Expression of estrogen receptor subtypes and neuronal nitric oxide synthase in neutrophils from women and men: Regulation by estrogen

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Abstract

Objectives: (a) To identify the subtype of estrogen receptor (ER) expressed in neutrophils from premenopausal women and in neutrophils from men under different estrogen conditions and (b) to analyze the association between the modifications in the expression of ER subtypes and neuronal nitric oxide synthase (nNOS) expression induced by estrogen. Methods: Neutrophils were isolated from pre-menopausal women during different stages of the menstrual cycle and from ten men for in vitro estrogen incubations. Results: Neutrophils from premenopausal women expressed both ERα and ERβ subtypes which were increased in the ovulatory phase of the menstrual cycle. Neutrophils derived from men also expressed ERα and ERβ but only ERα expression was enhanced by in vitro incubation with 17β-estradiol (10⁻⁸ mol/l). In vitro incubation of neutrophils from women with 17β-estradiol enhanced expression of both ER-α and ER-β subtypes. nNOS protein was overexpressed in neutrophils from premenopausal women during the ovulatory phase. 17β-Estradiol (10⁻⁸ mol/l) also increased nNOS protein expression in neutrophils derived from men. Mithramycin A (10⁻⁶ mol/l) and curcumin (10⁻⁶ mol/l), prevented the upregulation of nNOS and ERα in neutrophils derived from men, suggesting the involvement of AP-1 and Sp-1 transcription factors. Conclusions: Although the in vivo levels of circulating estrogen concentrations seem to be associated with overexpression of both ERα and ERβ in neutrophils from premenopausal women, which was further confirmed by the in vitro experiments with neutrophils from women, in vitro incubation of neutrophils from men with 17β-estradiol only increased ERα protein expression which was associated with enhanced expression of nNOS protein.

Keywords: Gender; Gene expression; Leukocyte; Nitric Oxide; Receptors

This article is referred to in the Editorial by X.-J. Du (pages 4–7) in this issue.

1. Introduction

Death from cardiovascular disease is relatively lower in premenopausal women compared with age-matched men [1]. However, after menopause, the risk of coronary heart disease increases dramatically [2,3]. Several reports have suggested that hormone replacement therapy reduces cardiovascular disease in postmenopausal women, although the data are controversial and the final causative mechanism remains unclear [4,5]. Traditionally, the major mechanisms responsible for the protective effect of female gender is believed to be due to an antiatherogenic action of estrogen on the lipid profile. More recently Ranki and co-workers [6,8] and Jovanovic et al. [7] have demonstrated that estrogens protect cardiac cells to metabolic injury in a gender and ageing-specific manner. However, new data suggest that estrogen may also exert its beneficial effects through a direct action on the vascular wall (for review, see Ref. [9]).

Time for primary review 14 days.
At present, there are two estrogen receptors (ER), identified ERα and ERβ, both of which are members of the superfamily of steroid hormone receptors. ERα and ERβ have considerable homology and, like all steroid hormone receptors, are transcription factors that alter gene expression when they are activated. Both ER form homodimers and may form heterodimers with each other, which have been shown to be active in experimental systems [10].

In spite of the growing interest in the action of estrogen on the endothelium less is known about the effect of estrogen on other cells activated in cardiovascular diseases such as neutrophils. Binding studies performed by Klebanoff [11] demonstrated the presence of ER on neutrophils. Moreover, we recently demonstrated that 17β-estradiol stimulated the expression of the neuronal nitric oxide synthase isoform (nNOS) in human neutrophils by an ER-dependent mechanism [12]. The increased expression of nNOS induced by 17β-estradiol in human neutrophils was associated with a reduction in their adhesive properties and favoured the antiplatelet effect of neutrophils [12,13]. However, at present there are no evidences about the type of ER expressed in human neutrophils. In this regard, although a great deal of progress has been made in cloning ER subtypes, very little is known about control of the expression of the ER subtypes. In this sense, estrogen itself has been demonstrated to upregulate ER expression in neonatal rats, ovine uterine cells and ewes endometrium and downregulate them in ewes liver and MCF-7 breast cancer cells under conditions of limited cell growth [14–18]. However, to date it is not known if estrogen regulates ER expression in human neutrophils. Therefore, the aims of the present study were (a) to identify the different ER subtypes expressed in neutrophils from premenopausal women and from healthy men, (b) to determine the involvement of estrogen in the regulation of their own ER, and (c) to analyze the association between the modifications in the expression of ER subtypes and nNOS expression induced by estrogen.

The molecular mechanism by which nuclear receptors modulate gene transcription has, in its simplest form, involved the binding of the ER to an estrogen responsive element (ERE) within the promoter of target cells. How-

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/56/1/43/293282)
ever, there are genes that do not contain a canonical ERE, including the 5′-flanking region of nNOS gene [19]. However, ER may form complexes with transcription factors such as Sp-1 and AP-1. Thus, the cooperative interaction of Sp-1 and AP-1 with ER proteins regulate several estrogen-inducible genes [20–22]. Therefore, our last aim was to determine the involvement of Sp-1 and AP-1 transactivation in the induction of nNOS by estrogen in neutrophils.

2. Methods

2.1. Neutrophil isolation

Human neutrophils were isolated as previously described [23,24] from peripheral blood from ten healthy pre-menopausal women (25–40 years old mean: 30±5) during different stages of the menstrual cycle and ten healthy men (25–40 years old mean: 32±6) for in vitro estrogen incubations. The protocols were approved by the Institutional Ethics Committee.

2.2. Neutrophils obtained from premenopausal women

To determine the in vivo effect of estrogen, neutrophils were obtained from the same donor (n=10) at two different points: (1) the ovulatory phase identified by the urinary peak of luteinizing hormone (LH) (by disposable test sticks (Donnatest Wyntek Diagnostic, San Diego, CA)) and (2) within the first 2 days of the follicular phase (menstruation). Serum estradiol concentrations were determined with an ELISA kit (Oxford Biomedical Research, Oxford, MI).

2.3. In vitro incubation of neutrophils from healthy men and women with estrogen

Neutrophils (5×10⁶ cells/tube) from ten men and ten
women during follicular phase were incubated with 17β-estradiol (10^{-8} \text{ mol/l}) or the solvent of 17β-estradiol (ethanol at final concentration <0.01%) for 6 h in an incubator at 37°C with 5% CO₂. The time of 6 h of incubation was chosen based in a previous work in which we observed a marked nNOS expression at this time after the addition of 10^{-8} \text{ mol/l} 17β-estradiol [12].

2.4. Determination of ER and nNOS expression by Western blot

After isolation, the neutrophils were lysed in Laemmli buffer [25] containing 2-mercaptoethanol. Proteins were separated on denaturing sodium dodecyl sulphate (SDS)–10% polyacrylamide gels (15 μg/lane) as previously described [26,27]. To verify that equal amounts of proteins had been loaded in the gel, we run and stained a parallel gel with identical samples with Coomassie to compare the intensities of the protein bands. ER protein expression was determined using the polyclonal antibody MC-20 (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody recognizes a common sequence of α and β ER subtypes [28]. The expression of α and β RE subtypes was further identified by specific monoclonal antibodies against each receptor (Santa Cruz Biotechnology). nNOS determination was also performed by Western blot as previously reported [12,26,27].

2.5. Electrophoretic mobility-shift assay

Nuclear protein extracts were prepared from neutrophils incubated for 30 min in the presence and in the absence of 17β-estradiol (10^{-8} \text{ mol/l}). Nuclear proteins were then mixed with an oligonucleotide corresponding to double-stranded Sp1 or AP1 consensus oligonucleotide (5′-ATT CGA TCG GGG GGG GGC GAG C-3′ for Sp1 and 5′-CGC TTG ATG AGT CAG CCG GAA-3′ for AP1, Promega). Each oligonucleotide was end-labelled using [γ-32P]ATP and T₄ polynucleotide kinase. Standard binding reactions were performed by incubating 10 μg of nuclear extracts in 20 μl of 10 mmol/l Tris–HCl, pH 7.5, containing 100 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 10 mmol/l MgCl₂, 20% (v/v) glycerol, and 50 000 dpm of [32P]-labelled Sp1 or AP1 oligonucleotides (approximately 1 pmol), for 20 min at room temperature, as reported [29]. After incubation, the samples were loaded onto a 4% polyacrylamide gel and run at a constant current of 100 V in 0.5× Tris–borate–EDTA (TBE) at 4°C, as described [29,30]. Gels were dried and placed on film at −20°C.

2.6. Statistical methods

Results are expressed as means±S.E.M. Each value corresponds to ten different experiments (neutrophils from ten men or from ten women, respectively) considering each donor as an independent experiment. The statistical significance was determined by ANOVA with Bonferroni’s correction for multiple comparisons or a Student’s t-test, paired or unpaired. A P value <0.05 was considered statistically significant.

3. Results

3.1. ER expression in human neutrophils

At ovulation, serum estradiol levels were 520±30 pg/ml (1.9×10^{-9} \text{ mol/l}). In the first 2 days of the follicular phase, serum estradiol concentrations fell to 160±10 pg/ml (5.9×10^{-10} \text{ mol/l}). Analysis of ER receptors using the polyclonal antibody MC-20 that recognizes both α and β ER subtypes showed an increased expression of ER in the

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Fig. 4. Representative Western blot demonstrating the expression of ERα (A) and ERβ (B) in neutrophils obtained from women during the follicular phase. Neutrophils were in vitro incubated with 17β-estradiol (10^{-8} \text{ mol/l}) for 6 h incubated with the solvent of 17β-estradiol, ethanol (final ethanol concentration <0.01%). Bottom: bar graph showing the densitometric analysis of the corresponding Western blot. Results are represented as mean±S.E.M. of ten different experiments. * P<0.05 with respect to neutrophils.
neutrophils obtained during the ovulatory phase compared with those found in the follicular phase (Fig. 1A). No changes in the expression of β-actin protein were observed in neutrophils from the follicular and ovulatory phases (Fig. 1A). Then, we analyzed the expression of each ER subtype expressed in the neutrophils from premenopausal women in the two phases of the menstrual cycle using specific monoclonal antibodies against ERα and ERβ, respectively. Neutrophils obtained during the ovulatory phase showed a higher expression of both ERα and ERβ subtypes than neutrophils obtained during the follicular phase (Fig. 1B).

Neutrophils from healthy men also expressed ER as it was observed by Western blotting using the MC-20 polyclonal antibody (Fig. 2) and incubation of neutrophils derived from men with 17β-estradiol (10⁻⁸ mol/l) enhanced the expression of ER (Fig. 2). 17β-estradiol (10⁻⁸ mol/l) failed to modify β-actin expression in neutrophils derived from men (Fig. 2). The dose of 10⁻⁸ mol/l 17β-estradiol was chosen based in previous studies in which we have demonstrated that this dose of 17β-estradiol increased nNOS protein expression in human neutrophils [12,13].

We further determined whether estrogen regulate the expression of their own receptors. For this purpose, ERα and ERβ subtypes expression was determined in the presence of two different ER antagonists, ICI 182780 (Ki in nmol/l for ERα: 0.2 and for ERβ: 0.08) and tamoxifen. (Ki in nmol/l for ERα: 3.4 and for ERβ: 2.5) Both tamoxifen (10⁻⁸ mol/l) and ICI 182780 (10⁻⁸ mol/l) reduced the expression of ER induced by 10⁻⁸ mol/l 17β-estradiol in neutrophils derived from men, suggesting the involvement of estrogen in the autoregulation of ER in neutrophils (Fig. 2). The ER antagonists were added 10 min before 17β-estradiol. The reduction in ER expression achieved with 10⁻⁸ mol/l ICI 182780 was of higher magnitude than that obtained with 10⁻⁸ mol/l tamoxifen (Fig. 2). Under basal conditions, neither ICI 182780 (10⁻⁸ mol/l) nor tamoxifen (10⁻⁸ mol/l) modified ER expression in neutrophils (Fig. 2).

Western blot analysis of the ER subtype expressed in neutrophils derived from men showed that, as occurred in neutrophils from premenopausal women, both ERα and ERβ subtypes were expressed (Fig. 3A and B). However, only the expression of the ERα subtype was enhanced by 10⁻⁸ mol/l 17β-estradiol (Fig. 3A). ICI 182780 (10⁻⁸ mol/l) almost completely inhibited the enhanced expression of ERα subtype induced by 17β-estradiol (Fig. 3A).

Fig. 5. (A) Representative Western blot demonstrating the expression of neuronal nitric oxide synthase (nNOS) in neutrophils from men incubated in the presence and in the absence of 17β-estradiol (10⁻⁸ mol/l) for 6 h. Additional experiments were performed in the presence and in the absence of the estrogen receptor antagonists tamoxifen (Tx, 10⁻⁸ mol/l) and ICI 182780 (ICI, 10⁻⁸ mol/l). The expression of β-actin is also shown. Bottom: bar graph showing the densitometric scanning of the Western blot. Results are represented as mean±S.E.M. of ten different experiments. * P<0.05 with respect to neutrophils incubated with the solvent of 17β-estradiol ethanol (final ethanol concentration <0.01%). ** P<0.05 with respect to 17β-estradiol in the presence of ICI 182780. (B) Representative Western blot demonstrating the expression of nNOS in neutrophils from premenopausal women obtained during the follicular (FOL) and ovulatory (OVUL) menstrual phases. The expression of β-actin in the neutrophils is also shown. The bottom shown the densitometric scanning of the Western blot. Results are represented as mean±S.E.M. of ten different experiments. * P<0.05 with respect to neutrophils during the follicular phase. (C) Western blot that demonstrates the specificity of the monoclonal antibody used in the experiments to recognize the nNOS protein. The monoclonal antibody did not recognize the endothelial isoform in human umbilical endothelial cells nor the inducible isoform in LPS-stimulated mouse macrophages, but it specifically recognized the nNOS isoform in homogenates of rat pituitary.
Therefore, to analyze whether the different ER expression in neutrophils from women and men was due to gender differences or to the in vivo and in vitro condition in which the experiments were performed, neutrophils from women during the follicular phase were in vitro incubated with 10^{-8} mol/l 17β-estradiol. 17β-Estradiol (10^{-8} mol/l) upregulated the expression of both ERα subtypes and ERβ subtype in neutrophils isolated from women (Fig. 4A and B).

### 3.2. Expression of nNOS protein in neutrophils: involvement of Sp-1 and AP-1 transcription factors

As previously reported [12,13], 17β-estradiol (10^{-8} mol/l) induced nNOS expression in neutrophils derived from men (Fig. 5A), an effect that was prevented by both 10^{-8} mol/l tamoxifen and 10^{-8} mol/l ICI 182780 (Fig. 5A). The level of inhibition of nNOS expression achieved with ICI 182780 was higher than with tamoxifen (Fig. 5A). In the absence of 17β-estradiol, the level of nNOS protein

Fig. 6. (A) Representative electrophoretic mobility-shift assay (EMSA) showing the binding of transcription factor Sp-1 to its 32P-labeled consensus sequence in nuclear extracts from neutrophils derived from men stimulated with 10^{-9} mol/l 17β-estradiol in the absence and in the presence of the Sp-1 inhibitor mithramycin A (10^{-5} mol/l). In the last lane is showed a competition experiment with unlabeled Sp-1 probe. (B) Representative EMSA showing the binding of transcription factor AP-1 to its 32P-labeled consensus sequence in nuclear extracts from neutrophils derived from men stimulated with 10^{-9} mol/l 17β-estradiol (10^{-9} mol/l) in the absence and in the presence of the specific inhibitor of AP-1 curcumin (10^{-6} mol/l). In the last lane is showed a competition experiments with unlabeled AP-1 probe.

Fig. 7. Representative Western blot demonstrating the expression of nNOS (A) and ERα (B) in neutrophils from men incubated in the presence (17βE₂) and in the absence (C, control) of 17β-estradiol (10^{-8} mol/l) and the specific inhibitor of Sp-1 mithramycin A (10^{-5} mol/l, MT) and AP-1 curcumin (10^{-6} mol/l, CM). Bottom: bar graphs showing the densitometric scanning of the Western blot. Results are mean±S.E.M. of ten different experiments. * P<0.05 with respect to control (C).
expression was not statistically modified by any of the two ER antagonists (Fig. 5A). During the ovulatory phase nNOS expression was higher than in the follicular phase (Fig. 5B). Fig. 5C shown the specificity of the nNOS antibody used.

The nNOS promoter did not contain a canonical estrogen response element (ERE), but binding sites for different transcription factors have been identified [19]. 17β-Estradiol stimulated DNA binding activity of both Sp-1 and AP-1 transcription factors in neutrophils derived from men (Fig. 6A and B). The presence of mithramycin A (10⁻⁶ mol/l) and curcumin (10⁻⁶ mol/l), two inhibitors of Sp-1 and AP-1, respectively [31,32], prevented the effect of 17β-estradiol (10⁻⁸ mol/l) on the DNA binding activity of Sp-1 and AP-1 (Fig. 6A and B). An excess of unlabelled Sp-1 and AP-1 probes prevented complex formation by nuclear extracts from 17β-estradiol-incubated neutrophils and labelled Sp-1 and AP-1, supporting binding specificity (Fig. 6A and B).

We then analyzed whether Sp-1 and AP-1 are involved in the upregulation of nNOS and ERα by 17β-estradiol. Western blot analysis showed that both mithramycin A (10⁻⁶ mol/l) and curcumin (10⁻⁶ mol/l) prevented the expression of nNOS protein induced by 10⁻⁸ mol/l 17β-estradiol in neutrophils derived from men (Fig. 7A). ERα subtype expression induced by 17β-estradiol was also reduced by both mithramycin and curcumin (Fig. 7B).

4. Discussion

Neutrophils play a detrimental role in the setting of cardiovascular diseases such as myocardial ischemia. In a previous work, we have shown that upregulation of nNOS protein expression by estrogen was involved in the regulation of the expression of CD11/CD18 adhesive proteins and the adhesiveness of these cells [12]. Moreover, 17β-estradiol-incubated neutrophils showed a greater ability to prevent platelet activation [10]. However, the mechanism by which estrogen upregulated NOS expression and the ER subtype expressed in neutrophils remains to be established.

In the present study we have shown that neutrophils from premenopausal women and healthy men expressed ERα and ERβ subtypes. However, whereas in premenopausal women the expression of ERα and ERβ was upregulated during ovulation (high serum estrogen concentration), and by in vitro incubation with 17β-estradiol in neutrophils obtained during follicular phase (low serum estrogen concentration), 17β-estradiol only upregulated the expression of the ERα subtype in neutrophils from men.

There are evidences demonstrating that estradiol induce the expression of ER during the menstrual cycle in the uterus of experimental animals and in different organs of nonmammalian vertebrates, although less is known in human cells [14–18]. In our study, the first suggestion that estrogen autoregulates ER expression in human neutrophils was the association observed between estrogen circulatory levels and the level of ER expressed in neutrophils from premenopausal women. Moreover, in neutrophils isolated from men, 17β-estradiol stimulated ER expression, an effect that was prevented by two different ER antagonists, tamoxifen and ICI 182780. Interestingly, at equimolar concentrations, ICI 182780 seems to be more potent to inhibit 17β-estradiol-induced ER expression than tamoxifen. McClelland et al. [33] have also reported a greater inhibition of estrogen-induced events by ICI 182780 than by tamoxifen, which could be related to the known agonistic/antagonistic effect of tamoxifen. In neutrophils derived from men, 17β-estradiol increased the expression of the ERα subtype but not the ERβ subtype. The upregulation of ERα in 17β-estradiol-stimulated neutrophils was prevented by ICI 182720. These results suggest that estrogen modulate ERα subtype expression but not ERβ subtype expression in neutrophils from men. However, in premenopausal women during the menstrual cycle associated with high estrogen levels (ovulation), both ERα and ERβ were upregulated in the neutrophils. These results may suggest a different gender-dependent regulation of ERα and ERβ in neutrophils from premenopausal women and men. It was further supported by the fact that in vitro incubation of neutrophils from women during the follicular phase with 17β-estradiol upregulated both ERα and ERβ subtype expression.

Unfortunately, there are no specific blockers of ERα and ERβ to establish the type of ER associated with nNOS expression. However, the experiments with neutrophils from men demonstrated that upregulation of nNOS expression was accompanied by an enhanced expression of the ERα subtype, suggesting the involvement of this ER subtype. In this regard, a case has been reported of a young man who has no functional ERα and has impaired brachial endothelium-dependent relaxation supporting the importance of ERα on the NO system [34]. Although nNOS encoding genes were originally considered to be constitutive it has become evident that their expression is regulated by a variety of stimuli including estrogen [12,35].

The mechanisms by which estrogen stimulated nNOS protein expression in neutrophils remains to be determined, given that the 5'-flanking region of nNOS gene does not contain a canonical estrogen response element [19]. There are two known mechanisms by which ER could activate gene expression. In the best understood mode of action, or classical pathway, the ER bind to a specific estrogen response element within target genes. In the second mode of action, the ER utilizes unspecified protein–protein interactions to enhance the activity of heterologous transcription of genes that contain binding sites for AP-1 and Sp-1 such as the Jun/Fos complex and c-myc [20–22].

In our study, 17β-estradiol stimulated the DNA binding activity of Sp-1 and AP-1 in neutrophils. Moreover, inhibition of DNA binding activity of Sp-1 and AP-1...
markedly reduced neutrophil nNOS expression induced by 17β-estradiol. Interestingly, blockade of Sp-1 and AP-1 transcription factors also reduced 17β-estradiol-induced overexpression of ERα in neutrophils from men, suggesting that these transcription factors are also involved in the autoregulation of ERα by estrogen. In this regard, Tang et al. [36] have identified a functionally important AP-1 site at position −3778 to −3744 in the promoter of ER in breast cancer cells. The present experiments did not allow us to determine whether SP-1 and AP-1 acts independently to enhance expression of ER and nNOS in neutrophils. Moreover, other transcription factors could also be involved in the regulation of eNOS and ER by estrogen. Therefore, further studies transflecting in neutrophils mutant ER and nNOS containing deletions in their DNA-binding site for AP-1 and SP-1 are needed.

In summary, neutrophils obtained from premenopausal women during the ovulatory phase of the menstrual cycle showed greater expression of both ERα and ERβ subtypes than during the follicular phase. In in vitro experiments, while 17β-estradiol upregulated both ERα and ERβ subtype expression in neutrophils for women, 17β-estradiol only stimulated the expression of the ERα subtype in neutrophils from men, an effect which was associated with an enhanced expression of nNOS protein. Sp-1 and AP-1 transcription factors are involved in the upregulation by estrogen of both nNOS and ERα in neutrophils.

The question then raised is why neutrophils coexpress ERα and ERβ subtypes. ERα and ERβ may be coupled to opposite effects one from the other on the neutrophil functionality as it has been recently reported in the vascular wall of mice [37]. Therefore, the different coexpression of ERα and ERβ in neutrophils from women and men by estrogen may contribute to a different response of their neutrophils as mentioned neutrophils derived from men by estrogen stimulation reduced neutrophils adhesion and platelet activation [12,13]. Interestingly, it has been reported premature coronary disease in a man with a disruptive mutation in ER probably of the α subtype [38]. Moreover, several observational studies have suggested that women taken estrogen have a lower risk of coronary heart disease [39]. However, randomized trials of administering estrogen in women with preexisting coronary heart disease have not confirmed the hypothetic cardiovascular benefits of estrogen [40,41]. Further studies are needed to analyze whether a different ERα and ERβ expression in neutrophils from women with and without preexisting coronary heart disease could contribute to these controversial results.

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