Expression of oestrogen receptor α and β in rat heart: role of local oestrogen synthesis

Christian Grohé, Stefan Kahlert, Kerstin Löbbert and Hans Vetter

Medizinische Universitäts-Poliklinik, University of Bonn, 53111 Bonn, Germany

(Requests for offprints should be addressed to Christian Grohé)

Abstract

The role of cardiac oestrogen receptor expression and local oestrogen synthesis in the pathogenesis of cardiovascular disease is poorly understood. Therefore we studied the effects of the oestrogen precursors androstendione and testosterone on the expression of cyp450 aromatase, oestrogen receptor α and β, and inducible NO synthase (iNOS) in neonatal rat cardiac myocytes. Here, we show that cyp450 aromatase is expressed in cardiac myocytes and incubation of cardiac myocytes with oestrogen precursors leads to sexual dimorphic transactivation of an oestrogen-responsive reporter plasmid. Furthermore, incubation with oestrogen precursors stimulated expression of oestrogen receptor α and β, and iNOS in a gender-specific fashion. These data suggest that local oestrogen biosynthesis of the heart is effective to activate oestrogen receptor α and β, and downstream target genes in a gender-based fashion and may therefore contribute to the beneficial effects of oestrogen in the pathogenesis of cardiovascular disease.

Introduction

A large array of cardiovascular diseases such as cardiac hypertrophy or cardiac remodeling after myocardial infarction reveal significant gender-based differences (Dahlberg *et al*.. 1990, Gardin *et al.et al.* 1995, Marcus *et al.* 1994). In this context, the changes of oestrogen plasma levels during the course of life have been attributed to play a role in the pathogenesis of this process. Although recent studies demonstrate that the heart is a target organ for oestrogen (Cabral *et al.* 1988, Malhotra *et al.* 1990, Grohé *et al.* 1996), the precise mechanisms whereby oestrogen may modulate cardiac hypertrophy and remodeling remain unclear. Previously, we have shown that cardiac myocytes and cardiac fibroblasts contain functional oestrogen receptors (Grohé *et al.* 1994, Grohé *et al.* 1997). Furthermore the cloning of a second oestrogen receptor type beta (Kuiper *et al.* 1996) and its expression in adult rat heart (Saunders *et al.* 1997) underline the importance of potential oestrogenic effects in the cardiovascular system. Recent observations suggest that local oestrogen synthesis may play an important role in the regulation of these effects seen in target tissues (Hickey *et al.* 1990). Local oestrogen biosynthesis requires the presence of the aromatase cyp450 enzyme, which metabolizes C_{19} steroids such as androstendione and testosterone to oestrogen by aromatization. Therefore, we sought to determine the expression pattern of cyp450 aromatase in cardiac myocytes and if the presence of oestrogen precursors such as androstendione and testosterone induces oestrogen receptor α and β expression. Previously, the constitutive isoform and the endothelial NO synthase have been identified as oestrogenic downstream target genes in the myocardium (Weiner *et al.* 1994). To further characterize the role of NO synthases in cardiac myocytes, we investigated if the inducible isoform of the NO synthase is also oestrogen-responsive in cardiac myocytes and if oestrogen precursors such as androstendione and testosterone regulate the expression of this NO synthase isoform.

Materials and Methods

Chemicals and solutions

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

Cell culture techniques

Cardiac myocytes from neonatal rat heart were prepared as described before (Grohé *et al.* 1997). Briefly, hearts of 1- to 2-day-old rats (Wistar-Kyoto strain) were isolated and digested in eight consecutive steps (Simpson *et al.* 1982) after separation by gender. 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 ***g*** for 5 min and the cell pellets were resuspended in 20 ml Ham's F10 supplemented with 10% HS and 10% FCS and plated on culture dishes. After 75 min the medium which contained the

Journal of Endocrinology (1998) **156,** R1–R7 © 1998 Journal of Endocrinology Ltd *Printed in Great Britain*
0022–0795/98/0156–00R1 $08.00/0 Accepted 12 December 1997

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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 3×10^4 cells/cm^2 for trans-fection. 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).

Immunoblotting

Lysates of neonatal cardiomyocytes (40 µg/lane) were analyzed by SDS-PAGE in a 7.5% gel and transferred to a nitrocellulose membrane as described before (Grohé *et al.* 1997). Protein content was measured with a standard Bradford assay. Immunoblotting was performed with a polyclonal aromatase antibody (1:500) (Mendelson *et al.* 1985), (kind gift of Dr E R Simpson), iNOS antibody (Calbiochem, Bad Soden, Germany; 1:2000, specific for the iNOS isoform and not crossreacting with the two other known members of the NO synthase family) and the following commercially available antibodies for oestrogen receptor α and β: monoclonal oestrogen receptor α antibody (Bio-mol, Hamburg, Germany, SRA-1000, 1:500) which was produced by immunization of BALB\C mice with a synthetic peptide comprising residues 287-300 (RAANLWPSPLMIKR) from the hinge region of the human oestrogen receptor α; polyclonal oestrogen receptor β antibody produced by immunization of New Zealand white rabbits with a synthetic peptide corresponding to the C-terminal amino acid residues 467-485 (CSSTEDSKNK ESSQNLQSQ) of the rat oestrogen receptor β (Dianova, Hamburg, Germany, PA1-310 1:500). Detection was performed with the Enhanced Chemiluminescence technique (ECL, Amersham). Densitometrical analysis of protein induction was performed on an Epson GT 8000-scanner with the analysis software ScanPak (Biometra, Göttingen, Germany).

Transfection assay

Neonatal rat cardiac myocytes were seeded at a density of 3×10^4 cells/cm² 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) or TK-*LUC* (the same plasmid as ERE-*LUC* but lacking the oestrogen responsive elements; kindly provided by Dr C Glass). After 24 h, the transfection medium was removed and cardiomyocytes were maintained in phenol red-free Ham's-F10 in the absence or presence of testosterone, androstendione and dihydrotestosterone (Sigma Chemicals; St Louis, MO, USA) and/or 4OH-androstendione (CIBA-Geigy, Basel, Switzerland), a competitive inhibitor of aromatase activity. Phenol red-free medium was used throughout all experiments as phenol red is known to act as a weak estrogen (Berthois *et al.* 1986). 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 (DeWet *et al.* 1987). In a subset of each transfection series, cells were transfected with pL7RH-Gal (SV40 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.

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.

Results

To determine the presence of the aromatase cyp450 protein in neonatal cardiac myocytes, a series of immunoblot experiments was performed. Immunoblotting with a monoclonal aromatase antibody detected the 54 kD aromatase protein in lysates derived from neonatal rat cardiomyocytes. The level of protein expression was low in lysates obtained from unstimulated cardiac myocytes. Incubation with 10^{-9} M 17β-oestradiol for 24 h lead to a marked increase in the abundance of aromatase protein (Fig. 1a). Control experiments with lysates derived from adult rat ovary and kidney revealed a band of the expected size in ovarian tissue and no detectable band in kidney tissue. In control experiments in which the primary antibody was omitted no bands were detected (data not shown). These data demonstrate that cyp450 aromatase is present in cardiac myocytes.

After our previous demonstration that cardiac myocytes contain functional oestrogen receptors (Grohé *et al.* 1994) and that cardiac myocytes contain cyp450 aromatase protein, we assessed the functional competence of cardiac myocytes to synthesize oestrogen from serum precursors using transient transfection experiments with a well characterized oestrogen-responsive reporter plasmid, ERE-*LUC* (Glass *et al.* 1988) in cardiac myocytes obtained from neonatal male and female rat heart.

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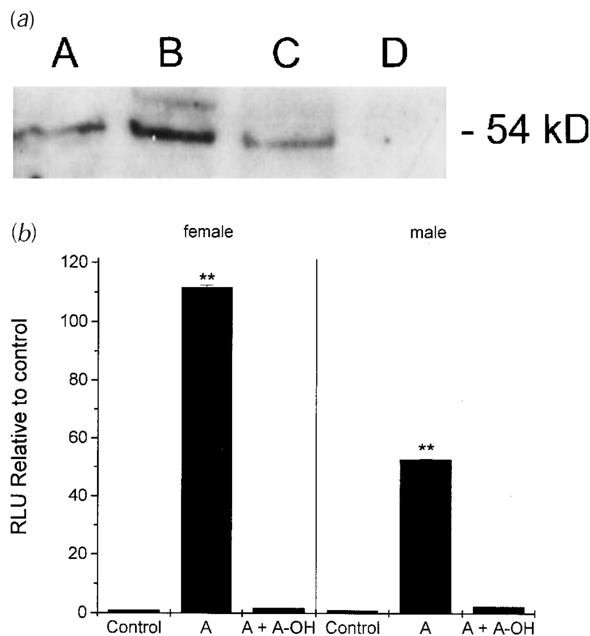

Figure 1 (*a*) Immunoblot analysis shows cyp450 aromatase protein in rat neonatal cardiac myocytes. Total cellular lysates of neonatal rat cardiac myocytes grown in the absence (A) or presence (B) of 10^{-9} M 17 -oestradiol (E_2) were subjected to SDS-PAGE, immunoblotted with an aromatase antibody, and visualized by a chemiluminescence technique. Note that the 54 kD protein is detectable in the absence of E_2, but is more abundant following E_2 treatment. Lysates from ovary (C) and nephrogenic tissue (D) served as positive and negative controls respectively. One of three similar studies is shown. (*b*) Bar graph shows transactivation of an oestrogen-responsive reporter construct after incubation of rat neonatal cardiac myocytes with androstendione. Neonatal cardiac myocytes obtained from female and male rats 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 androstendione (A) (10^{-8} M) or the aromatase inhibitor 4-OH-androstendione (A-OH) (10^{-7} M) and harvested after 24 h. Incubation with dihydrotestosterone (10^{-8} M), which cannot be converted to oestrogen served as a control (data not shown). Bars represent the mean luciferase activity with S.E.M. Luciferase activity is shown relative to control cells that were not exposed to hormones (**P<0.01).

Unpassaged rat cardiomyocytes transfected with ERE-*LUC* showed transactivation of the reporter construct after incubation with androstendione (10^{-8} M) in cells derived from male and female animals (Fig. 1b) in the absence of oestrogen and serum in phenol red free medium (female: 111.7-fold±0.73; male: 52.7-fold±0.27; n=9; P<0.01). Testosterone also stimulated ERE-LUC activation with significantly higher values in female cells compared with male cells (female: 50.39-fold±0.72; male: 20.22-fold±1.01; n=9, P<0.01) (Fig. 2). In contrast, the control plasmid lacking the oestrogen receptor-responsive element (TK-*LUC*) revealed no evidence of hormonal induction (data not shown). Coincubation experiments with the specific aromatase inhibitor 4OH-androstendione, when added to the experimental media, showed an inhibition of ERE-LUC activation by oestrogen precursors therefore demonstrating that aromatization is required to induce the oestrogen-responsive reporter plasmid. To further specify that the activation of ERE-LUC is mediated by aromatization of oestrogen precursors to oestrogen in cardiac myocytes, coincubation experiments with dihydrotestosterone, another product of androstendione and testosterone metabolism, which will not be converted to oestrogen were carried out. Incubation with dihydrotestosterone did not activate the oestrogen-responsive reporter plasmid. Control experiments with the oestrogen receptor antagonist ICI 182,780 completely inhibited the activation of ERE-LUC after incubation with oestrogen precursors (data not shown).

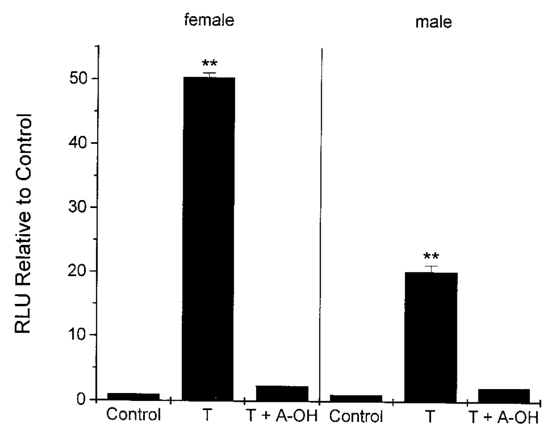

Figure 2 Bar graph shows transactivation of an oestrogen-responsive reporter construct after incubation of rat neonatal cardiac myocytes with testosterone. Neonatal cardiac myocytes obtained from female and male rats were transfected with a reporter plasmid (ERE-*LUC*) in which an oestrogen responsive element drives expression of the firefly luciferase gene. Cells were grown in the absence or presence of testosterone (10^{-8} M) (T) or the aromatase inhibitor 4-OH androstendione (10^{-9} M) (A-OH) and harvested after 24 h. Incubation with dihydrotestosterone (10^{-9} M) , which cannot be converted to estrogen served as a control (data not shown). Bars represent the mean luciferase activity with S.E.M. Luciferase activity is shown relative to control cells that were not exposed to hormones (**P<0.01).

A series of immunoblot analysis was performed to study the effects of oestrogen precursors on endogenous cardiac genes. Western blot analysis with a specific monoclonal oestrogen receptor antibody detected the 66 kD oestrogen receptor α protein in lysates derived from neonatal cardiomyocytes obtained from female and rat cardiac myocytes (Fig. 3a). Although the level of protein expression in the absence of incubation with oestrogen precursors (10^{-9} M) was low, incubation with androstendione (female: 25.2-fold; male: 8.0-fold; n=3, P<0.05) and testosterone (female: 35.7-fold; male: 17.43-fold; n=3, P<0.05), for 24 h lead to a marked increase in

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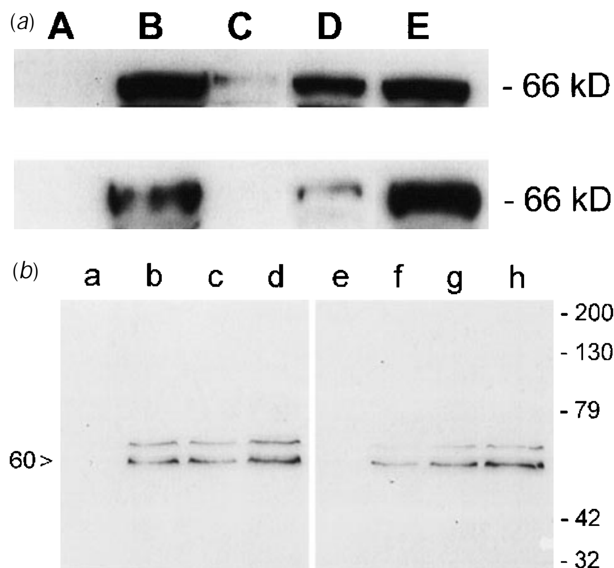

Figure 3 (*a*) Immunoblot shows activation of oestrogen receptor alpha expression by androstendione and testosterone. Total cellular lysates of male and female cardiac myocytes grown in the absence (A) or presence of 10^{-9} M testosterone (upper panel) or androstendione (lower panel) were subjected to SDS-PAGE, immunoblotted with an anti-oestrogen receptor alpha antibody, and developed with a chemiluminescence technique. The 66 kD estrogen receptor (ERα) protein migrated as indicated. Incubation with testosterone induced a 35.7-fold increase in cells of female (B) and a 17.4-fold increase in cells of male (D) origin. Incubation with androstendione induced a 25.2-fold increase in cells of female (B) and an 8.0-fold increase in cells of male (D) origin. Coincubation with the anti-estrogen ICI 182780 inhibited receptor induction (C). Lysate from adult rat ovary served as a positive control (E). One of three similar studies is shown. (*b*) Immunoblot shows activation of oestrogen receptor beta expression in neonatal rat cardiomyocytes by androstendione and testosterone. Total cellular lysates of female (left panel) and male (right panel) cardiac myocytes grown in the absence (a,e) or presence (b,f) of 10^{-9} M 17β-oestradiol, testosterone (c,g) or androstendione (d,h) were subjected to SDS-PAGE, immunoblotted with an anti-oestrogen receptor beta antibody, and developed with a chemiluminescence technique. The 60 kD estrogen receptor (ERβ) protein migrated as indicated. Incubation with testosterone induced a 12.4-fold increase in cells of female and an 11.4-fold increase in cells of male origin. Incubation with androstendione induced a 10.2-fold increase in cells of female and a 7.6-fold increase in cells of male origin. Coincubation with the anti-estrogen ICI 182780 inhibited receptor induction and lysate from adult rat ovary served as a positive control (data not shown). One of three similar studies is shown.

the abundance of oestrogen receptor α protein. Coincubation with an oestrogen receptor antagonist (ICI 182,780) inhibited the activation of the oestrogen receptor α protein after incubation with oestrogen precursors. Furthermore, a series of immunoblot analysis was carried out to determine the expression pattern of the oestrogen receptor in rat neonatal cardiomyocytes. Again, the level of expression of the receptor protein was low in the absence of oestrogen, incubation with androstendione (female: 10.2-fold; male: 7.6-fold; n=3, P<0.05) and testosterone (female: 12.4-fold; male: 11.4-fold; n=3, P<0.05), for 24 h lead to a marked increase in the abundance of oestrogen receptor protein. In contrast to the findings of oestrogen receptor α expression in cardiomyocytes, no significant gender-based difference was found in the expression pattern of oestrogen receptor β in neonatal rat cardiomyocytes. Lysates from adult rat ovary served as a positive control. In control experiments in which the primary antibody was omitted no proteins were detected (data not shown).

To determine whether oestrogen, besides its potential to upregulate the expression of the oestrogen receptor α and β, regulates the expression of downstream target genes, we assessed the level of the inducible NO synthase protein in male and female cardiomyocytes in the absence and presence of androstendione and testosterone. Immunoblot analysis of lysates from rat neonatal cardiomyocytes grown in the presence of androstendione and testosterone (10^{-9} M) identified a band with a molecular weight of 130 kD (Figure 4), corresponding to the expected size of the known isoform of the iNO synthase (Weiner *et al.* 1994). In the absence of oestrogen precursors only a weak signal was detected. Incubation with androstendione (female: 12.5-fold, male: 12.2-fold; n=3, P<0.05) and testosterone (female: 3.7-fold, male: 2.9-fold; n=3, P<0.05), for 24 h lead to a marked increase in the abundance of iNOS protein. Again, coincubation with an oestrogen receptor antagonist (ICI 182,780) inhibited the activation of iNOS protein expression after incubation with oestrogen precursors. Lysates from adult rat ovary served as a positive control. Control experiments in which the primary antibody was omitted were negative (data not shown).

Discussion

Oestrogen has been attributed to play an important role in the modulation of cardiac hypertrophy and cardiac remodeling after myocardial infarction as observations from clinical studies and experimental data from animal models suggest (Scheuer *et al.* 1987, Nabulsi *et al.* 1993, Marcus *et al.* 1994, Mendelsohn *et al.* 1994). These studies implicate that oestrogen may participate in attenuation of cardiac hypertrophy (Cabral *et al.* 1988) and contractile function (Scheuer *et al.* 1987). The steroid hormone family of oestrogens consist of a large array of different metabolites, which display significant differences in their plasma levels before and after the menopause. However, all oestrogen metabolites are synthesized by aromatization of C_{19} steroids such as androstendione and testosterone. We have previously shown that cardiomyocytes and myogenic cells contain functional oestrogen receptors (Grohé *et al.* 1994, Kahlert *et al.* 1997) and activate downstream target genes like c-fos, egr-1 and connexin 43

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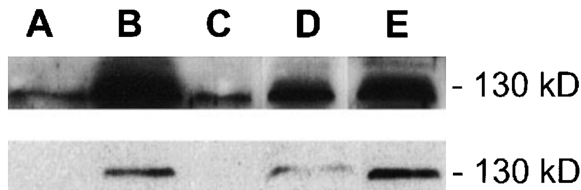

Figure 4 Immunoblot shows activation of inducible NO synthase expression by androstendione and testosterone. Total cellular lysates of male and female cardiac myocytes grown in the absence (A) or presence of 10^{-9} M testosterone (upper panel) or androstendione (lower panel) were subjected to SDS-PAGE, immunoblotted with an anti-NO synthase antibody, and developed with a chemiluminescence technique. The 130 kD iNO synthase protein migrated as indicated. Incubation with testosterone induced a 3.72-fold increase in cells of female (B) and a 2.9-fold increase in cells of male (D) origin. Incubation with androstendione induced a 12.5-fold increase in cells of female and a 12.2-fold increease in cells of male origin. Coincubation with the anti-estrogen ICI 182780 inhibited iNOS protein induction (C). Lysate from adult rat ovary served as a positive control (D). One of three similar studies is shown.

(Grohé *et al.* 1996). The recent cloning of a second oestrogen receptor (ER) called ERβ added another potential regulatory mechanism to explain the effects of oestrogen on the heart (Kuiper *et al.* 1996, Kuiper *et al.* 1997).

To further characterize the role of oestrogen in cardiovascular disease, we undertook this study to investigate if local oestrogen biosynthesis by aromatization of andro-stendione and testosterone may modulate oestrogen receptor expression and activation of oestrogenic downstream target genes in cardiac myocytes. In a first step, we studied the expression pattern of cyp450 aromatase in cardiac myocytes. This enzyme is predominantly expressed in reproductive and adipose tissue and controls the local oestrogen synthesis before and after the menopause (Hickey *et al.* 1990, Simpson *et al.* 1994). We here show that cyp450 aromatase, the enzyme which is responsible for the metabolism of C_{19} steroids to oestrogen is expressed in cardiac myocytes. In a next step, we investigated if cardiac myocytes can transactivate an oestrogen-receptor specific reporter plasmid in the presence of androstendione and testosterone in a gender-specific fashion. The transfection studies show that after incubation with oestrogen precursors, the reporter plasmid is activated in a gender-specific fashion and that incubation with andro-stendione leads to a signifcantly higher induction than testosterone. These data suggest that androstendione is metabolized to oestrogen to a higher degree than testosterone, as testosterone can also be metabolized to dihydrotestosterone by 5α-reductase. The specificity of the transactivation of ERE-LUC by metabolized oestrogen precursors could be demonstrated as control experiments with an aromatase inhibitor (4-OH androstendione) and an oestrogen receptor antagonist (ICI 182780) showed an inhibition of the transactivation of ERE-LUC. In addition, incubation with dihydrotestosterone also yielded no activation of ERE-LUC. Taken together, these data demonstrate that oestrogen precursors can activate an oestrogen-responsive reporter in cardiac myocytes in a gender-based fashion.

Furthermore, we studied if the presence of oestrogen precursors might influence the expression of the oestrogen receptor in the heart. Ostrogen receptor α protein was induced in a gender-specific fashion with a significantly higher expression in female cardiac myocytes compared with lysates obtained from male cardiac myocytes at the same level of stimulation (10^{-9} M). These findings may be explained by the recent observation that transcriptional regulation of steroid hormone receptors are influenced by coactivators and repressors such as RAC3 or SRC, whose expression patterns may display significant gender differences. In contrast to the expression pattern of ERα which is influenced by gender, the expression of ERβ in cardiac myocytes revealed no sexual dimorphic pattern. Interestingly another protein of slightly lower molecular weight was detected in ERβ immunoblots. This may be explained by recent findings from Chu and Fuller (1997) who describe a new splicing variant of ERβ in different tisues including ovary. It may therefore well be that such splicing variants can also be detected in the heart and are part of the differential gene activation machinery. In the light of the observation that ERα and ERβ can form heterodimers to initiate transcription (Cowley *et al.* 1997) and display significant differences in their response to antiestrogens (Paech *et al.* 1997), these regulatory mechanisms may be helpful to explain the complex nature of oestrogenic effects. The dual-specific activation pattern of the two oestrogen receptors in the heart may help to explain gender-based differences found in cardiovascular disease.

Finally, we examined if oestrogen receptor activation stimulated by oestrogen precursors altered the expression pattern of the inducible NO synthase in the heart. The different isoforms of the NO synthase family have been shown to play an important role in cardiac physiology (Balligand & Cannon 1997). The endothelial and the neuronal isoform have been shown to be induced upon stimulation by 17β-oestradiol in guinea pig heart on the mRNA level (Weiner *et al.* 1994, Hayashi *et al.* 1995). However, it remains controversial if the neuronal NO synthase is expressed in rat cardiac myocytes (Balligand & Cannon 1997). We therefore hypothesized that the expression pattern of the inducible isoform may also be modulated by oestrogen in the myocardium. Here we show for the first time that iNOS expression is influenced by oestrogen in a gender-based fashion. In our hands, androstendione was a stronger stimulus than testosterone to upregulate iNOS expression in rat cardiac myocytes. These data add oestrogen to a large array of cytokines and inflammatory paracrine substances which have been shown to modulate iNOs expression. As the mechanisms whereby the different NO

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synthase isoforms and their inhibition modulate cardiovascular disease are currently under investigation (Hu *et al.* 1994, Hou *et al.* 1995), our data contribute to our understanding that the inducible form of the NO synthase family is influenced by oestrogen and possibly by the changing plasma levels of oestrogen and its precursors throughout life.

In summary, our findings that cardiac myocytes can synthesize oestrogen and activate downstream target genes upon stimulation by oestrogen precursors in a gender-based fashion may help to explain the complex mechanisms which regulate the gender-based differences found in many cardiovascular diseases such as cardiac hypertrophy and cardiac remodeling after myocardial infarction.

Acknowledgements

The authors would like to thank Dr E R Simpson for the aromatase antibody and Dr C Glass for plasmids TK-*LUC* and ERE-*LUC*. This work was supported by a grant from the Deutsche Forschungsgemeinschaft Gr729/4-1 and Me 1502/1-1.

The authors would also like to thank Dr A E Wakeling (Zeneca Pharmaceuticals, Cheshire, UK) for sharing the pure anti-estrogen ICI 182780 and Susan Fitzpatrick and Keith Parker for helpful discussions.

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