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17β-Estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo

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Abstract

Objectives: NO production has been attributed to play a major role in cardiac diseases such as cardiac hypertrophy and cardiac remodeling after myocardial infarction which display significant gender-based differences. Therefore we assessed the effect of 17β-estradiol (E2) on estrogen receptor (ER) α and β and endothelial and inducible NO synthase in neonatal and adult rat cardiomyocytes. **Methods:** The presence of ERα and ERβ was demonstrated by immunofluorescence and western blot analysis as well as the expression pattern of inducible NO synthase (iNOS) and endothelial NOS (eNOS) in isolated cardiomyocytes from neonatal and adult rats. Furthermore, regulation of myocardial iNOS and eNOS expression by estrogen was evaluated in the myocardium from ovariectomized or sham-operated adult Wistar-Kyoto rats. **Results:** Incubation with E2 led to translocalization of the ER into the nucleus and increased receptor protein expression. E2 stimulated expression of iNOS and eNOS in both neonatal and adult cardiac myocytes. Coincubation with the pure anti-estrogen ICI 182.780 inhibited upregulation of ER and NOS expression. In ovariectomized rats myocardial iNOS and eNOS protein levels were significantly lower compared to sham-operated female animals. **Conclusion:** Taken together, these results show that E2 stimulates the expression of iNOS/eNOS in neonatal and adult cardiomyocytes in-vivo and in-vitro. These novel findings provide a potential mechanism of how estrogen may modulate NOS expression and NO formation in the myocardium.

Keywords: Estrogen receptor α, β; Inducible/endothelial NO synthase; Cardiac myocytes

1. Introduction

The incidence of cardiac diseases such as left ventricular hypertrophy and hypertensive heart disease displays significant differences in female patients before and after menopause [1,2]. In this context it has been hypothesized that estrogen may play an important role in the pathogenesis of this process as women after the menopause have an increased risk of developing cardiac diseases in coincidence with a declining level of 17β-estradiol [3]. Estrogen belongs to a class of steroid hormones, that regulate transcription in estrogenic target tissues upon binding to the respective intracellular estrogen receptor (ER). Two different subtypes of the ER have been described so far, ERα and ERβ [4,5]. In a classical mode of action these receptors induce transcription of downstream target genes by binding to enhancer elements in the responsive genes [6,7]. The binding affinity of the large array of estrogens to the respective estrogen receptors reveals a distinctive pattern that shows that certain estrogens vary in their affinity to bind either ERα or ERβ [8]. In addition, estrogens vary in their potential to activate either ERα or ERβ [9]. Furthermore, ERα and ERβ can form homo- as well as heterodimers to activate downstream target genes in the respective target tissues [10,11]. These recent observations suggest that estrogenic effects in

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a specific target tissue (i.e. the myocardium) depends not only on a specific estrogen ligand but also on the presence and distribution of ERα and ERβ.

In this context, it has been shown recently that NO production stimulated by estrogen may play an important role in the pathogenesis of gender-based differences found in cardiac disease [12]. 17β-estradiol increased the level of calcium-dependent NO synthase (NOS) activity in cardiac myocytes [13]. Cardiac NO synthesis has been attributed to play a crucial role in the regulation of cardiac muscle function [14,15]. The role of estrogen, however, in the regulation of NO synthesis in the myocardium is poorly understood. To further elucidate the underlying mechanisms how estrogen may exert a cardioprotective effect, we hypothesized that estrogen may also influence the expression of calcium-independent and calcium-dependent NOS isoforms (inducible NOS/endothelial NOS) which are expressed in cardiac tissue [16–18].

In a first step, we investigated, if rat adult myocytes contain functional estrogen receptors (α and β subtype), as these receptors are required to mediate estrogenic effects such as upregulation of downstream target genes. Furthermore, we investigated the regulation of iNOS and eNOS expression by estrogen in isolated neonatal and adult ventricular cardiomyocytes. Finally, we studied the myocardial expression of iNOS and eNOS in ovariectomized or sham-operated adult Wistar-Kyoto rats.

2. Methods

Materials: All chemicals were obtained from Merck (Darmstadt, Germany) if not otherwise specified.

Isolation and culture of adult cardiomyocytes: Procedures with experimental animals followed the guidelines of the National Institute of Environmental Health Sciences Animal and Use Committee. Cardiomyocytes were isolated after a modified protocol by Claycomb and Palazzo [19] from adult male and female Wistar-Kyoto rats weighing 200–350 g. Animals were lightly anaesthetized with diethylether and heparinized with 2500 units sodium heparin into the vena cava. The heart was rapidly dissected and mounted on the cannula of a Langendorff perfusion system. Retrograde perfusion was performed using a calcium-free perfusion buffer containing of 128 mM NaCl, 14 mM KCl, 190 μM NaH_2PO_4, 1 mM Na_2HPO_4, 1.5 mM $MgSO_4$, 10 mM Hepes, 5.5 mM D-Glucose, 2 mM sodium pyruvate according to the procedure of Powell [20]. Enzyme solution consists of about 1.3 mg/ml collagenase (0.452 U/mg, Boehringer Mannheim, Germany) and 5 mg/ml albumin, Fraction V, fatty acid free (Boehringer Mannheim). Thereafter, total ventricular tissue was chopped, minced into small pieces, incubated in 2% albumin solution (5 min) and passed through a mesh of 250 μm pore size. Cardiac fibroblasts and non-myocytes were separated from cardiac myocytes by three consecutive 2% serum albumin gradient steps. Freshly isolated cardiomyocytes were counted in a Fuchs-Rosenthal chamber and contained 80–85% rod-shaped ventricular myocytes, as assessed by visual control. Cells were seeded on culture dishes at a density of 2 ± 10^4 cells/cm^2 and cultured in medium 199 (Gibco; Eggenstein, Germany) supplemented with 2 mM carnitine, 5 mM taurin, 5 mM creatin, penicillin (100 U/ml) and streptomycin (100 U/ml) (CCT199 medium, pH 7.4).

Isolation and culture of neonatal cardiomyocytes: The hearts of 1–2 day old rats 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 as previously described [21]. Cell suspension was centrifuged at 400 *g* for 5 min and the cell pellet was resuspended in 20 ml of Hams' F10 supplemented with 10% horse serum (Biochrom; Berlin, Germany) and 10% estrogen-free fetal calf serum (FCS; c.c.pro; Neustadt, Germany) and plated on culture dishes. After 75 min the medium which contained the cardiomyocyte fraction of the digested tissue was removed. Cardiomyocytes were counted in a Fuchs-Rosenthal chamber and seeded on culture dishes at a density of 2×10^4 cells/cm^2. The neonatal cardiomyocyte culture contained 90–95% myocytes, as assessed by immunofluorescence staining with an antibody against troponin-t (CP05, Dianova; Hamburg, Germany; data not shown).

Treatment of cardiomyocytes: Serum-starved cells were stimulated with 10^{-9} M 17β-estradiol for 24 h to determine genomic effects. Controls were coincubated with 10^{-8} M ICI 182.780 (kind gift of Dr. A.E. Wakeling), a specific pure antiestrogen [22]. Unstimulated cells were incubated with 0.1% (v/v) ethanol, the solvent of 17β-estradiol. Moreover, a series of controls was performed with the stereoisomer 17α-estradiol, known to be biologically ineffective as an estrogen. After stimulation cells were rinsed with phosphate-buffered saline and lysed with 500 μl lysate buffer (50 mM NaCl, 50 mM NaF, 20 mM Tris, 10 mM EDTA, 20 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were clarified by centrifugation at 12 000 *g* for 10 min at 4°C. Protein content was measured with a standard Bradford assay.

Immuncytochemical analysis: Freshly isolated myocytes obtained from adult rats were suspended in CCT199 medium with 10% steroid-free fetal calf serum (Biochrom) and cultured on collagen-coated coverslips. After attachment of the cells to substrate, culture medium was replaced by serum-free CCT199 medium. Serum-starved cells were stimulated with 10^{-6} M 17β-estradiol±ICI 182.780 for 24 h. Control cells were incubated with 0.1% ethanol, the solvent of estrogen, and with an E2-BSA conjugate, 17β-estradiol-6-(*O*-carboxymethyl)oxime-BSA (10^{-6} M). E2-BSA is known as effective as free 17β-estradiol [23] but is

unable to pass the cellular membrane. Immunfluorescent staining was performed as previously described [21]. Cells were fixed in buffered 3.7% paraformaldehyde for 15 min, followed by 0.2% Triton X-100 in PBS for 10 min to permeabilize the membranes. Immunfluorescent studies of estrogen receptor were performed with a 1:50 dilution of a polyclonal rabbit antibody against a synthetic peptide identical to the C-terminal amino acid fragment of the rat estrogen receptor β (PA1-310, Dianova). The estrogen receptor α was immunolocalized using a 1:50 dilution of a mouse monoclonal antibody directed against the C-terminal domain of ERα (SRA-1010, Biomol, Hamburg, Germany). Antibodies were visualized by a goat anti-rabbit IgG coupled to rhodamine and goat anti-mouse IgG coupled to 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF) (Dianova, Hamburg, Germany). A double immunfluorescent study was carried out by incubating both antibodies at the same time on the same section. Confocal imaging was performed using a Leica TCS 4D laser scan microscope.

Western Blot Analysis: Total cell lysates (40 μg/lane) of each sample were subjected to SDS–PAGE on 10% gels for resolution of ERα/β and on 7.5% gels for resolution of NOS subtypes. Protein was transferred electrophoretically to a nitrocellulose membrane. Equal transfer among lanes was verified by reversible staining with Ponceau red. Immunoblotting was performed with polyclonal antibodies directed against the eNOS and the iNOS isoforms (1:1000; Calbiochem, Bad Soden, Germany). ERα and ERβ were detected with a monoclonal ERα antibody (SRA-1000, 1:500; Biomol) and a polyclonal ERβ antibody (PA1-310, 1:500; Dianova) respectively. To specify additional bands recognized by the iNOS antibody we preincubated the iNOS antibody with an specific anti-iNOS (1131–1144) blocking peptide (Calbiochem). Detection was performed with the enhanced chemiluminescence technique (ECL, Amersham, Braunschweig, Germany). Densitometrical analysis of immunoblots was performed on an Epson GT 8000 scanner with the analysis software ScanPak (Biometra, Göttingen, Germany).

In vivo experiments: Female Wistar Kyoto rats were ovariectomized or sham-operated 12–14 weeks after birth (Harlan Winkelmann, Borchen, Germany). Sham-operated male rats of the same age served as a control. Tissue samples were harvested ten weeks post surgery. Left and right ventricle were separated and rinsed once with ice-cold PBS. The tissue was frozen in liquid nitrogen and stored at −70°C until studied. Left ventricle was powdered with a mortar and pestle in lysate buffer and lysates were clarified as described above for the following western analysis. Serum estrogen levels were measured by a standard RIA following the manufacturerś protocol (DPC Biermann, Bad Nauheim, Germany).

Statistical analysis: All experimental data consist of a minimum of at least three independent experiments from three different animals. All reported values are mean +/− SEM. Statistical comparisons were made by students *t*-test. Statistical significance was assumed, if a null hypothesis could be rejected at the $P < 0.05$ level.

3. Results

3.1. Cardiac myocytes contain functional estrogen receptors of both subtypes

The presence of functional estrogen receptors is required to regulate estrogenic effects in respective target tissues. In a first step, we examined the expression of estrogen receptor α and β protein in ventricular cardiomyocytes obtained from adult female and male Wistar Kyoto rats. To study the cellular distribution of both estrogen receptor subtypes, double immunfluorescent staining of respective receptor proteins was carried out. In the absence of 17β-estradiol, confocal laser scan microscopy revealed a colocalization of the two estrogen receptor subtypes, ERα and ERβ, in the cytoplasm of the respective cardiomyocytes (Fig. 1A and B). In the presence of 17β-estradiol (E2, 10^{-6} M) for 24 h, ERα was detected predominantly in the nucleus, revealing that E2 induced a translocalization of the ER into the nucleus (Fig. 2A). Only a weak fluorescence signal was detected in the nucleus of cells which were cotreated with the ER-specific antagonist ICI 182.780 (10^{-7} M) (Fig. 2B). Stimulation with an E2-BSA conjugate (10^{-6} M, 24 h), which binds to the cellular membrane and does not diffuse intracellularly, did not cause a translocation of the ER into the nucleus (not shown).

The presence of ER protein in cardiomyocytes does not establish unequivocally the ability of these cells to respond directly to estrogen exposure. Therefore, to demonstrate the functional competence of the receptor, a series of immunoblot analyses was carried out to demonstrate estrogen-mediated effects on the expression of ERs. Immunoblot analysis was performed with specific antibodies directed against ERα or ERβ. Total cellular lysates of untreated cardiac myocytes obtained from female and male adult rat heart revealed a basal expression of both receptor subtypes. Stimulation of cardiomyocytes with E2 (10^{-9} M) for 24 h induced a marked increase of estrogen receptor protein (ERα: female 5.2±1.2-fold, male 3.6±0.6-fold; ERβ: female 21.3±3.7-fold, male 15.8±4.2-fold) (Fig. 3). The increase of ER expression could be observed in samples from animals of male and female gender, indicating that the regulation of ER expression by estrogen in the myocardium is gender-independent. In particular, the increase of ERβ protein in isolated adult cardiomyocytes after stimulation with E2 was 4-fold higher compared to the increase of the ERα. Coincubation with the pure antiestrogen ICI 182.780 completely inhibited the upregulation of both ER subtypes by estrogen.

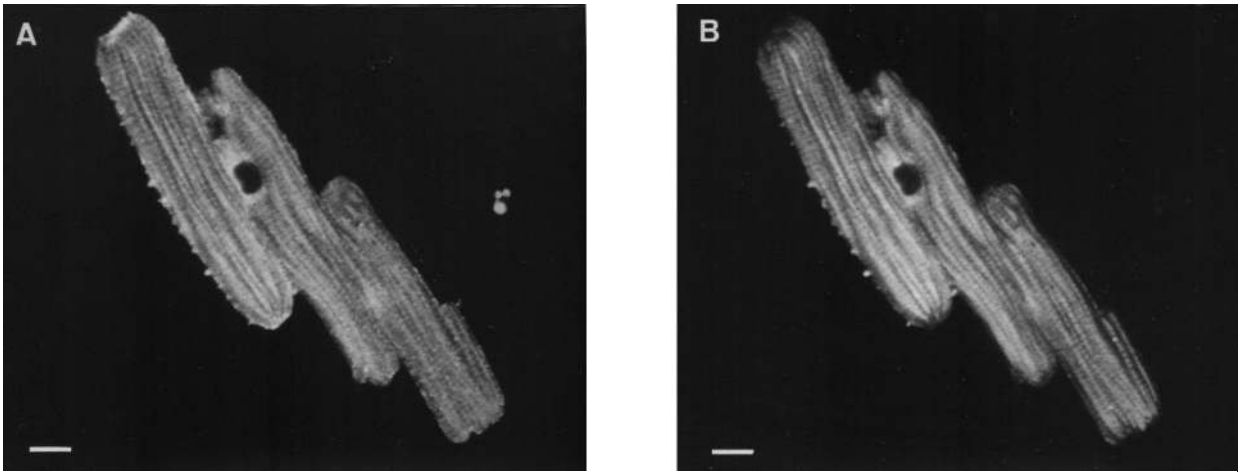

Fig. 1. Double-immunfluorescence labeling for ERα (A) and ERβ (B) in female adult rat cardiomyocytes. Cells cultured on collagen-coated glass coverslides in serum free medium CCT199 were double-immunostained with specific antibodies directed against the ERα (A) and ERβ (B). The subcellular distribution pattern of the receptor subtypes can be compared in the same cells by use of an anti-mouse secondary antibody coupled to DTAF and anti-rabbit antibody coupled to rhodamine. Confocal laser scanning microscopy revealed a colocalized, cytoplasmatic distribution of both ER subtypes in the absence of estrogen. Scale bar: 10 μm.

3.2. 17β-estradiol induced the expression of iNOS and eNOS in isolated cardiomyocytes

Recent observations suggest that NOS might be regulated by estrogen [13]. In an effort to elucidate the underlying mechanisms how NO synthesis in the myocardium is regulated by estrogen, we determined if estrogen stimulates the expression of the endothelial and inducible NO synthase (eNOS/iNOS) in cardiomyocytes. Therefore, we analyzed the expression pattern of eNOS and iNOS in the absence and presence of 17β-estradiol. Immunoblot analyses of lysates from adult and neonatal cardiomyocytes identified a band with a molecular weight of 130 kDa, corresponding to the expected size of iNOS (Fig. 4a) and eNOS (140 kD; Fig. 4b). In the absence of estrogen only a weak signal was detected. Incubation with physiologic concentrations of 17β-estradiol (E2, 10^{-9} M) for 24 h caused a marked increase in the abundance of the respective proteins as shown in compiled bar graphs in Fig. 5a and b (iNOS: 50.1±23.0-fold after 24h; eNOS: 15.7±4.3-fold after 24 h). The increase of eNOS/iNOS expression after stimulation with E2 was found in cardiomyocytes

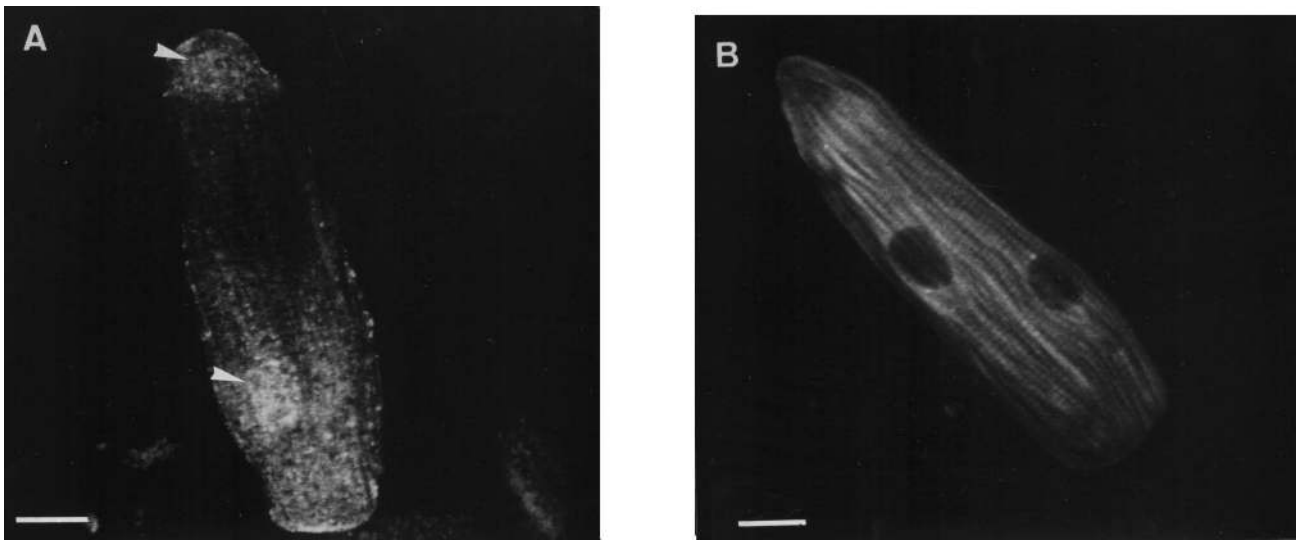

Fig. 2. Nuclear translocalization of the ERα after stimulation with 17β-estradiol. Isolated cardiomyocytes obtained from adult, female rat were cultured as described in Fig.1 but in the presence of 17β-estradiol (10^{-6} M) for 24 h. Cells were immunostained with an antibody directed specifically against the ERα and DTAF-conjugated secondary antibody. In the absence of 17β-estradiol, laser scan micrographs revealed a cytoplasmatic distribution of the ER as shown in Fig.1. After stimulation with 17β-estradiol receptor protein was found predominantly in the nucleus as indicated by arrows (A). Coincubation with the ER-specific antagonist ICI 182.780 inhibited the translocalization of the ER into the nucleus (B). Scale bar: 10 μm.

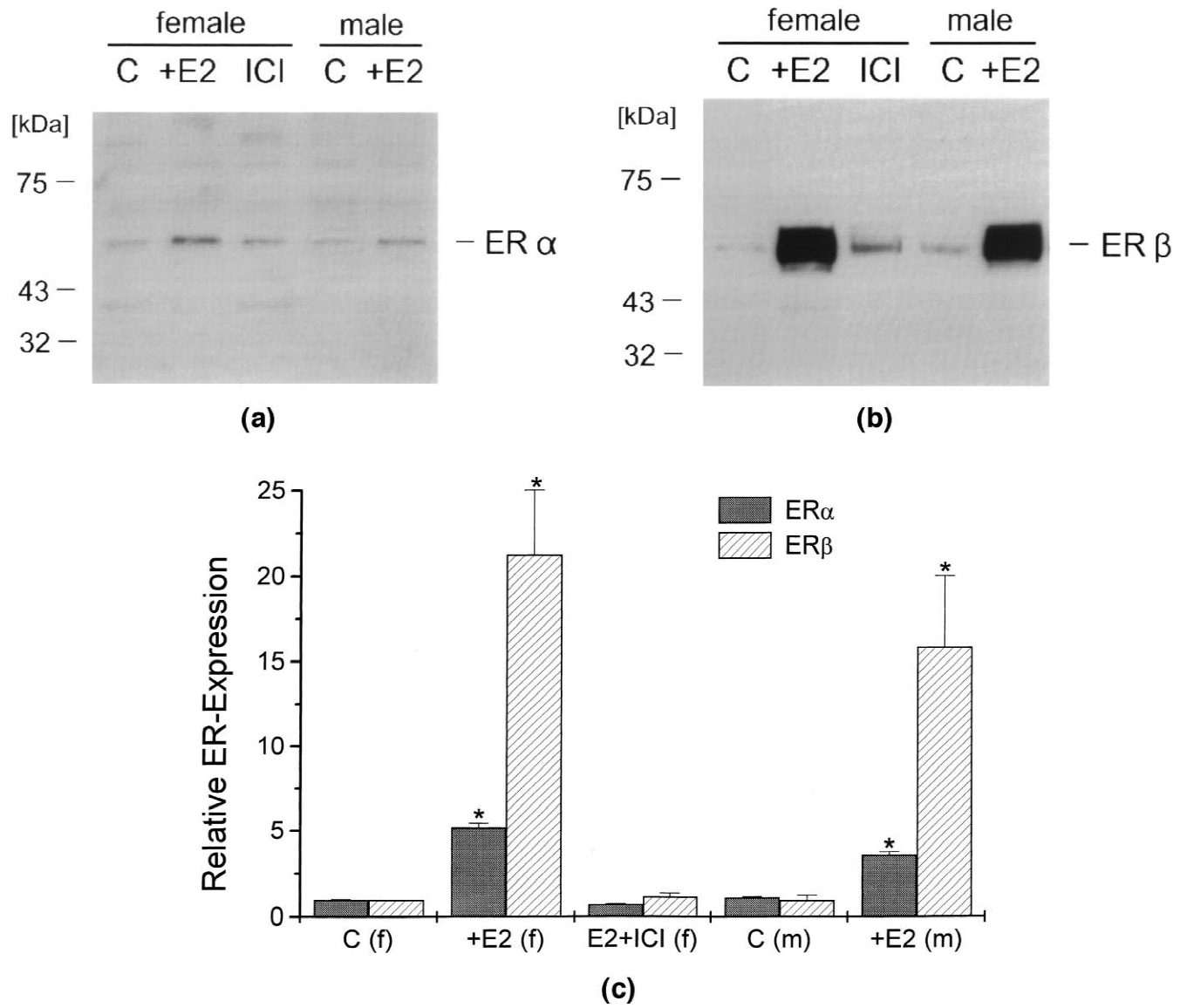

Fig. 3. Cardiac expression of ERα and ERβ protein is stimulated by 17β-estradiol. Cellular lysates of cardiomyocytes obtained from adult female and male rats were subjected to SDS–PAGE (40 μg protein per lane), immunoblotted with an anti-ERα (panel a) and anti-ERβ (panel b) primary antibody and visualized by a chemiluminescence technique. In representative immunoblots shown in panel a and b, only a weak signal at the expected size of 66 kDa (ERα) or 54 kDa (ERβ) could be detected in untreated control cells (c) from female and male animals. After stimulation with 17β-estradiol (E2, 10^{-9} M) for 24 h protein expression of both receptor subtypes increased. Coincubation with the anti-estrogen ICI 182.780 (ICI, 10^{-8} M) inhibited the upregulation of ERα and ERβ protein expression. Densitometrical analysis of three independent experiments are shown in panel c. * $P<0.05$ vs control.

obtained from male and female adult animals as well as in neonatal rats. Cotreatment with ICI 182.780 inhibited the activation of iNOS and eNOS protein expression by E2, which demonstrates that the ER is involved in mediating this process. Taken together, these data show that E2 regulates the expression of both eNOS and iNOS in adult and neonatal rat cardiac myocytes.

3.3. E2 stimulates NO synthase expression in vivo

Another set of experiments was carried out to further elucidate the role of estrogen in the regulation of NO synthases in the myocardium to extend our findings from an isolated cell model. Hearts from ovariectomized or sham-operated female and male adult Wistar-Kyoto rats were harvested ten weeks after surgery. The expression of both iNOS (Fig. 6a) and eNOS (Fig. 6b) was demonstrated by Western Blot analysis in cell lysates obtained from the free left ventricular wall. The expression of eNOS as well as iNOS was significantly higher in sham-operated female animals than in ovariectomized animals (eNOS: 2.1±0.3 fold; iNOS: 1.9±0.2 fold). No significant differences in NOS expression were found in lysates from ovariectomized females compared to male animals. The level of NOS protein decreased in parallel with the reduction of estrogen levels through ovariectomy as shown by measurement of

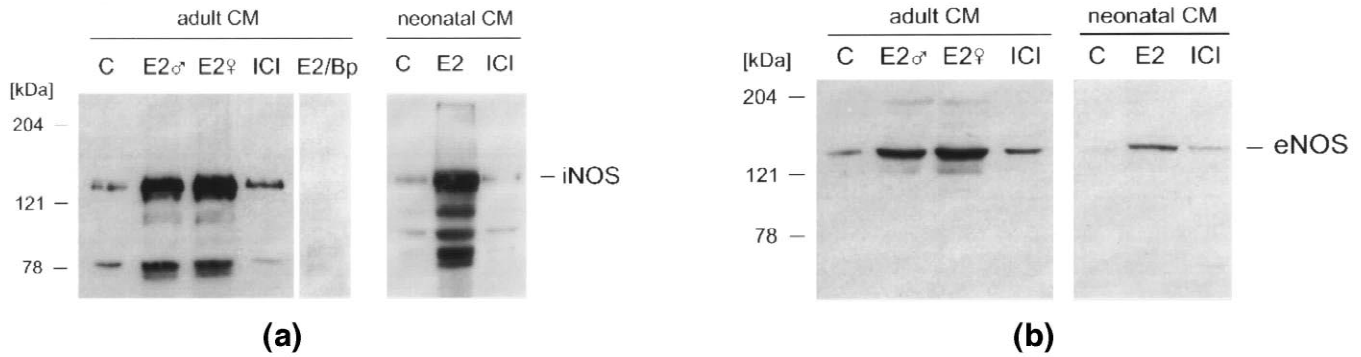

Fig. 4. β-estradiol stimulates iNOS (a) and eNOS (b) expression in isolated cardiomyocytes of adult and neonatal rats. Cardiomyocytes obtained from male and female adult rats and from neonatal rats were cultured in serum-free defined medium in absence or presence of 17β-estradiol (10^{-9} M). Cell lysates of cardiomyocytes (40 μg protein/lane) were subjected to SDS–PAGE, immunoblotted with specific primary antibodies directed against the iNOS protein (panel a) or eNOS protein (panel b), and developed with a chemiluminescence technique. In representative immunoblots shown in panel a and b, the protein was detectable at the expected size of 130 kDa (iNOS, panel a) and 140 kDa (eNOS, panel b). Note that the level of expression of both NOS proteins is low in the absence of 17β-estradiol (Control,C) but increases markedly in cells exposed to 17β-estradiol (E2, 10^{-9} M) for 24 h. Coincubation with ICI 182.780 (ICI) for 24 h inhibited the estrogen mediated increase in NO synthase expression. Other bands seen in the iNOS immunoblot are specific which was shown by a control using a specific iNOS blocking peptide (Bp).

serum levels. Estrogen level measured in sham-operated female rats was 53±35 pg/ml (n=8) compared to 10±3 pg/ml in male rats (n=8) and 5±1 pg/ml in ovariectomized animals (n=8). The specificity of the myocardial iNOS/eNOS expression was assessed by immunofluorescence (data not shown).

4. Discussion

The role of estrogen in gender-based differences found in cardiac disease remains to be clarified despite recent observations that show that estrogen exerts a large array of different genomic and non-genomic effects on both the myocardium and the vasculature including the endothelium [21,24–26]. According to the classical hypothesis cellular effects of estrogen have been attributed to be mediated by intracellular estrogen receptors that serve as transcription factors. The estrogen receptor is expressed in two forms, ERα and ERβ [4,5]. The recently cloned ERβ is highly homologous to the 'classical' ERα and has been shown to bind estrogens with a distinctive affinity and activates transcriptional expression of genes containing estrogen response elements in an estrogen-dependent manner [8].

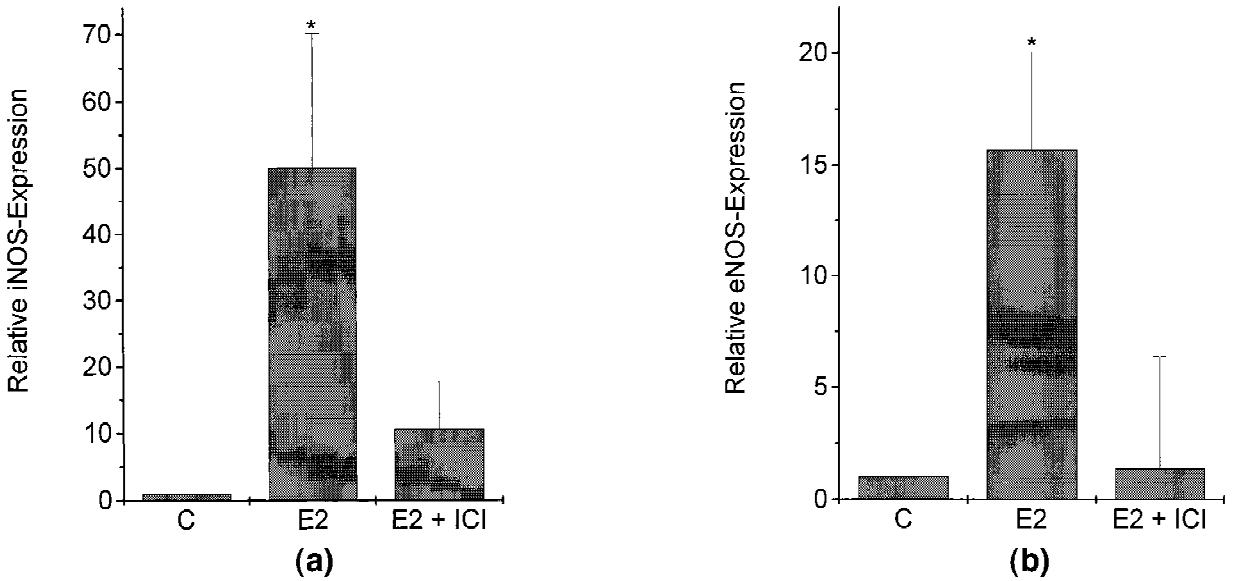

Fig. 5. Average densitometrical analyses of immunoblots representing the expression of iNOS (a) and eNOS (b) in rat adult cardiomyocytes. Summary findings of female adult cells are shown in the compiled bar graphs representing the expression of iNOS (a) and eNOS (b) relative to the untreated controls. Expression was determined by densitometrical analysis of immunoblots and are mean±SEM from three independent experiments from three different animals. * $P<0.05$ vs control.

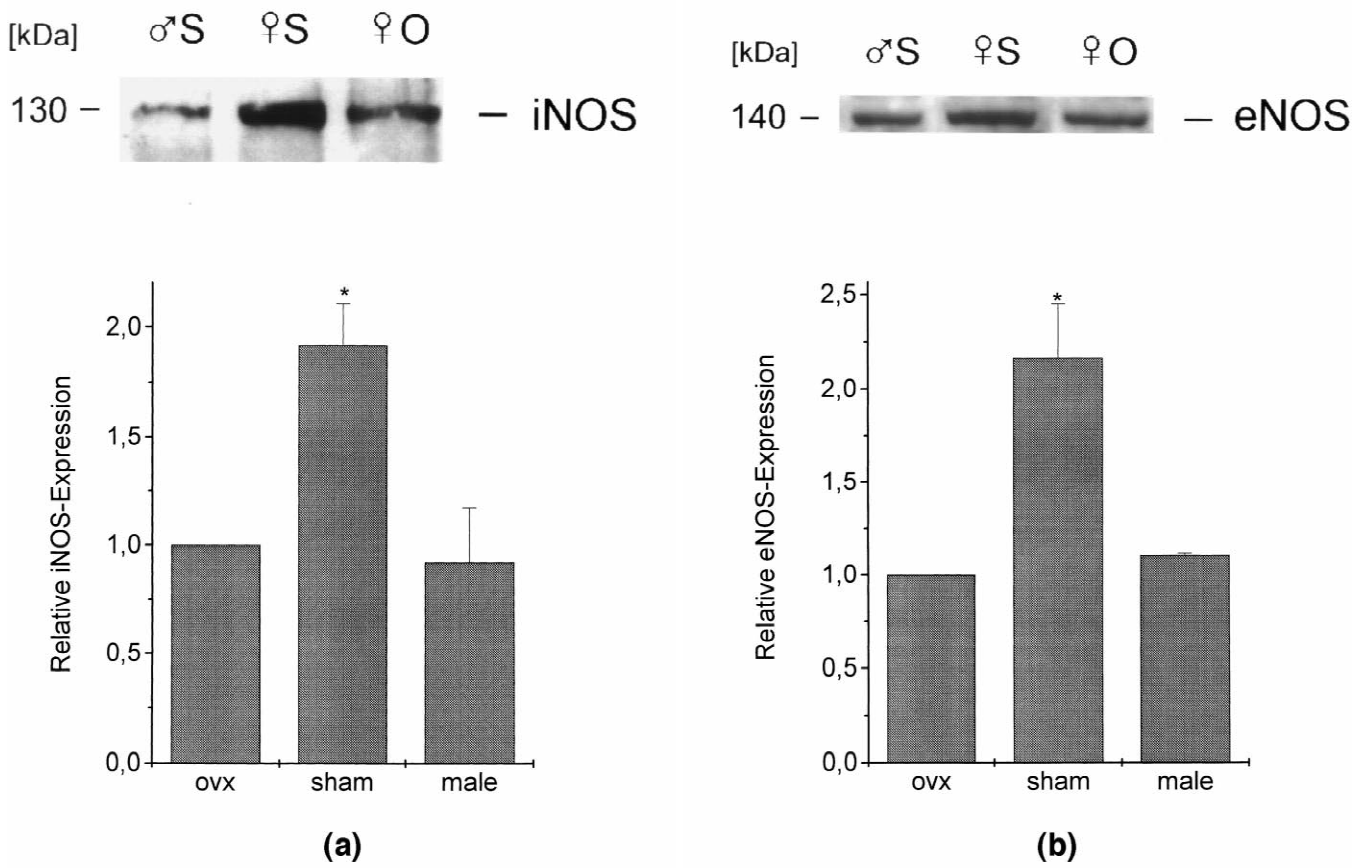

Fig. 6. Expression of iNOS and eNOS in left ventricular myocardium. Protein lysates from left ventricular cardiac tissue obtained from male sham-operated (♂S) and female sham-operated (♀S) and ovariectomized (♀O) rats were prepared 10 weeks after surgery. Western blot analysis was performed as described in Fig.4. Expression of iNOS (section a) and eNOS (section b) protein is significantly increased in lysates obtained from female sham-operated animals compared to ovariectomized or male animals as shown in representative immunoblots. Expression levels found in tissue obtained from male animals are not significant different to samples from ovariectomized animals. Summary findings of three independent experiments are shown in compiled bar graphs below representing the relative iNOS (section a) and eNOS (section b) expression normalized to ovariectomized samples. Expression was determined by densitometrical analysis of immunoblots and are mean±SEM. *$P<0.05$ vs control.

ERα and ERβ are capable of forming homodimers and heterodimers to stimulate downstream target genes [10,11]. This finding and the different transactivation properties at AP1 sites of ERα and ERβ [9] offer a complex pool of potential regulatory mechanisms to modulate cellular responses of the respective target tissue. Therefore, we undertook this study to dissect potential regulatory pathways involved in mediating estrogenic effects in the myocardium. In the present study we demonstrate that adult cardiomyocytes contain functional receptors of both subtypes. Immunofluorescent staining confirmed the presence of receptor protein and further demonstrated the colocalized intracellular distribution pattern of the two receptor subtypes. Protein distribution is altered by estrogen exposure leading to translocalization into the nucleus, a behaviour characteristic of the ER in classical target tissues. Immunoblot experiments did not only demonstrate the presence of ERα and ERβ protein but estrogen exposure markedly stimulated expression of ER in cardiomyocytes, confirming the ability of these cells to respond directly to estrogen. Here we show for the first time that the responsiveness of ERβ expression to estrogen was markedly higher compared to ERα in cardiac myocytes. Given that ERα homodimers and ERα/ERβ heterodimers are preferentially formed over ERβ homodimers [10] our observation suggest that the higher expression level of ERβ in cardiac myocytes results in a higher probability of forming ERα/ERβ heterodimers and ERβ homodimers. Further studies, however, are required to investigate if ERα/ERβ heterodimerization and homodimerization of ERβ plays an important role the transcriptional regulation of cardiac target genes. In vivo this may have a profound effect on the cellular response to E2 and provide a substrate for actions of tissue-selective estrogens.

The relevance of functional estrogen receptors in the adult myocardium is defined by the potential downstream target genes involved in the pathogenesis of heart disease. In this context it has been shown that treatment with E2 increases the activity of calcium-dependent NO synthase (eNOS) in guinea pig heart [13], however the regulation of

calcium-dependent NO synthase and calcium-independent isoform of NOS by estrogen in the myocardium is poorly understood. The physiologic and pathophysiologic roles of NO formation in cardiac diseases are currently under investigation [27,28] and the mechanisms by which NO influences myocardial contractility are the subject of ongoing research but still remain largely unexplored. We therefore investigated the expression of calcium-dependent and calcium-independent NOS in cardiomyocytes in absence and presence of estrogen. Here we show that estrogen regulates the expression of both, eNOS and iNOS, in neonatal and adult cardiac myocytes. Stimulation with physiologic doses of E2 led to a significant upregulation of both isoforms. The complete inhibition of this activation by the anti-estrogen ICI 182.780 demonstrates that this process is due to an genomic effect mediated through cardiac estrogen receptors. The mechanisms of anti-estrogens such as ICI 182.780 have been extensively studied [22,29,30]. Transcriptional activation by the ER is mediated by two separate activation functions (AF-1 and AF-2). Estrogenic agonists as well as antagonists bind to the ligand binding domain of the estrogen receptor. The binding of agonists such as E2 triggers AF-2 activity, whereas the binding of antagonists such as ICI 182.780 does not. As ICI 182.780 is a complete ERα and ERβ antagonist, it inhibits transcriptional activation of these receptors. This implicates that we observed a de novo synthesis of NOS protein not a posttranscriptional activation. ER probably activates eNOS gene transcription by binding to specific estrogen response elements which have been described in the 5′ flanking region of the endothelial NOS gene [31,32]. The increased myocardial expression of both isoforms, eNOS and iNOS, upon stimulation by estrogen reported here extends the findings of MacRitchie showing the upregulation of eNOS mRNA by estrogen in pulmonary artery endothelial cells (PAECs) [33].

To further clarify the role of estrogen in the regulation of NOS in the adult myocardium we carried out an additional set of in vivo experiments. We focused on left ventricular myocardium in these experiments to specifically investigate the organic substrate of pathological conditions such as left ventricular hypertrophy and left heart failure. Although in vivo studies are more difficult to interpret due to the effects of estrogen on the vasculature [12,34,35] we suggest a possible role of cardiac NO production to alter cardiac myocyte function. Our data show that ovariectomy significantly reduced the expression of both, eNOS and iNOS, in the adult left ventricular myocardium compared to sham-operated animals in correspondence to a lower serum estrogen level. Taken together, our data suggest that NO production by either eNOS or iNOS and consequently the activation of the soluble form of guanylate cyclase with a resulting increase in cGMP is influenced by estrogen in vivo and in vitro. NO and cGMP have been found to decrease cardiac myocyte L-type calcium current and myofibrillar contraction through several different mechanisms (for review see [14,15]). Our data contribute to these findings and add a new mechanism how NO might influence cardiac muscle function.

In summary, the experiments presented in this paper demonstrate that estrogen mediates genomic effects in the heart. E2 stimulates the expression of both receptor subtypes, ERα and ERβ, as well as eNOS and iNOS expression in adult myocardium. These novel findings provide a potential mechanism how estrogen may modulate NOS expression and NO formation in the myocardium.

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