent in the mouse heart. However, it appears that ERα expression is chamber specific and that, in certain cases, hormone levels can modulate the subcellular location of SSHRs.

Introduction

Cardiovascular diseases are the leading cause of mortality for both men and women throughout most of the industrialized world [1-7]. Interestingly, women tend to develop heart disease later in life than men [8, 9]. In fact, the incidence of cardiovascular diseases only begins to steadily increase in women after the onset of menopause [10]. It is believed that the late onset of cardiovascular disease in women is partially attributable to sex differences in sex steroid hormones [8]. Specifically, endogenous estrogen may have a cardioprotective effect in premenopausal women [9]. Furthermore, evidence suggests that the male sex hormones may play a role in the development of certain types of cardiovascular disease [11, 12]. Thus it appears

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that various sex steroid hormones may be involved in the prevention and development of cardiovascular disease.

The biological effects of sex hormones are mainly mediated by sex steroid hormone receptors (SSHR) [13, 14]. In fact, all the sex steroid hormones act as ligands for their specific receptors; estrogen activates estrogen receptors (ERα and ERβ) and androgen activates androgen receptors (AR). Numerous studies have shown that SSHRs are present in the heart [15-19], specifically, ERα, ERβ and AR are expressed in cardiac myocytes [13, 20-23]. In addition, immunostaining has revealed that ERα and ERβ are located in the cytoplasm and nuclei in cardiac tissue [16-18]. However, these studies were conducted on either left ventricle tissue samples [17, 18] or on the whole heart [16]. In addition, it is not clear whether SSHR distribution is uniform in the different subcellular fractions (total, sarcolemmal, cytosolic and nuclear) or if certain subcellular compartments are enriched in one or more SSHR. The knowledge regarding SSHR distribution and abundance within the atria also is limited. Furthermore, it is not apparent whether SSHR expression differs between the atria and ventricles. Therefore, the specific objectives of this study were: 1) to characterize the subcellular distribution and expression of SSHRs in the atria and ventricles from male and female mice and to determine if there were any sex differences, 2) to determine if SSHR expression differed between the atria and the ventricles, and 3) to determine the effect of hormonal changes on SSHR expression and distribution in the hearts of female mice. The characterization of the expression and distribution of SSHRs in the atria and the ventricle could help identify how these receptors and sex steroid hormones contribute to the development of cardiovascular disease.

Materials and Methods

Animals

Male and female CD1 mice (Charles River Laboratories, St-Constant, Quebec, Canada) were used to examine the distribution and expression of SSHR (male n=30, female n=30) and to determine the effect of hormonal regulation on SSHR expression (sham-operated females n=45, ovariectomized females n=64). Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Hearts were quickly removed and placed in ice-cold tyrode solution containing (in mM) 130 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.33 Na_2HPO_4, 10 Hepes, 5.5 glucose and 1 $CaCl_2$ (pH 7.4). Ventricles and atria were then separated, frozen in liquid nitrogen and stored at -80°C. All experiments were performed in accordance to the guidelines of the Canadian Council on Animal Care.

Ovariectomy

Twenty-one day old female mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A 1 cm incision was made in the skin and back muscles parallel to the midline of the animal. Next, the ovaries were located and the oviduct, including the ovarian blood vessels, was ligated and the ovary removed. The incision in the back musculature was closed with nylon thread (Ethicon 4.0) and the skin was sutured with stainless steel wound clips (MikRon Autoclip). Throughout the surgical procedure mouse body temperature was maintained at 37°C with a heating pad. Age-matched sham-operated mice underwent a similar procedure except the ovaries and oviducts were not removed.

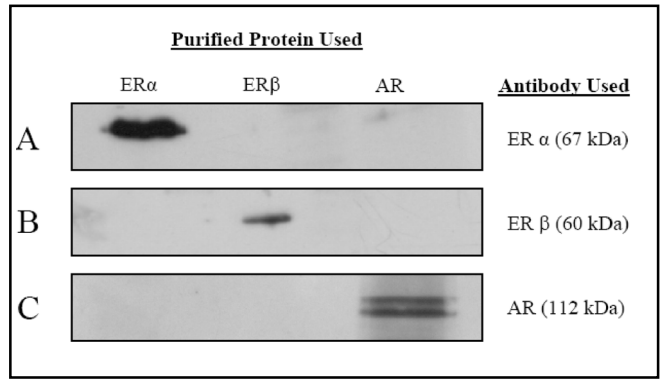
Ovariectomized mice and sham-operated mice were sacrificed at 1, 3 and 6-weeks post surgery. The hearts were removed as described above and the uterus was removed and weighed. In addition, blood was collected from the thoracic cavity before coagulation occurred. The blood was centrifuged for 20 minutes at 2000 g and 4°C. The supernatant (serum) was removed and stored at -20°C until its time of use. Serum concentrations of 17β-estradiol were measured with a radioimmunoassay (Maisonneuve-Rosemont Hospital, Montreal).

Subcellular Fractionation

Four different subcellular fractions (total, cytosol, sarcolemma, and nuclear) were isolated from the hearts of 2-3 month old male and female mice as described previously [24]. These procedures were adapted from those published by Jones et al. [25] and Boivin and Allen [26]. Briefly, frozen hearts (5 hearts per isolation) were placed in a homogenizing solution that contained (in mM) 20 Tris (pH 7.4), 1 EDTA, 0.01 leupeptin, 1 Na_3VO_4, 0.6 benzamidine, 1 PMSF and 15 μg/mL aprotinin. Samples were homogenized for 20 seconds (3 times) at high speed (Polytron PowerGen model 125) at 4°C. The homogenate was then aliquoted into 3 tubes. The first tube was adjusted to 1% (v/v) with Triton X-100, incubated for 2 hours at 4°C to allow the proteins to solubilize, then centrifuged for 10 minutes at 10 000 g and 4°C to remove cell debris, nuclei and mitochondria. This supernatant is referred to herein as the total protein fraction. The second tube was centrifuged for 20 minutes at 200 000 g and 4°C. The supernatant is referred to as the cytosolic fraction. The third tube was centrifuged for 10 minutes at 10 000 g and 4°C. The supernatant obtained was centrifuged again for 20 minutes at 200 000 g and 4°C. The pellet was then resuspended in the homogenizing buffer supplemented with 0.6 M KCl to dissociate myofibrillar proteins. A second and third centrifugation were performed (200 000 g for 20 minutes at 4°C), with the pellets being resuspended using homogenizing buffer, to wash out the KCl. The final pellet was resuspended in the homogenizing buffer and employed as the enriched sarcolemmal fraction [25]. All fractions were frozen in liquid nitrogen and stored at -80°C until needed.

The protocol for isolating nuclear membranes, adapted from Jones et al. [25], was recently described by Lizotte et al. [24]. Hearts (10/isolation) were crushed into a fine powder under liquid nitrogen, the powder was suspended in Tris-buffered saline (TBS) solution containing (in mM) 25 Tris, 135 NaCl, 2.5

Fig. 1. Specificity of SSHR antibodies: Purified ERα (A), ERβ (B) and AR (C) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated antibodies.



KCl (pH 7.4), and homogenized at low speed for 30 seconds using a Polytron PowerGen model 125. The homogenate was centrifuged for 15 minutes at 450 g and 4°C to remove cell debris and the resulting supernatants mixed with an isotonic solution comprised of (in mM): 10 Hepes (pH 7.4), 1.5 $MgCl_2$, 10 KCl, 0.5 DTT, 0.2 Na_3VO_4 and 25 µg/mL leupeptin. The solution was then centrifuged at 2 000 g for 15 minutes at 4°C to pellet the nuclei. Pellets were then resuspended in a hypertonic solution containing (in mM) 300 Hepes, 1400 KCl, 30 $MgCl_2$, 0.2 Na_3VO_4 and 25 µg/mL leupeptin, incubated on ice for 20 minutes to allow the nuclei to shrink, and then centrifugated for 15 minutes at 2 000 g and 4°C. The final pellet was resuspended in a storage solution composed of (in mM) 20 Hepes, 420 NaCl, 1.5 $MgCl_2$, 0.2 EDTA, 0.5 PMSF, 0.5 DTT, 0.2 Na_3VO_4, 25% (v/v) glycerol and 25 µg/mL. Isolated nuclei were frozen in liquid nitrogen and stored at -80°C until needed.

Enzymatic assays

We have previously characterized each of the subcellular fractions examined in this study using specific markers for different cellular organelles [24]. In brief, glucose-6-phophate dehydrogenase activity was used as a marker for the cytosol [27], Na^+/K^+-ATPase activity was assayed as a marker for the sarcolemma [28], and a Ca^{2+}-ATPase assay was used to determine the mitochondrial Ca^{2+}-ATPase (azide-sensitive), the plasma membrane Ca^{2+}-ATPase activity and the sarco-endoplasmic reticulum Ca^{2+}-ATPase activity (thapsigargin-sensitive) [29, 30]. ATPase activity was assessed by measuring inorganic phosphate release employing a modified version of the Fiske and Subbarow method [31]. Overall, the results showed that the different protein fractions were highly enriched with minimal cross-contamination [24].

Protein determination

Protein concentrations were determined by the Bradford method [32] using bovine serum albumin as the standard.

Western blots

Equal quantities of protein were separated by SDS-PAGE electrophoresis (10-100 µg/lane of protein). This technique was performed using 4% acrylamide stacking gels layered over 5-15% acrylamide-gradient separating gels. Following SDS-PAGE, proteins were electrophoretically transferred onto nitrocellulose membranes for 1.5 hours at 350 mV. Prior to blocking, membranes were stained with Ponceau S to confirm uniform protein transfer. Membranes were incubated for 1.5 hours at room temperature in TBS buffer containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dried milk (TBST-NFDM 5%) to block non-specific binding sites and then overnight at 4°C in TBST-NFDM 1% containing polyclonal antibodies against either the C-terminal amino acids 586-600 of rat ERα (67 kDa; 1:10 000; Upstate Biotechnology, Lake Placid, USA), the N-terminal amino acids 1-150 of human ERβ (60 kDa; 1: 7 500; Santa Cruz Biotechnology, CA, USA) or the C-terminal amino acids of human AR (112 kDa; 1:1 000; Santa Cruz Biotechnology, CA, USA). Membranes were then washed, incubated for 3 hours in the presence of the appropriate horseradish peroxidase-conjugated secondary antibody, washed again, and immune complexes visualized by enhanced chemiluminescence. To ensure that the optical density was not measured on saturated samples, different exposure times (1, 3, 5 and 15 minutes) were employed. Samples where light was unable to penetrate the band of interest were considered saturated and were not used for analysis. Immunoreactive bands were quantified by densitometry (Multi-Analyst program; Bio-Rad, CA, USA). Receptor abundance was normalized to an experiment-specific control (specific details are provided in the appropriate figure legends) and relative protein abundance was plotted for comparison.

Specificity of SSHR antibodies

The specificity and cross-reactivity of the antibodies used for the Western blot analysis were tested against purified ERα, ERβ and AR proteins (Sigma Chemical Co., St. Louis, MO, U.S.A.). Figure 1 shows that each antibody recognized the corresponding protein of interest; ERα (A), ERβ (B) and AR (C). To evaluate whether each antibody reacted with more than a single receptor it was tested against each purified protein. Panels A-C show that ERα, ERβ and AR reacted with their corresponding receptor, but did not react with the other purified proteins. Taken together, these data show that the antibodies used were specific for their corresponding receptor and did not cross-react with the other two receptors examined.

Statistical Analysis

Three samples (5 hearts/preparation, except for the nuclear fractions which used 10 hearts/preparation) were used to calculate mean abundance. Unpaired Student t-tests as well one-way analysis of variance (ANOVA) with a Tukey post test were used when appropriate. Statistical analysis was performed with Origin 5.0 (OriginLab, Northhampton, MA). Results are expressed as mean ± standard error (SEM) and were considered statistically significant when p-values were less than 0.05.

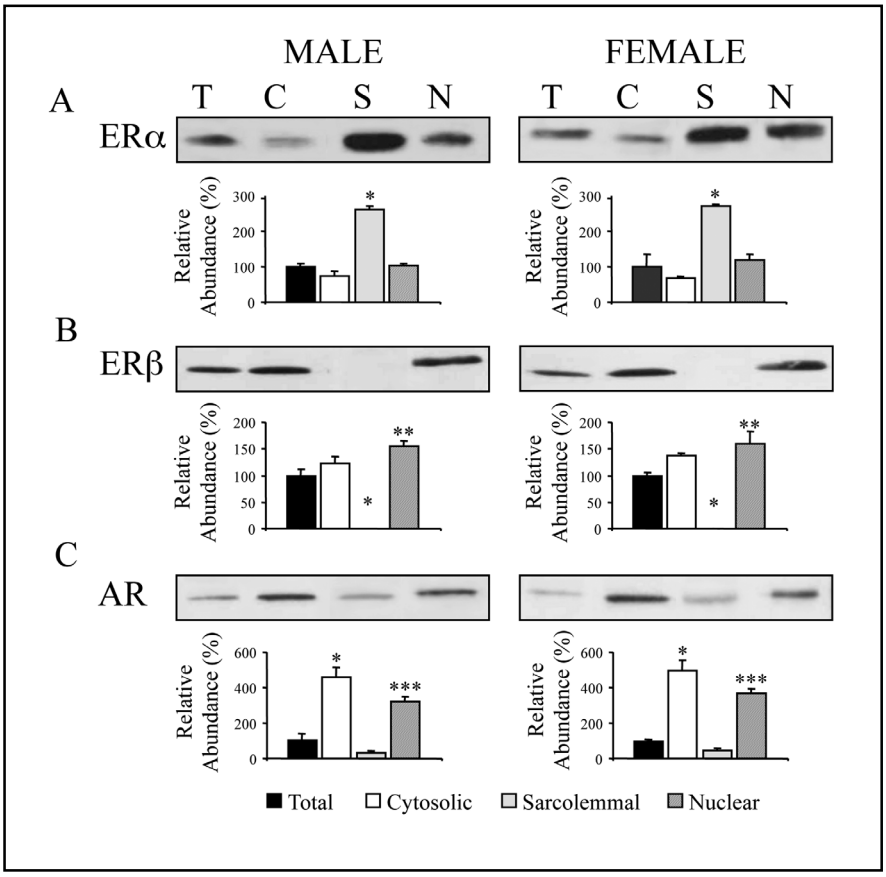


Fig. 2. Subcellular distribution of SSHRs in male and female ventricles: Cardiac ventricles from 2-3 month old male and female mice were subfractionated and the fractions [total (T), cytosolic (C), sarcolemmal (S) and nuclear (N) proteins; 100 µg of protein/lane] were subjected to immunoblotting using antibodies specific to either ERα, ERβ or AR. Uniform loading and transfer were verified by staining with Ponceau S. Representative immunoblots are shown above the corresponding histograms and show the mean abundance of the receptor of interest in each fraction. The abundance of each receptor in the total protein fraction was taken as 100% and that of the other fractions normalized to this value. Values are means of 3 separate determinations (5 mice/each). * $p<0.05$ vs all; ** $p<0.05$ vs total; *** $p<0.05$ vs total and sarcolemmal.

Results

Subcellular distribution of SSHRs in male and female mouse heart

Previous studies have shown that SSHRs are abundant in numerous different tissues [9, 33, 34] . In the heart, ERα and ERβ and AR are expressed in cardiac myocytes [13, 20-23]. However, the subcellular distribution of SSHRs within the heart is not clear. Therefore, the first objective of this study was to characterize the subcellular distribution of SSHRs in atrial and ventricular tissue. In a previous publication we described protocols for the subcellular fractionation of mouse heart and characterized the purity of the resulting fractions using specific markers for various cellular organelles [24]. In the present study, we have used the aforementioned fractionation techniques to study the expression and subcellular distribution of SSHRs in the heart.

Western blot analysis demonstrated the presence of ERα, ERβ and AR in ventricular tissue from both male and female mice (Figure 2). Figure 2, panels A-C, show representative examples of the total SSHR immunoreactivity in unfractioned ventricular tissue plus subcellular distribution of ERα, ERβ and AR in the cytosolic, sarcolemmal and nuclear fractions isolated from male (left panel) and female (right panel) ventricular tissue. Identical amounts of protein were loaded for each experiment illustrated in this figure. In both males and females, ERα (panel A) and AR (panel C) were detected in all four protein fractions. In contrast, ERβ (panel B) was detected in the total, cytosolic and nuclear fractions, but was not in the sarcolemmal fraction, in both male and female hearts. Next, we determined the relative abundance of the different receptors in male and female ventricular tissue. Figure 2A shows that the abundance of ERα was significantly greater in the sarcolemmal fraction compared to all other fractions in both male and female tissues. In contrast, ERβ was not detected in the sarcolemmal fraction from the ventricles of either male or female mice (figure 2B), but was present in similar quantities in both the cytosolic and nuclear fractions. In comparison to all other fractions, AR was the most abundant in the cytosolic fraction (figure 2C). In addition, AR was significantly more abundant in the nuclear fraction compared to the sarcolemmal and total fractions. Overall these data suggest that ERα localize primarily to the sarcolemma, whereas ERβ and AR are primarily found in the nucleus and in the cytosol.

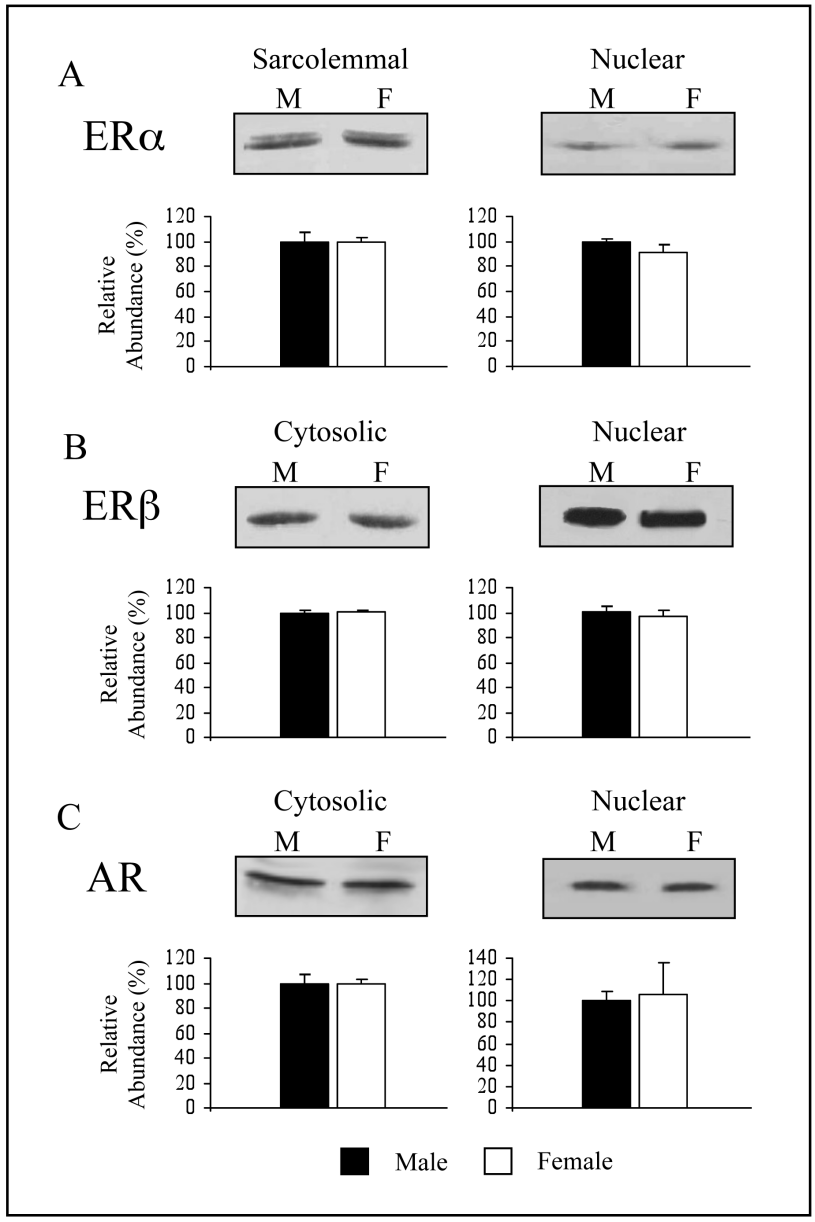


Fig. 3. The abundance of SSHRs was similar in male and female ventricular tissue: Sarcolemmal (ERα), cytosolic (ERβ and AR) and nuclear proteins (ERα, ERβ and AR) were isolated from male and female mouse ventricles, resolved by SDS-PAGE, and transferred to nitrocelulose membranes. Membranes were probed with antibodies to either ERα, ERβ or AR. Samples from male and female mice (100 μg of protein/lane) were analyzed on the same gel. Shown are representative immunoblots and histograms of the distribution of ERα, ERβ and AR in the indicated subcellular fractions from male and female mice. Values are means of 3 separate determinations (5 mice/each). The relative abundance was calculated using values from males as 100%. Uniform loading and transfer were confirmed by staining with Ponceau S.

Subsequent experiments examined the subcellular distribution of the different SSHRs in male and female atrial tissues. These experiments employed the total, cytosolic and sarcolemmal fractions since isolation of a nuclear fraction from atria was technically impractical (100 pairs of mouse atria would be needed to yield 50 μg of nuclear fraction). The results showed that, as in ventricles, ERα was primarily found in the atrial sarcolemmal fraction, whereas ERβ and AR were predominant in the cytosolic fraction (data not shown).

Comparison of SSHR expression in male and female ventricular and atrial tissues

We next compared the relative abundance of SSHRs in ventricular tissue from male and female mice to determine if sex affects receptor expression. For this experiment male and female ventricular proteins were resolved on the same gel so that receptor expression could be compared between the sexes. Figure 3 shows representative Western blots and the mean abundance of ERα, ERβ and AR in the different cellular fractions. Figure 3A shows that ERα expression was comparable in males and females in both the sarcolemmal and nuclear fractions. Similarly, in the cytosolic and nuclear fractions, ERβ (figure 3B) and AR (figure 3C) expression levels did not differ between males and females. We next compared SSHR expression in protein fractions from male and female atrial tissues. Only the protein fractions where the receptors were most abundant were used for these experiments (e.g. sarcolemmal or cytosolic). Similar to

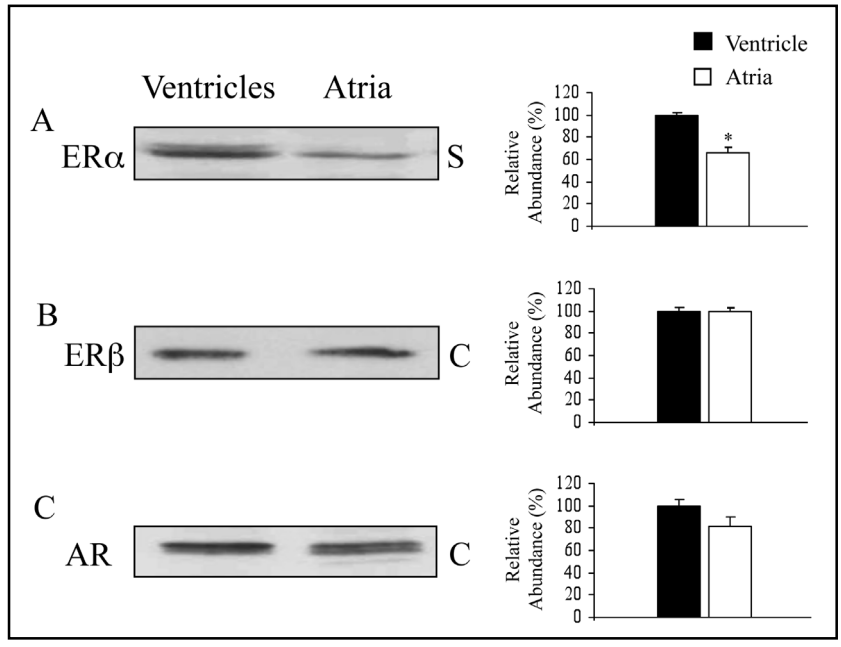


Fig. 4. Comparison of SSHR expression in ventricular and atrial tissue isolated from female mice: SSHR expression in the sarcolemmal (S) and cytosolic fractions (C) from ventricular and atrial tissue was examined by immunoblotting. Sarcolemmal fractions were probed with anti-ERα whereas cytosolic fractions were probed with either anti-ERβ or anti-AR. Protein loading was 10 μg for ERα and ERβ and 50 μg for AR. Ventricular and atrial samples were run simultaneously on the same gel to allow comparison of relative abundance between both chambers. Panels A-C show representative Western blots (left) and mean protein abundance (right) for ERα, ERβ and AR in ventricular and atrial protein fractions. For each receptor, the relative abundance in the ventricular samples was taken as 100%. Values are means of 3 separate determinations (5 mice/each; *$p<0.05$). Uniform loading and transfer were confirmed by staining with Ponceau S.

receptor expression in the ventricles, ERα, ERβ and AR abundance was comparable in protein fractions from male and female atrial tissue (data not shown). Overall, these results suggest that sex does not affect the abundance of ERα, ERβ and AR in mouse atrial or ventricular tissues.

Comparison of ventricular and atrial expression of SSHRs

It has been reported previously that the expression levels of the SSHRs can differ between tissues [35]. Thus, we compared the abundance of the SSHRs between the atria and the ventricles to determine if there were any chamber-specific differences. Representative Western blots for ERα (A; sarcolemmal fraction), ERβ (B; cytosolic fraction) and AR (C; cytosolic fraction) are shown in figure 4. The results showed that in female mice the mean abundance of ERα was significantly greater in the ventricle than in the atria (panel A). In contrast, the abundance of ERβ and AR were similar in female atrial and ventricular tissue (panels B and C, respectively). A similar distribution of ERα, ERβ and AR in the atria and ventricles was observed in male mice. Thus, these results suggest that the level of ERα expression, is chamber-specific in the mouse heart whereas ERβ and AR expression are not chamber specific.

Hormonal regulation of SSHRs

A) Influence of sexual maturity on SSHR distribution and expression. It is possible that the changes in hormone levels that occur with sexual maturity influence the expression and/or distribution of SSHRs. Therefore, we sought to characterize the effects of hormonal variation on the expression levels of ERα, ERβ and AR in female mouse ventricles. The results showed that the subcellular distribution and expression levels of ERα, ERβ and AR were similar in the ventricles of 21-day old mice (pre-sexual maturity) and 2-3-month old mice (sexually mature) (data not shown). Thus, the distribution and expression of SSHRs in mouse ventricle do not appear to be influenced by hormonal changes that occur with sexual maturity.

B) Influence of ovariectomy on SSHR distribution and expression. In the next series of experiments, 21-day old mice were ovariectomized in order to characterize the effect(s) of a sudden change in sex steroid hormone levels on ventricular ERα, ERβ and AR distribution and expression. Six weeks after ovariectomy there was a significant increase in body weight and a significant reduction in uterine weight in ovariectomized mice compared to the age-matched sham-operated mice (Table 1). As expected, ovariectomy resulted in marked decreases in serum 17β-estradiol compared to sham-operated mice (Table 2). The results also showed that the subcellular distribution of ERα, ERβ and AR were similar in both the sham and the ovariectomized animals (data not shown). Although ovariectomy did not affect SSHR distribution, it is possible that it may have altered receptor expression. Thus, we also examined the effects of ovariectomy on receptor abundance in ventricular tissues. Figure 5 (panels A, B, and C) shows the results

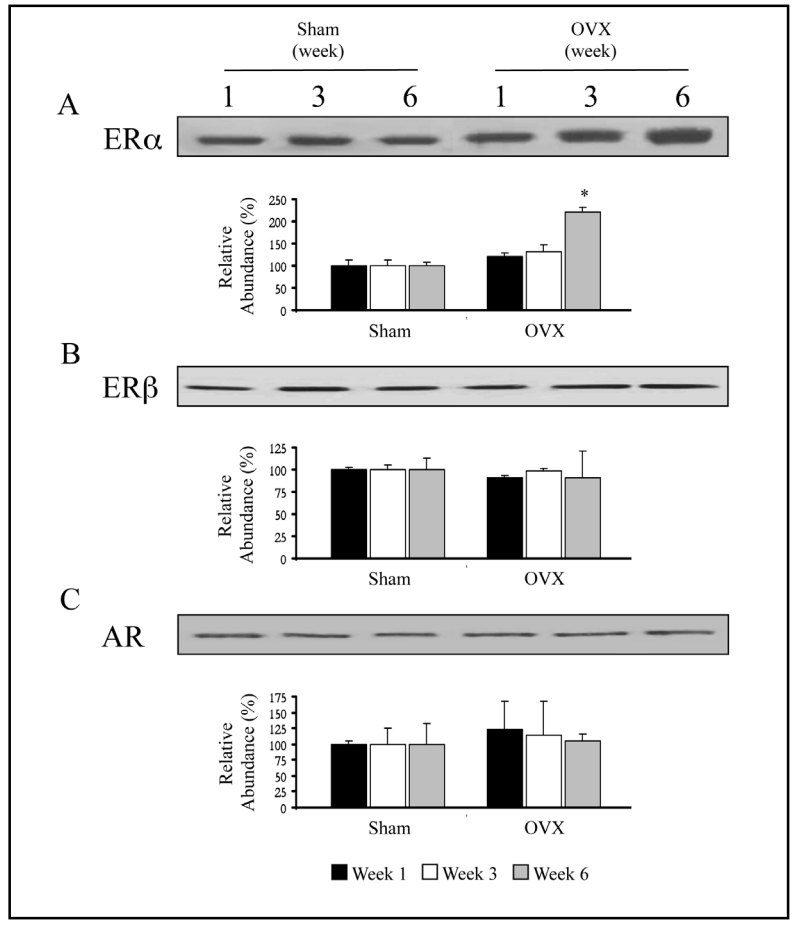


Fig. 5. The effect of ovariectomy on SSHR expression: Sarcolemmal or cytosolic fractions (100 µg of protein/lane) were prepared from ventricular tissue isolated from sham-operated (sham) or ovariectomized (OVX) mice 1, 3 or 6 weeks post-surgery. The sarcolemma fraction was probed for ERα and the cytosolic fraction was probed for ERβ and AR. Panels A-C show representative Western blots (top) and mean relative abundance (bottom) for ERα, ERβ and AR distribution and expression in ventricular tissue from sham and OVX mice. Three samples were analyzed for each time point (both sham and OVX). Each sample comprises the ventricular tissue from 5 hearts (*p<0.05 vs 6 week sham). Relative abundance was calculated using the sham-operated time point as 100% (e.g. OVX 1-week was normalized by Sham 1-week). Uniform loading and transfer were confirmed by staining with Ponceau S.

of quantification of ventricular ERα, ERβ and AR at one, three and six weeks post-surgery. Panel A shows that six weeks after surgery, the quantity of ERα immunoreactivity in the ventricular sarcolemmal fraction was significantly increased in ovariectomized mice compared to the sham-operated group. Interestingly, cytosolic ERβ (panel B) and AR (panel C) levels were similar in sham-operated and the ovariectomized mice. In the nuclear fraction, levels of ERα, ERβ and AR did not differ between the sham-operated group and the ovariectomized mouse group (data not shown). Overall, these results suggest that a reduction in hormone levels, as seen with ovariectomy, had a selective and marked effect on sarcolemmal ERα content in mouse cardiac ventricles.

Finally, we characterized the effect of ovariectomy on SSHR expression in atrial tissue. Interestingly, the levels of ERα and ERβ and AR were similar in sham-operated and ovariectomized mice (data not shown). Thus, it appears that altered hormone levels that result from ovariectomy did not affect SSHR expression in the mouse atria.

	Sham-Operated (n=39)	Ovariectomy (n=37)
Body weight (g)	26.3 ± 0.3	28.0 ± 0.3*
Uterine weight (mg)	130.6 ± 7.2	10.6 ± 0.7*

Table 1. Physical characteristics of ovariectomized mice (6 weeks post surgery). * p<0.05

Group	17β-estradiol (pM)
Male (n=8)	56 ± 7
Female Control (n=7)	390 ± 117
OVX 1-week (n=10)	122 ± 19
OVX 3-weeks (n=7)	154 ± 27
OVX 6-weeks (n=3)	133 ± 28

Table 2. Serum levels of 17β-estradiol in male, female and ovariectomized female mice.

Discussion

This study showed that the SSHRs ERα, ERβ and AR were differently distributed in subcellular protein fractions from mouse heart. Moreover, the pattern of subcellular distribution for each SSHR was similar in the atria and ventricles and did not differ between sexes. Further examination revealed that ERβ and AR expression was similar in both chambers of the heart, whereas ERα expression was significantly higher in the ventricles compared to the atria. Interestingly, ovariectomy resulted in a significant increase in ERα immunoreactivity associated with the ventricular sarcolemmal fraction. This suggests that changes in hormone levels, such as those that occur during menopause, may modulate ERα expression in the ventricle. Overall, these results suggest that SSHR distribution and expression in the mouse heart are not influenced by sex. Thus, it is unlikely that sex-related differences in mouse cardiac function [36-39] are mediated by sex differences in SSHR distribution and expression. However, it is possible that the changes in SSHR expression in response to altered hormone levels could affect cardiac function and possibly contribute to the development of cardiac disease.

Subcellular distribution of SSHRs

In non-cardiac tissue, ERα is localized predominantly in the sarcolemmal and nuclear fractions [40]. Similarly, the present study has shown that, in the myocardium (atria and ventricles), ERα was predominantly located in the sarcolemma, but also present in the nucleus. Studies that have examined cardiomyocytes with immunohistochemical techniques also have shown the presence of ERα in the sarcolemma and the nucleus as well as in the cytosol and intercalated discs [16-18]. The present study also found that ERα was present in the cytosol, but in markedly lower abundance in comparison with the sarcolemma. In contrast, work by Förster et al. [41] did not find any evidence of ERα in mouse heart. It is possible that differences in results between the present study and Förster et al. [41] reflect differences in sensitivity of the antibodies used in each study. Overall, the general consensus appears to be that ERα are present in the heart.

This study also showed that ERβ localized primarily to the nucleus and the cytosol in cardiac tissue. Similarly, other studies have shown that ERβ is expressed in cardiac myocytes [10, 13, 18, 20, 42, 43], associating with the nuclei in both human and rat hearts [18, 21, 22]. In contrast, Förster et al. found no evidence of ERβ in the murine heart [41]. The differences in results between the present study and those reported by Förster et al. [41] may be attributable to differences in antisera as well as visualizing techniques. Of note, it also has been reported that ERβs are localized in the mitochondria of human cardiomyocytes [44]. In the present study the abundance and distribution of SSHRs were not examined in organelles, such as mitochondria. Hence, it is possible that ERβs are localized in the mitochondria as well as the cytosolic and nuclear fractions in mouse cardiac tissue.

It is well known that when estrogen binds with its receptor it can result in the alterations in gene expression [45]. These changes are considered genomic effects. However, estrogen also can produce non-genomic effects. In this scenario, estrogen binds to a receptor in the sarcolemma (e.g. estrogen receptor) and activates signaling cascades, which can modulate intracellular signalling (e.g. PI3-kinase, MAPK and NOS) [45]. The present study found that ERα was most abundant in the sarcolemma and nuclear fractions. ERα in the nuclear fraction suggests the receptor can produce genomic effects whereas the ability of estrogen to modulate the function of sarcolemmal proteins [46, 47] suggest that ERα can produce non-genomic effects. ERβ was located primarily in the nucleus and cytosol. The prominent nuclear location of ERβ suggests that activation of this receptor subtype would be more likely to produce a genomic response than a non-genomic response. However, as mentioned above, Yang et al. reported that ERβs also localize to the mitochondria in cardiomyocytes [44]. This suggests that ERβ may mediate non-genomic actions of estrogen on mitochondrial function, such as protection against ATP depletion and antioxidant effects [44]. Overall, it appears that the localization of ERα and ERβ within the cell is important in determining the action of the receptor.

Previous work has shown that ARs are present in atrial and ventricular tissue of different species [19, 23]. Similarly, this study showed that ARs were expressed in mouse atrial and ventricular tissue. Although earlier work has shown that ARs are expressed in cardiac myocytes, the location of the receptors within the cells was not examined [23]. The present study has shown that ARs were predominantly expressed in the cytosol and in the nucleus in mature heart tissue. Similarly, ARs also are expressed in the cytosol and in the nuclei in rat liver cells [48].

Similar to estrogen, androgens also can mediate their effects via non-genomic or genomic mechanisms [49]. Recent work has shown that activation of plasma

membrane androgen receptors in rat cardiomyocytes results in a rapid non-genomic intracellular Ca^{2+} release [49]. However, in mouse cardiac tissue the level of AR expression in the sarcolemma was very low. This suggests that the non-genomic effects of AR activation may be nominal in mouse cardiac tissue. Interestingly, AR can control gene expression via the activation of intracellular AR-mediated signalling pathways [50]. The present study has shown that AR expression was high in both the cytosolic and nuclear fractions from mouse cardiac tissue. The location of the ARs suggests that they may alter cardiac function in the mouse through genomic pathways. However, the relationship between AR location and the pathway(s) by which ARs exert their effects needs to be investigated further.

Since the development of cardiac disease is influenced by sex it is possible that sex also may affect SSHR distribution. Interestingly, this study showed that SSHR distribution and expression were similar in atrial and ventricular tissue from male and female mice. Similarly, Mahmoodzadeh et al. [17] showed that cardiac ERα expression does not differ between human males and females. Together these findings suggest that sex does not influence SSHR distribution and expression levels.

Atrial and ventricular patterns of SSHR expression

The subcellular distribution of sex steroid receptors can differ between tissues of the same species [35]. Nevertheless, previous studies have not determined whether the expression of SSHRs differs between the atria and the ventricles in mouse heart. This study showed that ERβ and AR did not differ between heart chambers, but that ERα expression was lower in the atria than in the ventricles. This is in contrast to what has been observed in the rat heart, where ERα is higher in the atria than in the ventricles [51]. However, the present study examined ERα protein levels whereas work by Jankowski et al. [51] examined ERα mRNA levels. Thus, it is possible that differences in translation also may contribute to the observed differences in ER.

SSHRs and hormonal variations

This study has shown that both 17β-estradiol and uterine weight were significantly reduced at 6-week post ovariectomy. These results are in agreement with several other studies that have shown estrogen levels and uterine weight decrease after ovariectomy [52-54]. This study also showed that sarcolemmal ERα expression was markedly increased in ventricular tissue from ovariectomized mice (6-weeks post-surgery). Interestingly, several studies have shown that ERα mRNA expression decreases in MCF7 cells treated with estrogen [55, 56], suggesting that estrogen downregulates ERα expression. Thus, a reduction in estrogen levels, such as occurs following ovariectomy, could result in the upregulation of ERα receptors. This increase in ERα expression could alter cardiac function via non-genomic and genomic pathways and result in the development of cardiac pathology. It also is possible that the increased ventricular ERα is an attempt to compensate for the reduction in estrogens levels. In theory, increasing the ratio of receptors to hormone could help maintain SSHR signalling in the presence of declining estrogen levels.

It should be noted that several studies have reported that ERα expression is decreased in the heart after ovariectomy [43, 51]. However, these studies examined SSHRs in rat ventricular tissue, whereas the present study examined mouse ventricular tissue. Furthermore, we have observed that SSHR expression was not altered in 37-day old mice that underwent ovariectomy, whereas SSHR expression was altered when mice were ovariectomized at 21-days of age (unpublished data). This suggests that the age of the mice is very important for ovariectomy experiments. Thus, 21-day old mice were used for the ovariectomy experiments in the present study. Hence, it is possible that the differences observed between this study and work by Jankowski et al. [51] and Xu et al. [43] result from both differences in the species and age of the animals used for the experiments.

Overall the findings from the present study could be important for several reasons. First, these results suggest that SSHR expression may be modulated by hormonal variations under certain conditions, which could alter how cardiac cells function (e.g. ion channel function). Changes in cellular function could then alter cardiac performance and contribute to sex-related differences in the development of certain heart diseases. Second, many experiments that examine hormone replacement or ovariectomy are carried out at 4 weeks after the initiation of treatment or post-surgery. However, the present study shows that this time point may be too early to observe any significant changes in the effects of sex steroid hormones or in SSHR expression.

Limitation

Cardiac myocytes represent approximately 75% of the volume of the myocardial mass [57]. Thus, the results presented here likely reflect the distribution and abundance of SSHRs in cardiac myocytes. However, cardiac

myocytes only represent approximately 30% of all myocardial cells [57], with non-myocyte cells (e.g. smooth muscle cells, endothelial cells, fibroblasts) comprising the other 70%. Of the non-myocyte cells 90% are cardiac fibroblasts [58]. Non-myocyte cells play important roles in the heart and their function important can be regulated by sex steroid hormones [58]. Consequently, the distribution and abundance of SSHRs in these cell types also is important. It is possible that the results presented in this study also reflect SSHR abundance and distribution in non-myocytes as well. In support of this hypothesis one study has shown that ERβ is localized in the cytosol and nucleus of fibroblasts [58]. Since the primary objective of the present study was to characterize the distribution and abundance of sex steroid hormone receptors in the heart, this study did not examine distribution and abundance in specific cell types. However, this is an important question that needs to be addressed in future studies.

Conclusions

Overall, this study has shown that the subcellular distribution of SSHRs does not differ between males and females in the atria or in ventricles. This suggests that SSHR receptor distribution/expression are unlikely involved in sex-related differences in cardiac function observed in mice. However, this study also showed that a reduction in hormone levels increased the abundance of ERα associated with the sarcolemma in mouse ventricles. Thus, it is possible that the decline in hormone levels observed in older female mice could alter SSHR expression. It is plausible that changes in receptor expression could result in altered cardiac cellular function and the development of disease. In theory, it also is possible that similar changes occur in humans, specifically that menopause-related alterations in SSHR expression may contribute to the development of heart disease.

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