Cardiac myocytes and fibroblasts contain functional estrogen receptors

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Abstract Gender-based differences found in cardiovascular diseases raise the possibility that estrogen may have direct effects on cardiac tissue. Therefore we investigated whether cardiac myocytes and fibroblasts express functional estrogen receptors. Immunofluorescence demonstrated estrogen receptor protein expression in both female and male rat cardiac myocytes and fibroblasts. Nuclear translocation of the estrogen receptor protein was observed after stimulation of cardiomyocytes with 17β-estradiol (E_2). Cells transfected with an estrogen-responsive reporter plasmid showed that treatment with E_2 induced a significant increase in reporter activity. Furthermore, E_2 induced a significant increase in expression of the estrogen receptors α and β, progesterone receptor and connexin 43 in cardiac myocytes. Cardiac myocytes and fibroblasts contain functional estrogen receptors and estrogen regulates expression of specific cardiac genes. These data suggest that gender-based differences in cardiac diseases may in part be due to direct effects of estrogen on the heart.

Key words: Cardiomyocyte; Estrogen receptor α; Estrogen receptor β; Gene expression; Cardiac fibroblast

1. Introduction

There are significant gender-based differences in the incidence of a wide variety of cardiovascular diseases, including coronary artery disease (CAD) [1,2], sudden death [3], and hypertension [4–7]. Although attention has recently focused on gender-specific characteristics of CAD, the importance of gender in the development of hypertensive heart disease is less well understood. Women have a lower prevalence of left ventricular hypertrophy (LVH) than men [4,7]. For many years, this was attributed largely to differences between men and women in body size and risk factor profiles (e.g. hypertension). Analysis of the Framingham Heart Study data, however, showed that left ventricular mass is significantly greater in men than in women even after indexing for body surface area [5]. Furthermore, even amongst patients with known hypertension, male gender correlates strongly with the development of LVH [6].

The results of several recent studies demonstrating clinically relevant gender-based differences in the pathophysiology of hypertensive heart disease have raised new questions regarding the mechanisms responsible for the observed differences. For example, the CARDIA study demonstrated that the higher prevalence of LVH in men remains even after correction for a large number of risk factors, and further demonstrated that these differences in left ventricular size begin early in life (i.e. prior to menopause [7]). Taken together these studies suggest intrinsic factors may modulate the response to pathophysiologic factors that induce LVH.

We, and others, have hypothesized that the steroid sex hormone estrogen contributes to the reduced propensity for the development of LVH in females. This hypothesis is supported by data derived from several different animal models. Rat models of pulmonary hypertension demonstrate that ovariectomy potentiates the induction of right ventricular hypertrophy while estrogen replacement attenuates this response [8]. Furthermore, estrogen treatment inhibits the development of LVH in sinoaortic denervated rats [9]. Thus, these studies suggest that circulating estrogens contribute to the observed gender-based differences in the development of LVH.

Although the specific mechanisms by which estrogen exerts its cardioprotective effects are unknown, two general pathways are likely: (1) effects of estrogen on systemic factors; and (2) direct effects of estrogen on cardiac cells. Estrogens are known to alter several systemic factors that may play a role in the development of hypertensive heart disease. For example, estrogen replacement therapy is associated with reduced circulating levels of both angiotensin converting enzyme activity [10] and insulin [11]. We hypothesized that in addition to these systemic effects of estrogen, direct effects of estrogen on cardiac tissue might also contribute to the observed gender-based differences in cardiac diseases.

Nearly all of the known biological effects of estrogen are mediated by the estrogen receptor, an intracellular protein that regulates gene expression in an estrogen-dependent fashion [12,13]. Though previous studies demonstrated binding of radiolabeled estrogen to cardiac tissue [14,15], the presence and functional integrity of estrogen receptors in the heart have not yet been established. Therefore, we sought to determine whether cardiac myocytes and/or fibroblasts express functional estrogen receptors. In the present study, we demonstrate, for the first time, that both cardiomyocytes and cardiac fibroblasts contain estrogen receptor protein and that the receptor is capable of estrogen-mediated gene activation. These data identify the heart as a target organ for direct effects of estrogen on the cardiovascular system.

2. Materials and methods

All chemicals were obtained from Merck, Darmstadt, Germany if not otherwise specified.

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2.1. Cell culture techniques

rats Wistar-Kyoto strain rats, 1–2 days old, were separated by gender and hearts were isolated and digested with 10 ml of Spinner solution (116 mM NaCl, 5.3 mM KCl, 8 mM NaH_2PO_4, 22.6 mM $NaHCO_3$, 10 mM HEPES, 5 mM D-Glucose, pH 7.4) containing 0.1% collagenase (Cytogen; Berlin, Germany) for 10 min at 37°C in eight consecutive steps (modified after [16]). After each digestion, the medium containing the suspended cardiac cells was removed and an equal volume of Spinner/collagenase solution was added. The cardiac cell suspension was mixed with an equal volume of Ham's F10 (Gibco BRL; Eggenstein, Germany) supplemented with 10% horse serum (HS; Biochrom; Berlin, Germany), 10% estrogen-free fetal calf serum (FCS; c.c.pro; Hamburg, Germany) and 25 μg/ml gentamicin (Gibco BRL; Eggenstein, Germany) and stored at 4°C. Heart tissue was digested until the cells were completely suspended. Suspensions were centrifuged at $400\times g$ for 5 min and the cell pellets were resuspended in 20 ml of Ham's F10 supplemented with 10% HS and 10% FCS and plated on culture dishes. After 75 min the medium which contained the cardiomyocyte fraction of the digested tissue was removed. The adherent fraction of the plated cells consisted of cardiac fibroblasts. The dishes were gently rinsed three times to remove remaining cardiomyocytes. Cardiomyocytes were counted in a Fuchs-Rosenthal chamber and seeded on culture dishes at a density of 2×10^4 cells/cm^2 for immunofluorescent staining and 3×10^4 cells/cm^2 for transfection. The culture medium for cardiac fibroblasts was exchanged for DMEM (Gibco BRL; Eggenstein, Germany) supplemented with 20% estrogen-free FCS and 25 μg/ml gentamicin. The cardiomyocyte culture contained 90–95% myocytes, as assessed by immunofluorescence staining with an antibody against troponin-t (CP05, Dianova; Hamburg, Germany; data not shown). Purity of cardiac fibroblast culture was assessed by repeated differential plating and microscopic evaluation.

2.2. Immunoblotting

2×10^6 neonatal cardiomyocytes and cardiofibroblasts were lysed in 2 ml of the following buffer: 50 mM NaCl, 20 mM Tris (pH 7.4), 50 mM NaF, 50 mM EDTA, 20 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM sodium orthovanadate (Na_3VO_4), 1% Triton X-100, 1 mM PMSF, 0.6 mg/ml leupeptin and 10 μg/ml aprotinin. Total cell lysates (50 μg/lane) were analyzed by SDS-PAGE in a 7.5% gel and transferred to a nitrocellulose membrane. Protein content was measured with a standard Bradford assay. Immunoblotting was performed with either a monoclonal anti-estrogen receptor α antibody (SRA 1000, 1:1000 dilution, Biomol; Hamburg, Germany) or anti-estrogen receptor β antibody (1:500, Dianova; Hamburg, Germany), anti-progesterone receptor antibody (SRA 1100, 1:1000 dilution, Biomol; Hamburg, Germany) or anti-connexin 43 antibody (C13720 Transduction Laboratories 1:250, Dianova, Hamburg, Germany) followed by detection with the enhanced chemiluminescence technique (ECL, Amersham). Densitometric analysis of protein induction was performed on an Epson GT 8000-scanner with the analysis software ScanPak (Biometra; Göttingen, Germany).

2.3. Immunofluorescent staining of the estrogen receptor

Neonatal cardiac myocytes were grown on uncoated glass coverslips for immunofluorescent staining. Cellular staining was performed as previously described with minor modifications [17]. Cells were fixed with 2% paraformaldehyde and phosphate-buffered saline. Immunofluorescent studies of the estrogen receptor were performed with a 1:20 dilution of a polyclonal rabbit antibody (kind gift of K. Yoshinaga, NIH, Bethesda, MD) against a synthetic peptide identical to an 18 amino acid fragment from the hinge region of the rat estrogen receptor [18]. Progesterone receptor was detected with a monoclonal mouse antibody against a synthetic peptide identical to amino acids 523–536 of the chicken progesterone receptor (SRA 1100, 1:500 dilution, Biomol; Hamburg Germany). A goat anti-rabbit IgG conjugated DTAF was used as a secondary antibody (Dianova; Hamburg; Germany). Studies in which the primary antibody was omitted served as negative controls. A Zeiss MC405 microscope equipped with a Zeiss Plan-Apochromat 63/1.4 was used for conventional fluorescence microscopy. The confocal imaging system consisted of a Zeiss Axiophot fluorescence microscope, a BioRad MRC-600 confocal scanner and a Silicon Graphics Workstation. The images were recorded using a Zeiss Neofluar 63/1.3 objective. The system was equipped with an argon/krypton mixed gas laser. Image processing was done on a Silicon Graphics Workstation using 'Imaris' (Bitplane AG; Zürich, Switzerland), a 3D multi-channel image processing software specialized for confocal microscopic images [19–21]. Cells were studied following 24 h incubation in the absence or presence of 10^{-6} M 17β-estradiol.

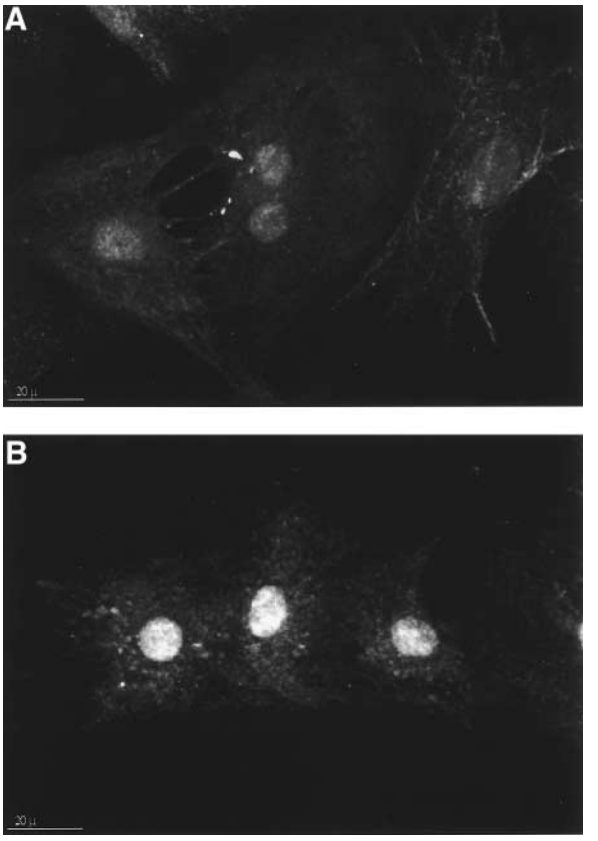

Fig. 1. Rat neonatal cardiac myocytes express estrogen receptor protein. Unstimulated (upper) and 17β-estradiol-stimulated (lower) cardiac myocytes grown on glass coverslips were immunostained with an anti-estrogen receptor primary antibody and a DTAF-conjugated secondary antibody. In the absence of estrogen, confocal laser scan microscopy revealed a mixed cytoplasmic and nuclear distribution of the estrogen receptor. After 17β-estradiol treatment the estrogen receptor protein was found predominantly in the nucleus. Magnification 630×.

2.4. Transfection assay

Neonatal rat cardiac myocytes and fibroblasts were seeded at a density of 3×10^4 cells/cm^2 for transfection. Cells were transfected using a liposome-conjugated transfection technique according to the manufacturer's instructions (DOTAP; Boehringer-Mannheim, Germany). Cells were transfected with a constitutively expressed β-galactosidase plasmid (see below) and either ERE-*LUC* (containing three copies of the vitellogenin estrogen-responsive element driving expression of the luciferase gene (kind gift of C. Glass)) or TK-*LUC* (the same plasmid as ERE-*LUC* but lacking the estrogen responsive elements; also the gift of C. Glass). After 24 h, the transfection medium was removed and cardiomyocytes were maintained in phenol red-free Ham's F10 with angiotensin II (100 nM; Sigma Chemicals; St. Louis, MO), isoproterenol (100 μM; Sigma Chemicals; St. Louis, MO), L-phenylephrine (100 μM; Sigma Chemicals; St. Louis, MO), insulin (10 μg/ml; Serva; Heidelberg, Germany), transferrin (10 μg/ml; Serva; Heidelberg, Germany), ascorbic acid (100 μM; Sigma Chemicals; St. Louis, MO), vitamin B12 (1.5 μM; Serva; Heidelberg, Germany), sodium selenite (20 nM) and BrdU (100 μM; Sigma Chemicals; St. Louis, MO) in the absence or presence of 17β-estradiol (Sigma Chemicals; St. Louis, MO) and/or ICI 182780, a pure estrogen antagonist (kind gift of Dr. A. Wakeling, Zeneca Pharmaceuticals, Cheshire, UK). Cardiac fibroblasts were maintained in DMEM plus 1% estrogen-free FCS. Phenol red-free medium was used throughout all ex-

periments as phenol red is known to act as a weak estrogen [22]. An equal volume of vehicle alone (0.1% ethanol) was added to control cells. Following 24 h of incubation in the experimental conditions, cells were harvested and luciferase activity was determined on a luminometer (C-Gem, Optocom 1) as described [23,24]. In a subset of each transfection series, cells were transfected with pL7RH- Gal (SV-40 promoter including a nuclear localization signal driving the β-galactosidase cDNA). Transfection efficiency was determined by staining of the transfected cells. Cells were washed with phosphate buffered saline and then fixed in 0.5% glutaraldehyde for 10 min followed by three more washes. They were then incubated overnight at 37°C in a staining solution containing 15 mM $K_3Fe(CN)_6$, 15 mM $K_2Fe(CN)_6{\cdot}3H_2O$, 0.15 mM $MgCl_2$, 1% DMSO and 1 mg/ml X-Gal. Nuclei of cells stained for β-galactosidase activity were counted and the results of each luciferase determination were normalized for the transfection efficiency.

2.5. Statistical analysis

All reported values are mean ± S.E.M. Statistical comparisons were made by Student's *t*-test. Statistical significance was assumed if a null hypothesis could be rejected at the $P < 0.05$ level.

3. Results

To determine estrogen receptor expression and the intracellular location of the estrogen receptor, immunofluorescent staining was carried out. In the absence of 17β-estradiol, estrogen receptor was detected in both the cytosolic and nuclear compartments of cardiac myocytes and fibroblasts (Figs. 1 and 2, respectively). Incubation of the cells in 10^{-6} M 17β-estradiol for 24 h induced nuclear translocation of the receptor protein. In control experiments in which the primary antibody was omitted, no staining was detected (data not shown). Because of the complex regulation of steroid hormone receptor activation [25,26], the presence of estrogen receptor protein does not establish unequivocally the ability of these cells to respond directly to estrogen exposure. Therefore, to demonstrate the functional competence of the receptor, transient transfection experiments were carried out using a well characterized estrogen-responsive reporter plasmid, ERE-*LUC* [27]. Unpassaged rat cardiomyocytes transfected with ERE-*LUC*

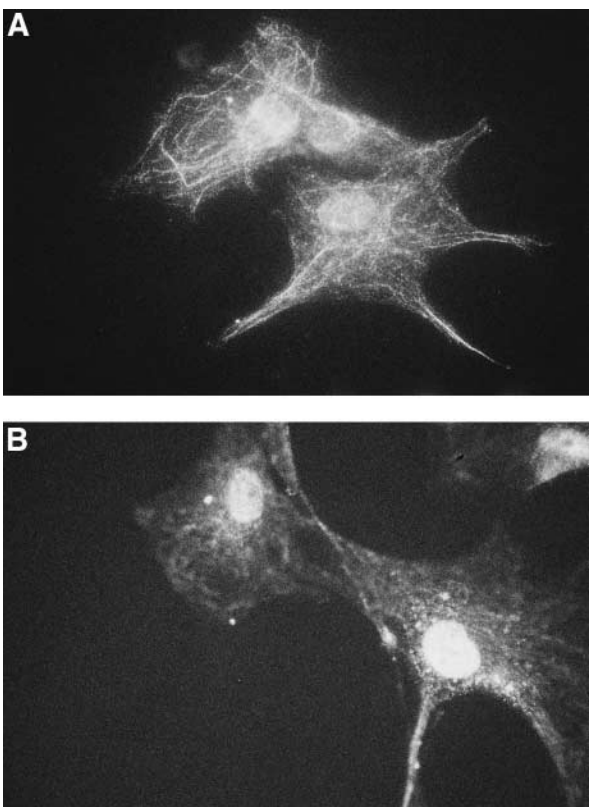

Fig. 2. Rat neonatal cardiac fibroblasts express estrogen receptor protein. Unstimulated (upper) and 17β-estradiol-stimulated (lower) cardiac fibroblasts grown on glass coverslips were immunostained with an anti-estrogen receptor primary antibody and a DTAF-conjugated secondary antibody. As with cardiomyocytes, in the absence of estrogen, the estrogen receptor demonstrated a mixed cytoplasmic and nuclear distribution. After 17β-estradiol treatment the estrogen receptor protein was found predominantly in the nucleus. Magnification 630×.

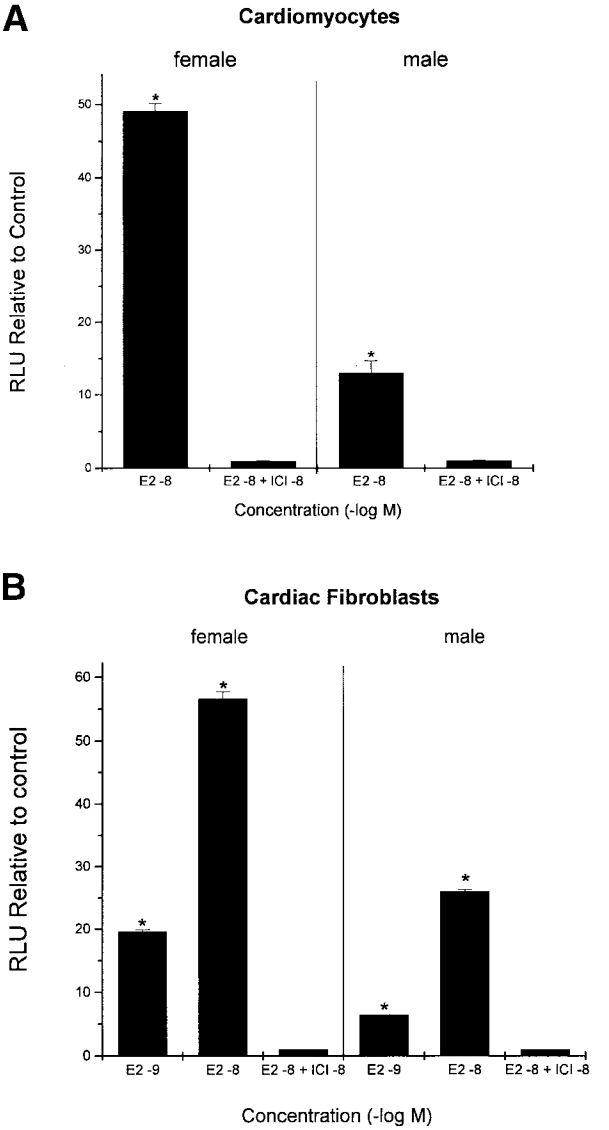

Fig. 3. Rat neonatal cardiac myocytes and fibroblasts contain transcriptionally competent estrogen receptor. Rat neonatal cardiac myocytes (upper panel) or cardiac fibroblasts (lower panel) were transfected with a reporter plasmid (ERE-*LUC*) in which an estrogen-responsive element drives expression of the firefly luciferase gene. Cells were grown in the absence or presence of 17β-estradiol (E_2) or the anti-estrogen ICI 182780 (10^{-8} M) and harvested after 24 h. Bars represent the mean luciferase activity with S.E.M. Luciferase activity is shown relative to control cells that were not exposed to hormones.

showed dose-dependent transactivation of the reporter construct after incubation with 17β-estradiol (Fig. 3). Maximal activation of the reporter (male: 13 ± 1.68-fold, female 49.1 ± 1.10-fold; $n = 9$, $P < 0.01$) occurred at an estrogen concentration of 10^{-8} M. Similarly, cardiac fibroblasts transfected with ERE-*LUC* also showed dose dependent activation of the reporter (male: 24.47 ± 0.23-fold, female 60.55 ± 1.08-fold; $n = 9$, $P < 0.01$). In contrast, the control plasmid lacking the estrogen receptor-responsive element (TK-*LUC*) revealed no evidence of hormonal induction in either cell type (data not shown). The specificity of the estrogen-induced activation of ERE-*LUC* was demonstrated further by the inhibition of activation observed when the specific estrogen antagonist ICI 182780 was added to the experimental media (Fig. 3). Taken together, these data demonstrate that the estrogen receptor in cardiomyocytes and cardiac fibroblasts is functionally intact. Western blot analysis with specific monoclonal estrogen receptor antibodies directed against the estrogen receptor α and estrogen receptor β isoform detected the 66/60 kDa estrogen receptor protein in lysates derived from neonatal cardiomyocytes (Fig. 4A,B). In addition, we could also demonstrate expression of the estrogen receptor in immunoblot analysis derived from male and female cardiac fibroblasts (data not shown). Although the level of protein expression in both cell types was low in the absence of 17β-estradiol, incubation in 10^{-9} M 17β-estradiol for 24 h led to a marked increase in the abundance of estrogen receptor protein. Immunostaining with a different primary antibody (NIH 715) showed the same result and control experiments in which the primary antibody was omitted were negative (data not shown). Thus, these studies confirm the immunofluorescent analysis demonstrating estrogen receptor protein in cardiac cells.

In non-cardiac cells, estrogen-mediated regulation of progesterone receptor gene expression is well known [28]. To determine whether estrogen, besides its potential to upregulate the expression of the estrogen receptor, regulates the expression of endogenous cardiac genes, we assessed the level of

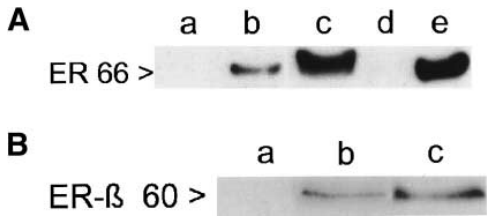

Fig. 4. A: Estrogen induces increased estrogen receptor α protein expression. Total cellular lysates of male and female cardiac myocytes grown in the absence (a) or presence of 10^{-9} M 17β-estradiol were subjected to SDS-PAGE, immunoblotted with an anti-estrogen receptor antibody, and developed with a chemiluminescence technique. E_2 induced a 6.4-fold increase (b: male) and a 14.8-fold increase (c: female) in expression of the estrogen receptor α in cardiac myocytes. The 66 kDa estrogen receptor (ER) protein migrates as indicated by the arrow. Coincubation with the anti-estrogen ICI 182780 inhibited receptor induction by estrogen (d). Lysate from adult rat ovary served as a positive control (e). One of three similar studies is shown. B: Estrogen induces increased estrogen receptor β protein expression. Total cellular lysates of male and female cardiac myocytes grown in the absence (a) or presence of 10^{-9} M 17β-estradiol were subjected to SDS-PAGE, immunoblotted with an anti-estrogen receptor antibody, and developed with a chemiluminescence technique. E_2 induced a significant increase (b: male/c: female) in expression of the estrogen receptor β in cardiac myocytes, respectively. The 60 kDa estrogen receptor (ER) protein migrates as indicated by the arrow. One of three similar studies is shown.

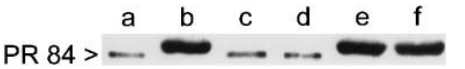

Fig. 5. Estrogen induces increased expression of the endogenous progesterone receptor gene in rat neonatal cardiomyocytes. Total cellular lysates of female (a,b,c) and male (d,e) rat neonatal cardiomyocytes grown in the absence or presence of 10^{-9} M 17β-estradiol were separated by SDS-PAGE, immunoblotted with an anti-progesterone receptor antibody and developed with chemiluminescence techniques. The 84 kDa progesterone receptor (PR) could be detected in unstimulated cells (a,d). Estrogen exposure increased progesterone receptor protein content (b: female, 17.5-fold; d: male, 16-fold). Coincubation with the anti-estrogen ICI 182780 inhibited the induction (c). Lysate from adult rat ovary served as a positive control (f). One of three similar studies is shown.

progesterone receptor protein in male and female cardiomyocytes in the absence and presence of 17β-estradiol. Immunoblot analysis of lysates from rat neonatal cardiomyocytes grown in the presence of 10^{-9} M 17β-estradiol identifies a band with a molecular weight of 84 kDa (Fig. 5), corresponding to the expected size of the A isoform of the rat progesterone receptor [29]. In the absence of 17β-estradiol only a weak signal was detected. Quantification of the intensity of the signal representing the progesterone receptor in three separate experiments demonstrated that estrogen exposure resulted in a increase in progesterone receptor protein expression (male: 12.6, female: 13.4; Fig. 5). Furthermore, immunoblot analysis of lysates from cardiac myocytes demonstrated a band of 43 kDa, which corresponds to the size of the gap junction protein connexin 43 [30]. Connexin 43 expression was upregulated after incubation with 10^{-9} M E_2 (male: 6.2-fold, female: 24.8-fold). Again, in the absence of 17β-estradiol only a weak signal was detected (Fig. 6). In addition, we could also demonstrate expression of the progesterone receptor and connexin 43 in immunoblot analysis derived from neonatal male and female cardiac fibroblasts (data not shown). Quantification of the intensity of the signal representing connexin 43 expression in three separate experiments demonstrated that estrogen exposure resulted in the protein expression mentioned before ($n = 3$; $P < 0.05$). These data demonstrate that estrogen can modulate expression of endogenous cardiac genes and reveals gender-based differences in the activation of those genes.

4. Discussion

Although several clinical studies have demonstrated that women have a reduced incidence of LVH when compared to men, the mechanism(s) responsible for these observations is unclear. A large body of experimental data derived from animal studies implicates estrogen in contributing to this effect [9,31–33]. As noted above, estrogen deficiency potentiates, and estrogen replacement attenuates the development of both right and left ventricular hypertrophy in rat models [8,9,34]. The contractile function of hearts taken from ovariectomized female rats is reduced compared with normals, and this deficit is reversed in mice treated with estrogen replacement [35]. These effects on cardiac contractility are mirrored by estrogen-mediated changes in expression of contractile proteins such as myosin heavy chain [32,36]. Estrogen treatment prevents the decrease in the ratio of V1 to V3 myosin isoforms in hypertensive rats [36]. Additional mechanisms, suggested by in vivo animal models, by which estrogen may regulate the

Fig. 6. Estrogen induces increased expression of the connexin 43 gene in rat neonatal cardiomyocytes. Total cellular lysates of male (a,b,c) and female (d,e,f) rat neonatal cardiomyocytes grown in the absence or presence of 10^{-9} M 17β-estradiol were separated by SDS-PAGE, immunoblotted with an anti-connexin 43 antibody and developed with chemiluminescence techniques. The 43 kDa connexin 43 (CNX 43) expression was difficult to detect in unstimulated cells (a), estrogen exposure increased connexin 43 protein content in myocytes (lane d: male: 6.2-fold; lane e: female: 24.8-fold). Coincubation with the anti-estrogen ICI 182780 inhibited the induction (c,f). Lysate from adult rat brain served as a positive control (g). One of three similar studies is shown.

contractile function and metabolic state of the myocardium, include: increasing glucose uptake [37,38] and augmenting expression of nitric oxide synthase [39]. These studies demonstrate clearly that estrogen plays a role in modulating the cardiac phenotype. Traditionally, effects of estrogen on cardiovascular diseases have been attributed to the systemic effects of estrogens. For example, until recently the anti-atherogenic effects of estrogens were largely attributed to the beneficial effects of estrogen on lipid metabolism. More recently attention has shifted to include investigation of direct effects of estrogen on the vascular wall and the potential contribution of this additional pathway to the atheroprotective effects of estrogen [40]. For example, vascular smooth muscle cells are now known to express estrogen receptors [24,41,42], which are functionally intact. In addition, estrogen has been shown to inhibit the response to vascular injury in the mouse carotid artery, even without alterations in systemic lipids [43]. These data suggest that vascular tissue is a target organ for direct effects of estrogen. We hypothesized, similarly, that the heart may also be a target organ for estrogen action and therefore sought to determine whether cardiac cells express estrogen receptors. In the present study we demonstrate expression of functionally intact estrogen receptors in both cardiac myocytes and fibroblasts.

Previous attempts to demonstrate estrogen receptors in cardiac tissue have been confined to demonstration of uptake into cardiac cells of radiolabeled estrogen following intravenous administration [14,15,44]. Although these reports clearly demonstrated specific estrogen binding to heart tissue, they did not identify the estrogen receptor specifically as responsible for the observed binding activity, nor did they demonstrate any biological effects resulting from estrogen binding. In the present study, we applied both immunoblotting and immunofluorescent staining to demonstrate that cardiac cells express estrogen receptor protein. The immunoblot experiments demonstrate the presence of estrogen receptor protein of the expected molecular weight [45]. The immunofluorescent staining confirmed the presence of estrogen receptor protein, and further demonstrated that the intracellular distribution of the protein is altered by estrogen exposure, a behavior characteristic of estrogen receptors in other tissues [46].

As with other nuclear receptors, transcriptional activation of the estrogen receptor is a complex process that requires the functional integrity of both the receptor itself and of a number of other associated proteins [25,26]. A number of mutant estrogen receptors in breast cancer tissues are detectable by immunologic methods, but are known to be transcriptionally silent [47]. In addition, human vascular smooth muscle cells express an estrogen receptor isoform that is also detected by anti-estrogen receptor antibodies, but which is not capable of mediating alterations in gene expression [48]. Thus, demonstration of the presence of estrogen receptor protein in heart tissue is not sufficient to suggest a functional role for the protein. We therefore also sought to determine whether the estrogen receptor in cardiac myocytes and fibroblasts is capable of transcriptional transactivation and found it capable of high level transactivation of a classical estrogen responsive element in transient transfection experiments with cardiac cells. The specificity of this activation is demonstrated both by the lack of activation of the backbone vector (which does not contain the estrogen-responsive elements) and by inhibition of the response by the receptor antagonist ICI 182780. The activation observed in these experiments is similar but somewhat more robust than that found in other non-reproductive cells such as vascular smooth muscle cells [24].

Although the transient transfection experiments demonstrate estrogen-mediated activation of an exogenous gene, a convincing demonstration of the estrogen-responsive nature of cardiac cells should include the demonstration of estrogen-regulated expression of endogenous genes. We chose to investigate estrogen effects on three genes known to respond to E_2 in non-cardiac cells: estrogen receptor, the progesterone receptor and the gap junction protein connexin 43 expression. Estrogen exposure markedly stimulated estrogen receptor, progesterone receptor and connexin 43 expression in both cardiac myocytes and fibroblasts, confirming the ability of these cells to respond directly to estrogen. Although the physiologic significance of progesterone receptors in the heart is unknown, the addition of progesterone to estrogen treatment in ovariectomized mice has been shown to increase the density of both muscarinic and β-adrenergic receptors [33]. In addition, we show that connexin 43 expression in neonatal cardiac myocytes and fibroblasts is upregulated after estrogen incubation. These observations extend our knowledge about connexin 43 expression in adult heart, which revealed no inducibility by estrogen in contrast to adult reproductive tissue which was shown to be estrogen sensitive [39]. In the light of recent observations that suggest that E_2 influences coupling and uncoupling of cardiac myocytes [48], the modulation of this gap junction protein by estrogen may well be of physiologic importance in a large array of cardiac diseases. Interestingly, the inducibility of the endogenous genes revealed significant gender-based differences, which leads to the suggestion that mechanisms other than steroid binding may play a role in these processes. In this context, recent studies have demonstrated the presence of a novel variant of the estrogen receptor [49–51] called ER β. This novel estrogen receptor (ER β) may also play a role in the complex regulation of estrogenic effects in cardiovascular disease as its presence, which was shown in this study, suggests [52].

In summary, we have shown that rat neonatal cardiomyocytes express functional estrogen receptors, and further, that estrogen exposure modulates gene expression in these cells and these findings support the hypothesis that the heart is a target organ for estrogen action.

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