Activation of estrogen receptor β is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes

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Abstract Physiological effects of estrogen on myocardium are mediated by two intracellular estrogen receptors, ERα and ERβ, that regulate transcription of target genes through binding to specific DNA target sequences. To define the role of ERβ in the transcriptional activation of both endothelial (eNOS) and inducible nitric oxide synthase (iNOS) in cardiac myocytes, we used the complete ERβ-specific antagonist *R*,*R*-tetrahydrochrysene (*R*,*R*-THC). *R*,*R*-THC inhibited activation of iNOS/eNOS promoter-luciferase reporter constructs (iNOS/eNOS-Luc) in a dose-dependent fashion in COS7 cells selectively transfected with ERβ, but failed to influence ERα-mediated increase of iNOS/eNOS-Luc. In neonatal rat cardiomyocytes transfected with eNOS-Luc or iNOS-Luc, incubation with 17β-estradiol (E2, 10^{-8} M) for 24 h stimulated expression of eNOS and iNOS. *R*,*R*-THC (10^{-5} M) completely inhibited this effect. Furthermore, eNOS and iNOS protein expression in cardiac myocytes induced by E2 was completely blocked by *R*,*R*-THC as shown by immunoblot analysis. Taken together, these results show that ERβ mediates transcriptional activation of eNOS and iNOS by E2.

Key words: Estrogen receptor; Nitric oxide synthase; Cardiomyocyte

1. Introduction

Numerous studies underline the importance of estrogenic hormones in gender-based differences found in the pathogenesis of cardiac diseases [1–4]. The regulatory mechanisms of estrogen effects on the myocardium, however, are still poorly understood. Recent studies suggest that nitric oxide (NO) plays an important role in estrogen-mediated effects on myocardium. NO is generated by a family of NO synthases (NOS) which catalyze the conversion of the amino acid L-arginine to citrulline [5]. Estrogen has been shown to increase expression and activity of NOS in the myocardium [6,7], and this may represent a possible mechanism for the protective role of estrogens on the heart. Effects of estrogen are mediated by estrogen receptors (ERs) that act as ligand-activated transcription factors [8,9]. Two different ERs, ERα and ERβ, have been described so far [10,11] and we have shown that both of these receptors are expressed and functional in neonatal and adult cardiac myocytes [7,12]. ERβ seems to be the predominant receptor in the myocardium and thus might be an important transducer of estrogen action. Responsiveness of ERβ expression to 17β-estradiol (E2) in cardiomyocytes is markedly higher compared to ERα [7]. E2 action on myocardium has been shown to induce non-genomic effects [13,14] and to activate a number of target genes via ER [7,12]. The functional distinctions between ERα and ERβ in this process are not known. However, some differences in signaling mechanisms of the ERs have already been revealed. The ability of ER to enhance transcription of target genes has been attributed to binding to classical estrogen response elements (ERE) and to utilizing transactivation functions (AF-1 and AF-2) to recruit coactivator proteins of representatives of the SRC/p160 coactivation protein family [15,16]. AF-1 and AF-2 domains regulate transcription independently and synergistically, depending on the promoter and cellular context [17,18]. Different regulatory functions of ERα and ERβ may be in part due to different AF-1 domains. Studies using receptor chimeras where the A/B region of the ERs, which contain the AF-1 domain, were interchanged have demonstrated that differences in transcriptional potency as well as response to anti-estrogens rely on the nature of this domain [19,20]. Furthermore, ERα and ERβ have different effects on cellular AP-1 activity [21,22] and nuclear factor κB activity [23], in some cases producing opposite effects even within the same cell line. Due to the already published differences between ERα and ERβ and because of the described predominant ERβ responsiveness to E2 in cardiomyocytes, a detailed analysis of the functional distinctions between ERα and ERβ is necessary for a complete understanding of estrogen action in the myocardium.

A separate investigation of the roles of ERα and ERβ in the myocardium has become much easier due to the recent development of novel, non-steroidal ligands that show subtype-selective differences in ligand binding and transcriptional potency for the two ERs. Specifically, it has been shown that the *R*,*R*-enantiomer of tetrahydrochrysene, *R*,*R*-THC, acts as a complete and selective antagonist on ERβ [24]. The mode of action of *R*,*R*-THC to reveal antagonistic properties is in part explained by its quantitative differences in recruitment of coactivator proteins such as SRC-1, -2, -3. This implies that

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while generally similar to estradiol, this ligand induces ER conformations that differ somewhat from that induced by estradiol [24].

In an effort to further characterize the specific role of ERβ in cardiac disease, we used *R,R*-THC as a pharmacological probe to investigate the influence of ERβ on the expression of the inducible and endothelial NOS (iNOS, eNOS) isoforms in neonatal rat cardiac myocytes. Dissecting the distinctive role of the ER subtypes in the selected target tissues such as the cardiovascular system has important implications in the use of receptor-specific antagonists and agonists in clinical medicine. These clinical implications have been recently underlined in the observation by Weihua et al. [47], which showed the importance of ERβ regulation in other sex hormone-responsive tissues such as the prostate. In this report, we show that ERβ is a prerequisite for E2-induced increase of eNOS and iNOS expression on the transcriptional and translational level in cardiac myocytes.

2. Materials and methods

2.1. Materials

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

2.2. Cell culture

COS7 were used to selectively express transfected ERs as this cell line expresses neither ERα nor ERβ. COS7 were obtained from American Type Culture Collection (Manassas, VA). COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Karlsruhe, Germany) supplemented with 10% charcoal-stripped, estrogen-free fetal calf serum (FCS; c.c.pro, Hamburg, Germany). All media contained 25 μg/ml gentamicin (Life Technologies). Phenol red-free medium was used throughout all experiments as phenol red is known to act as a weak estrogen [25].

2.3. Isolation and culture of neonatal cardiomyocytes

Procedures with experimental animals followed the guidelines of the German animal protection law. The hearts of 1–2-day-old male and female rats (Wistar–Kyoto strain) 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 [13]. Cell suspension was centrifuged at $400\times g$ for 5 min and the cell pellet was resuspended in 20 ml of Ham's F10 supplemented with 10% horse serum (Biochrom, Berlin, Germany) and 10% estrogen-free 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 at a density of $\sim 2\times 10^4$ cells/cm^2 in phenol red-free DMEM supplemented with 10% charcoal-stripped, estrogen-free FCS.

2.4. Treatment of COS7 and cardiomyocytes

Serum-starved cells were treated with E2 (10^{-7}–10^{-10} M) ± *R,R*-THC (10^{-5}–10^{-7} M) for 24 h. Control cells were incubated with 0.1% (v/v) ethanol, the solvent of E2. Additional controls were coincubated with 10^{-8} M ICI 182.780 (Tocris, Bristol, UK), a specific inhibitor for the two known ERs [26].

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 phenylmethylsulfonyl fluoride, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were clarified by centrifugation at $12\,000\times g$ for 10 min at 4°C. Protein content was measured with a standard Bradford assay.

2.5. Western blot analysis

Total cell lysates (40 μg/lane) of each sample were subjected to SDS–PAGE on 7.5% gels for resolution of eNOS and iNOS. Protein was transferred electrophoretically to nitrocellulose membranes. Equal transfer among lanes was verified by reversible staining with Ponceau red. Immunoblotting was performed with antibodies specific for eNOS (rabbit polyclonal, 1:1000, #482726, Calbiochem, Bad Soden, Germany) and iNOS (rabbit polyclonal, 1:2000, #482728, Calbiochem). Detection was performed with the Enhanced Chemiluminescence technique (NEN Life Science, Cologne, Germany). Densitometric analysis of immunoblots was performed with the Gel Doc 1000 imaging system using the analysis software Multi-Analyst (Bio-Rad Laboratories, Munich, Germany).

2.6. Transient transfections

COS7 cells and neonatal cardiomyocytes were grown to an approximate density of 70% and transfected using a liposome-conjugated transfection technique according to the manufacturer's instructions (DOTAP; Roche Diagnostics, Mannheim, Germany). COS7 cells were transiently transfected with the respective ER expression vectors (human ERα, HEG0, kind gift from Dr. P. Chambon and rat ERβ, pCMV29, kind gift from Dr. G. Kuiper) and the reporter plasmids ERE-Luc that contain three copies of an estrogen-responsive element from the *Xenopus* vitellogenin gene linked to a luciferase gene under control of a thymine kinase promoter (kindly provided by Dr. C. Glass). Additionally cells were cotransfected with an eNOS promoter construct containing 1.6 kb upstream of the human eNOS gene (eNOS-Luc, kindly provided by Dr. C.J. Lowenstein) [27] or an iNOS promoter containing 1122 kb upstream of human iNOS gene (iNOS-Luc, kindly provided by Dr. N.E. Madias) cloned upstream of a luciferase reporter gene in a pGL2-Basic plasmid [28]. 2 μg total DNA per assay was found to give maximal efficiency. As an internal control of transfection efficiency 2 ng of β-galactosidase expression plasmid pL7RH-Gal was cotransfected. After transfection cells were washed, medium was changed to serum-free with addition of E2 ± *R,R*-THC as described in Section 2.4. Cells were lysed 48 h after transfection and assayed for luciferase and β-galactosidase activity as described previously [29].

3. Results

Although it has been shown before that E2 stimulates the expression of eNOS and iNOS in cardiac myocytes [7], the specific roles of ERα and ERβ in this process are not known. In order to define the influence of both ERs on the transcriptional regulation of NOS we used the ER subtype-selective ligand *R,R*-THC.

As an initial step we examined the transactivation properties of ERα and ERβ at a classical ERE in response to *R,R*-THC. COS7 cells which are devoid of ERs were cotransfected with expression plasmids for ERα or ERβ together with a reporter plasmid that contained an ERE linked to luciferase (ERE-Luc). These cells were treated with increasing concentrations of *R,R*-THC, or with E2 for comparison. Maximal transactivation of ERE-Luc in ERβ- and in ERα-transfected cells was seen at a dose of 10^{-8} M E2. *R,R*-THC did not show any agonistic activity in the presence of ERα in COS7 cells and did not influence E2-mediated transactivation by ERα (Fig. 1A). Although agonistic properties of *R,R*-THC on ERα have been shown in HEC-1 and CHO cells [24,30], in COS7 cells we could not observe any agonistic activity on ERα. These differences in agonistic properties of *R,R*-THC may depend greatly on the cellular background and promoter context and/or on different protocols used in cell and ligand treatment. In ERβ-transfected cells *R,R*-THC clearly exhibited antagonistic activity in a dose-dependent manner and completely inhibited E2-mediated transcriptional activation by ERβ at a dose of 10^{-5} M (Fig. 1B).

To control whether *R,R*-THC is also capable of influencing the E2-dependent iNOS and eNOS transactivation, COS7 cells were selectively transfected with expression plasmids for

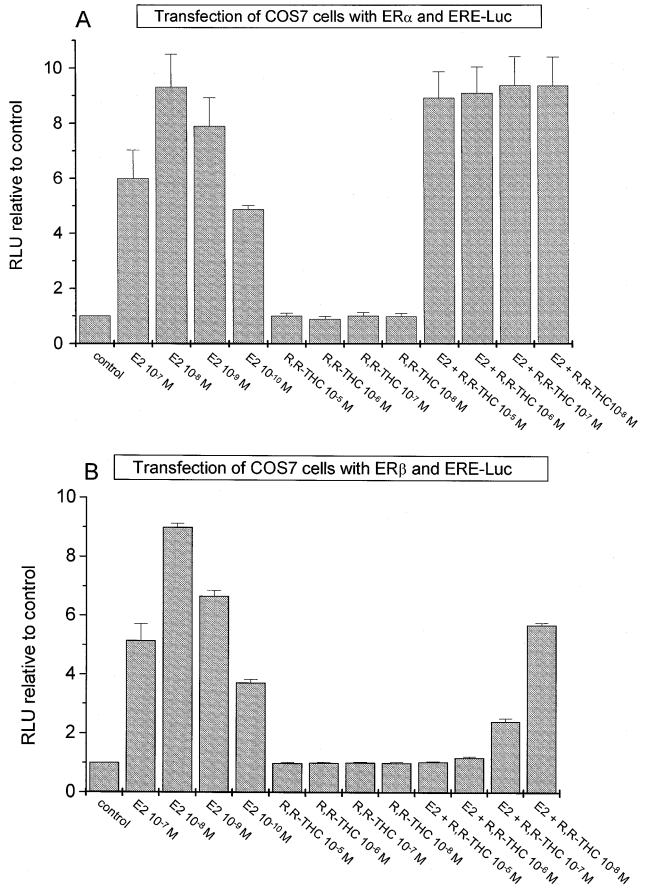

Fig. 1. Transcriptional activation of ERE-Luc by ERα (A) and ERβ (B) in response to E2 and *R,R*-THC in COS7 cells. ER-negative COS7 cells were cotransfected with either ERα or ERβ expression vectors together with an estrogen-responsive reporter gene (ERE-Luc) and treated with E2 and *R,R*-THC for 24 h in increasing concentrations. Additionally cells were cotreated with E2 (10^{-8} M) and increasing concentrations of *R,R*-THC (10^{-8}–10^{-5} M). Luc activity was determined and plotted as fold activation over the individual Luc activity measured in cells treated with vehicle alone. Values represent the mean ± S.E.M. for at least three independent transfections performed in triplicate.

ERα or ERβ and for iNOS-Luc or eNOS-Luc promoters as reporter constructs. These cells were incubated with E2 or vehicle alone for 24 h (Fig. 2). E2 (10^{-8} M) stimulated the expression of iNOS-Luc (maximum: 8.38 ± 0.02-fold compared to vehicle-treated control) and eNOS-Luc (maximum: 7.87 ± 0.02-fold compared to vehicle-treated control) in the presence of ERα as well as in presence of ERβ. Coincubation with *R,R*-THC inhibited activation of iNOS-Luc and eNOS-Luc expression in a dose-dependent fashion in cells selectively transfected with ERβ, but failed to influence ERα-mediated increase of iNOS/eNOS-Luc. Complete inhibition of both eNOS-Luc and iNOS-Luc expression in ERβ-transfected cells was obtained at a dose of 10^{-5} M. In cells which were cotreated with ICI 182.780 (10^{-8} M), a specific inhibitor of the two known ERs, activation of the reporter constructs was also prevented. Neither *R,R*-THC nor ICI 182.780 could transactivate the promoter constructs. In the absence of ERα or ERβ activation of iNOS-Luc and eNOS-Luc was low and could not be increased by E2 (data not shown).

After the demonstration that *R,R*-THC acts as a complete ERβ antagonist and selectively inhibits ERβ-mediated transactivation of iNOS-Luc and eNOS-Luc in a dose-dependent fashion, we used neonatal cardiomyocytes to describe the role of ERβ in the heart.

Neonatal rat cardiac myocytes which are known to express both ERα and ERβ were transfected with iNOS-Luc and eNOS-Luc and incubated with E2 (10^{-8} M) for 24 h. E2 stimulated the expression of eNOS-Luc (maximum: 9.46 ± 0.09-fold) (Fig. 3A) and iNOS-Luc (maximum: 9.41 ± 0.06-fold) expression (Fig. 3B). Coincubation with *R,R*-THC (10^{-5}–10^{-7} M) inhibited the transactivation of both promoter constructs in a dose-dependent fashion suggesting a specific role for ERβ in E2-mediated activation of NOS. Treatment with *R,R*-THC alone had no effect on eNOS and iNOS promoter constructs. Control experiments using ICI 182.780 (10^{-8} M) demonstrated a complete inhibition of E2-mediated activation of both reporter constructs in cardiac myocytes.

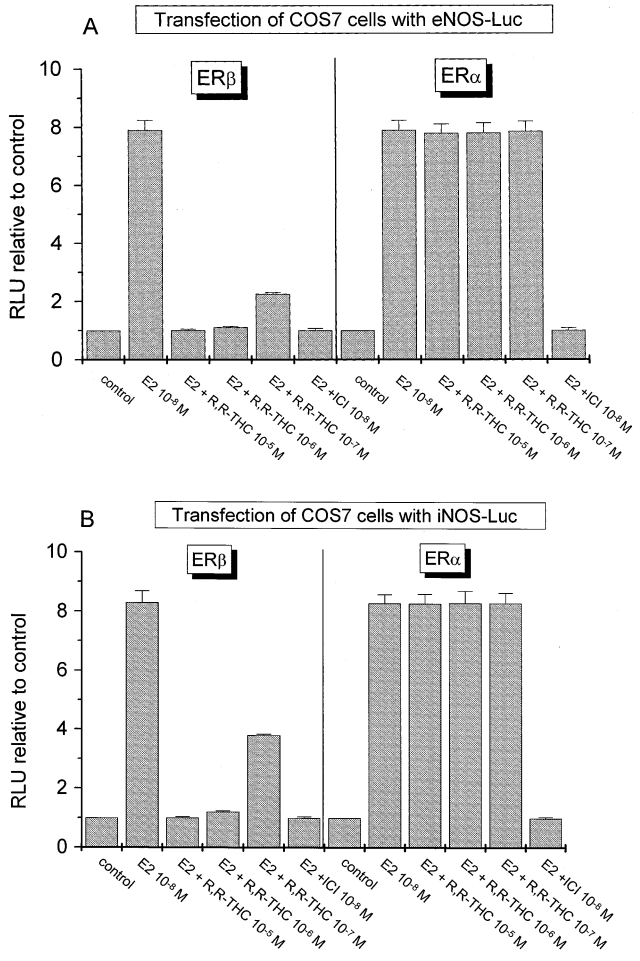

Fig. 2. Transcriptional activation of eNOS and iNOS promoter-luciferase reporter constructs by ERα and ERβ in response to E2 and *R,R*-THC in COS7 cells. ER-negative COS7 cells were cotransfected with either ERα or ERβ expression vectors together with an eNOS or iNOS promoter reporter gene (A: eNOS-Luc, B: iNOS-Luc) and treated with E2 (10^{-8} M) and *R,R*-THC for 24 h in increasing concentrations (10^{-5}–10^{-7} M). Additional cells were cotreated with the complete ER antagonist ICI 182.780 (ICI, 10^{-8} M). Luc activity was determined and plotted as fold activation over the individual Luc activity measured in cells treated with vehicle alone. Values represent the mean ± S.E.M. for at least three independent transfections performed in triplicate.

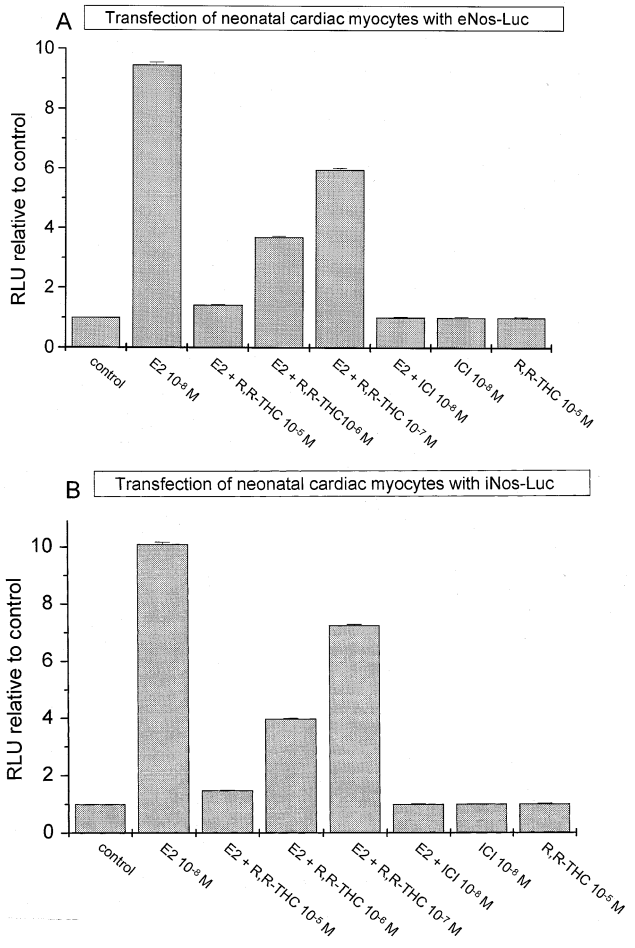

Fig. 3. Transcriptional activation of eNOS and iNOS promoter-luciferase reporter constructs in response to E2 is inhibited by *R,R*-THC in neonatal rat cardiac myocytes. Neonatal cardiac myocytes which contain both ER subtypes were transfected with an eNOS or iNOS promoter reporter gene (A: eNOS-Luc, B: iNOS-Luc) and treated with E2 (10^{-8} M) alone and together with *R,R*-THC for 24 h in increasing concentrations (E2: 10^{-8} M, THC: 10^{-5}–10^{-7} M). Additional cells were cotreated with the complete ER antagonist ICI 182.780 (ICI, 10^{-8} M). Luc activity was determined and plotted as fold activation over the individual Luc activity measured in cells treated with vehicle alone. Values represent the mean ± S.E.M. for at least three independent transfections performed in triplicate.

To determine if ERβ-mediated effects on the iNOS and eNOS promoter were present also on the protein level an immunoblot analysis was performed. We therefore analyzed the expression pattern of eNOS and iNOS in the absence and presence of E2 and *R,R*-THC in cardiac myocytes. Immunoblot analyses of lysates from cardiomyocytes identified a band with a molecular weight of 140 kDa, corresponding to the expected size of eNOS (Fig. 4A) and iNOS (130 kDa; Fig. 4B). In the absence of estrogen a signal was detected, but incubation with physiological concentrations of E2 (10^{-8} M) for 24 h led to a marked increase in the abundance of the respective proteins (iNOS: 2.3 ± 0.59-fold after 24 h; eNOS: 2.15 ± 0.24-fold after 24 h). Cotreatment with *R,R*-THC inhibited activation of protein expression of eNOS as well as iNOS. Coincubation with the specific pure estrogen antagonist ICI 182.780 also inhibited the upregulation of both NOS proteins by E2. Treatment with *R,R*-THC and ICI 182.780 alone did not affect the expression of eNOS and iNOS.

4. Discussion

The ability of the two estrogen receptor subtypes to display distinct or even opposing transcriptional activities is an emerging paradigm in estrogen signaling. Although ERα and ERβ share a high degree of amino acid homology there are major differences between these receptors for instance with respect to their tissue distribution, their ligand binding specificity and also with respect to the phenotype of the corresponding knock-out mice [31–33]. Both receptors are ex-

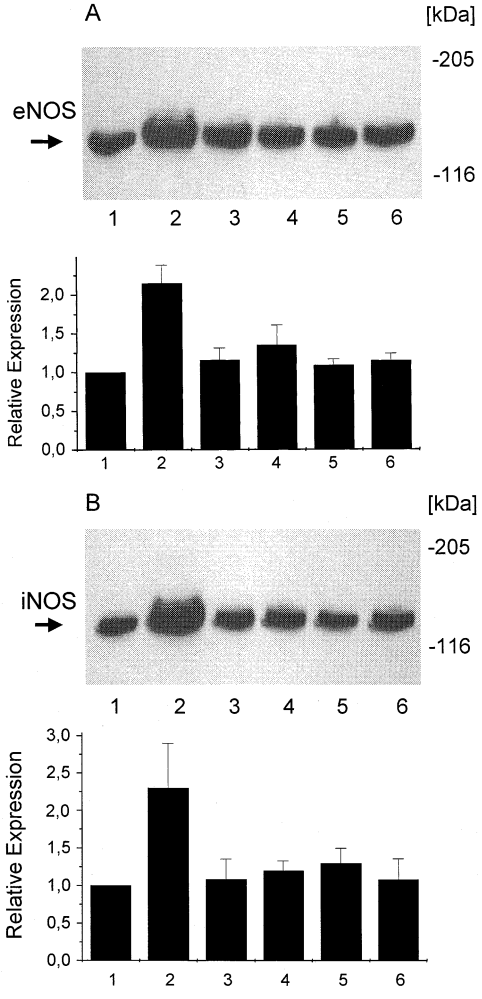

Fig. 4. *R,R*-THC inhibits E2-stimulated eNOS and iNOS expression in neonatal rat cardiac myocytes. Neonatal rat cardiac myocytes were cultured in serum-free defined medium in the absence or presence of E2 (10^{-8} M). Cell lysates of cardiomyocytes (40 μg protein/lane) were subjected to SDS–PAGE and immunoblotted with specific primary antibodies directed against the eNOS protein (A) and iNOS protein (B). In representative immunoblots shown in 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 E2 (Control, 1) but increases markedly in cells exposed to E2 (10^{-9} M) for 24 h (2). Coincubation with *R,R*-THC (10^{-5} M) completely inhibited E2-induced expression of eNOS and iNOS (3). Cotreatment with ICI 182.780 (10^{-8} M) for 24 h also inhibited the estrogen-mediated increase in NOS expression (4). *R,R*-THC (5) and ICI 182.780 (6) alone had no effect on protein expression. Summary findings are shown in the compiled bar graphs representing the expression of iNOS and eNOS relative to the untreated controls. Expression was determined by densitometric analysis of immunoblots and results are mean ± S.E.M. from three independent experiments.

pressed and functional in cardiac tissue [12,13], supporting a direct role for estrogen in cardiac physiology.

In the present work we show for the first time different transactivation properties between the two receptors in a cellular background which contains both receptors. We were able to demonstrate that NOS, as important physiological estrogen target genes in the heart, are regulated via ERβ. In particular, our data show that endogenous ERβ is necessary to mediate an upregulation of iNOS and eNOS in neonatal rat cardiac myocytes. The promoters of iNOS and eNOS have been cloned and analyzed in detail. This analysis revealed the existence of several half-palindromic ER binding sequences on the promoter [34,35], which make it plausible that estrogen can transcriptionally regulate NOS genes, leading to enhanced NO production. NO has been claimed to influence a variety of physiological parameters in the heart such as contraction, relaxation and heart rate [36–39].

Up to now the investigation of how ERα and ERβ contribute to the transcriptional activation of eNOS and iNOS has been hampered by the lack of specific ERβ antagonists. Therefore, the recent development the a new non-steroidal ligand *R*,*R*-THC, which acts as an ERβ-specific antagonist, allows us to further dissect the respective roles of the ERs in this process.

The molecular basis underlying the reported different transcriptional properties between the ERs is poorly understood at the present time. ERβ displays extensive sequence similarity to ERα, however there are significant differences in the C-terminal ligand binding domain and in the N-terminal transactivation domain [11]. These structural differences probably account for differential binding of several estrogenic chemicals and several phytoestrogens to ERα and ERβ protein [40] and may also account for different transcriptional activity. Transcriptional activation is strongly influenced by cell and promoter context [19]. Therefore it is to be expected that the two receptors might have distinct functions and interact with different sets of proteins such as SRC/p160 or MAD2, which has been identified as a protein that interacts specifically with ERβ but not with ERα [41]. Furthermore, ERβ has been identified to play an important role in the control of growth by an interaction with other nuclear receptors [47], which demonstrates the need to investigate the tissue-specific properties of ER subtypes in a hormone-sensitive cellular context which expresses both receptors endogenously.

In our experiments, inhibition of ERβ by *R*,*R*-THC prevented upregulation of eNOS and iNOS expression. What does that mean in the context of ER signaling? The antagonistic action of *R*,*R*-THC on ERβ will prevent activated homodimers of ERβ and will prevent an activated ERβ component in heterodimers. As *R*,*R*-THC blocked the upregulation of iNOS and eNOS expression in cardiac myocytes, ERα homodimers do not seem to be able to induce this expression. Thus heterodimers containing activated ERβ or ERβ homodimers seem to be necessary in cardiomyocytes to activate E2-induced NOS expression. Coexpression of ERα and ERβ results in the preferential formation of heterodimers instead of homodimers [42,43]. It has been shown that transcriptional activation could occur if only one of the ER subtypes within the heterodimer was bound by E2 [44]. Colocalization and subsequent heterodimerization in cardiomyocytes may therefore result in receptor activity distinct from that of homodimers. In accordance with our observations, alterations in transcriptional activity resulting from coexpression of ERα and ERβ have been described recently, and ERβ has been found to act as a dominant regulator of estrogen signaling [45,46].

The use of *R*,*R*-THC has been shown to be instrumental in the dissection of the role of ER subtypes in tissues which express both receptor subtypes endogenously. In this context it is important to note that the ERβ bound with *R*,*R*-THC fails to recruit p160 coactivator proteins [30]. The recruitment of different coactivator proteins in selective target tissues adds a new dimension in the regulatory network involved in response to estrogen. The extent of conformational changes induced by the different use of different ligands and the ability to recruit different coactivator proteins of ER subtypes plays an increasing role in the regulatory network of hormone action. The effort to understand the complexity of estrogenic action on the myocardium is substantiated by the use of these novel ligand compounds with their ability to promote transactivation and coactivator recruitment. The understanding of these mechanisms is critical and these observations will ultimately lead to the use of receptor-specific selective estrogen receptor modulators in clinical medicine.

In conclusion, we demonstrated that in cardiac myocytes ERβ is essential to regulate the activity of NOS. However, the particular roles of ERα and ERβ homo- and heterodimerization and their distinct pattern in coactivator recruitment, which ultimately leads to NOS activation in the myocardium, remain to be further investigated. We suggest that coexpression of both receptors may lead to a regulatory mechanism with antagonist and agonist features. This principle may well play an important role in the cellular response to physiological and pathophysiological responses such as oxidative stress or ischemia in the myocardium. These findings may help to elucidate the distinct roles of ERα and ERβ in the pathogenesis of gender-based differences found in cardiac disease.

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