17β-Estradiol inhibits NADPH oxidase activity through the regulation of p47\textit{phox} mRNA and protein expression in THP-1 cells

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Abstract

In this report, we demonstrate that NADPH oxidase is activated by tumor necrosis factor-α (TNF-α) plus interferon-γ (IFN-γ) in human monocytic cells (THP-1 cells) differentiated with phorbol ester (PMA) and that physiological concentration of 17β-estradiol inhibits NADPH oxidase activity in THP-1 cells stimulated with TNF-α plus IFN-γ. This effect is mediated by estrogen receptor based on estrogen receptor antagonist (ICI 182, 780) that diminishes inhibition by 17β-estradiol. This inhibition is specific in 17β-estradiol because 17α-estradiol, testosterone and progesterone do not inhibit NADPH oxidase activity. Activation of NADPH oxidase induced by TNF-α plus IFN-γ is caused by up-regulation of p47\textit{phox} (cytosolic component of NADPH oxidase) expression. 17β-Estradiol prevents the up-regulation of p47\textit{phox} mRNA and protein expression. This prevention of p47\textit{phox} expression depends on the inhibition of NF-κB activation. Our results implicate that 17β-estradiol has an anti-atherosclerotic effects through the improvement of nitric oxide (NO) bioavailability caused by the regulation of superoxide (O2\textit{•−}) production.

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1. Introduction

Estrogen replacement therapy in postmenopausal women has been reported to reduce the risk of cardiovascular disease [1]. Although estrogen replacement promotes a more favorable lipid profile, this effect does not sufficiently explain the total anti-atherosclerotic benefit. For example, estrogens have been suggested to have direct protective actions on the vasculature.

Nitric oxide (NO) excreted from endothelial cells—an important physiological factor of vascular homeostasis—is synthesized by endothelial nitric oxide synthase (eNOS). The impaired NO release is associated with endothelial dysfunction in atherosclerosis [2,3].

Peroxynitrite (ONOO•−), the reaction product of NO and superoxide (O2\textit{•−}), is more toxic than either NO or O2\textit{•−} alone. ONOO•− is capable of nitrating critical tyrosine residues, which may alter protein function. For example, ONOO•− has been observed to cause the specific nitration of Tyr34 in manganese-superoxide dismutase (MnSOD), thereby altering the function of the enzyme [4]. The release of ONOO•− was increased in atherosclerosis [5], and we previously reported that ONOO•− could impair the NO-mediated vascular response during the regression of dietary cholesterol-induced atherosclerosis [6].

The neutrophil protein complex NADPH oxidase plays a vital role in the nonspecific host defense against pathogens by generating large quantities of O2\textit{•−} during the respiratory burst [7]. NADPH oxidase is the major source of O2\textit{•−} in vascular tissues and consists of the membrane-bound heterodimeric flavocytochrome b558 (gp91\textit{phox} and p22\textit{phox}) and four cytosolic proteins (p47\textit{phox}, p67\textit{phox}, p40\textit{phox} and Rac1/2). These components must assemble in the membrane for the enzyme to become active. Assembly of the complex is regulated through the phosphorylation of p47\textit{phox} [8].

Abbreviations: PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; ONOO•−, peroxynitrite; O2\textit{•−}, superoxide; NO, nitric oxide; phox, phagocyte oxidase
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Our aim in this study was to investigate the effects of 17β-estradiol on phagocyte NADPH oxidase activity and on the protein expression of p47<sup>phox</sup> and p67<sup>phox</sup> in phorbol ester (PMA)-differentiated human monocytic cells (THP-1 cells).

2. Materials and methods

2.1. Chemicals

17β-Estradiol, 17α-estradiol, progesterone, bis-N-methylacridinium nitrate (Lucigenin), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Testosterone was purchased from Wako Pure Chemical (Osaka, Japan). ICI-182,780 was kindly provided by Zeneca Pharmaceuticals. Monoclonal p47<sup>phox</sup> and p67<sup>phox</sup> antibodies were purchased from Transduction Laboratories (Lexington, KY). Human recombinant tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were purchased from Roche Diagnostics (Indianapolis, IN). All of the other reagents and solvents used in this study were of analytic reagent grade.

2.2. Cell culture

The human monocytic cells (THP-1 cells) were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> using RPMI-1640 medium containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). To measure NADPH oxidase, we seeded onto 15-cm culture dishes at a minimum of 6 × 10<sup>6</sup> cells per plate. To perform Western blot, reverse transcription-polymerase chain reaction (RT-PCR) and electrophoresis mobility shift assay (EMSA), we seeded onto 6-cm culture dishes at a minimum of 1 × 10<sup>6</sup> cells per plate. In order to differentiate THP-1 cells to the macrophage-like cell, we incubated them for 48 h with 100 nM PMA in RPMI-1640 phenol red-free medium containing 10% charcoal stripped fetal calf serum.

2.3. NADPH oxidase activity

The differentiated THP-1 cells were incubated for 24 h in incubation medium containing indicated concentrations of sex steroid hormones, TNF-α (10 ng/ml) and IFN-γ (100 U/ml). The activity was measured by the Lucigenin method with chemiluminescence leader (BLR-201, ALOKA) as previously described [9]. The protein concentration was determined by a Dc protein assay kit (Bio-Rad, CA).

2.4. Western blotting

The differentiated THP-1 cells were incubated for 24 h with the indicated concentrations of 17β-estradiol, TNF-α (10 ng/ml) and IFN-γ (100 U/ml). Ten-microgram proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to PVDF-membrane. The blots were placed in blocking solutions (Tris-buffered saline (10 mM Tris (pH 8.0) and 150 mM NaCl), 0.05% Tween-20 and 5% nonfat milk) for 1 h. Blots were incubated for 1 h with p47<sup>phox</sup> or p67<sup>phox</sup> monoclonal antibodies, washed with Tris-buffered saline and 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody. Bound IgGs were visualized using an enhanced chemiluminescence detection system (Pierce, IL) according to the manufacturer’s protocol. Band intensities were quantified with the NIH-image system.

2.5. RT-PCR

The differentiated THP-1 cells were incubated for 6 h with the indicated concentrations of 17β-estradiol, TNF-α
(10 ng/ml) and IFN-γ (100 U/ml). Total RNA was isolated by TRizol reagents (Invitrogen). Total RNA, 0.5 μg, was reverse-transcribed to the first strand cDNA and these cDNAs were amplified by PCR performed with One Step RNA PCR Kit (Takara Shuzo, Otsu, Japan) using the following primers: p47phox sense primer 5′-caccgagatctacgagttcata-3′ and p47phox anti-sense primer 5′-ctttcatctgacagaaccaccaac-3′ [10] Internal control for equal cDNA loading in each reaction was assessed using the following primers: h-actin sense primer 5′-gacctggctggccgggacctg-3′ and h-actin anti-sense primer 5′-cacatctgctggaaggtgga-3′. The amplified products were resolved by 2% agarose gel electrophoresis. Ethidium bromide-stained band intensities were quantified with the NIH-image system. p47phox band intensities were compared with band intensities of h-actin.

2.6. EMSA

The differentiated THP-1 cells were incubated for 1.5 h with the indicated concentrations of 17β-estradiol, TNF-α (10 ng/ml) and IFN-γ (100 U/ml). Nuclear extracts of THP-1 cells were extracted with the NE-PER nuclear extraction reagent (Pierce). Double-stranded oligonucleotide encompassing the kappaB (κB) 5′-ggggatttccc-3′ (Santa Cruz Biotechnology, Inc, CA) was end-labeled using γ-32P-ATP, 7000 Ci/mmol, and T4 polynucleotide kinase (Promega, CA). Five-microgram nuclear protein extract was incubated for 30 min at room temperature in binding buffer (25 mM HEPES (pH 7.9), 100 mM KCl, 5% Ficoll-400, 2% glycerol, 0.025% Igepal CA-630, 0.05 mM EDTA, 0.05 mM EGTA, 2 mM DTT, 0.5 mM PMSF) containing 10-μg bovine serum albumin and 1-μg polydI–dC. Radiolabeled consensus oligonucleotide (100,000 cpm) was subsequently added to each sample, and incubated at room temperature for an additional 30 min. Protein–DNA complexes were subsequently resolved in a 5% native Tris/taurine-buffered gel. Gels were dried and exposed to autoradiographic film at −80 °C.

2.7. Statistical analysis

Data were obtained from three or four different experiments. Each value represents the mean ± S.E. Statistical significance was assessed by the Student’s t test, and differences between treatment groups were considered significant at P < 0.05.

3. Results

To determine the effects of estrogen on NADPH oxidase in human monocytic cells (THP-1 cells), we compared
Fig. 5. The effect of 17β-estradiol on p67phox and p47phox protein expression in THP-1 cells. The open and solid bars represent band intensities of p67phox and p47phox protein expression. 1: 24-h incubation with TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) in PMA-differentiated THP-1 cells; 2, 3 and 4: in the presence of 10⁻⁸M (lane 2), 10⁻⁹M (lane 3) and 10⁻¹⁰M (lane 4) 17β-estradiol. Band intensities were calculated and p67phox or p47phox band intensities were compared with TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) induced THP-1 cells (lane 1). Data represent the mean ± S.E. of four separate experiments (*P < 0.05 vs. lane 1).

NADPH oxidase activity in nondifferentiated and PMA-differentiated THP-1 cells. Fig. 1 shows that PMA-induced differentiation alone induced NADPH oxidase activity up to 2.2-fold. And we also detected that TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) stimulated NADPH oxidase activity up to 3.5-fold in differentiated THP-1 cells compared with nondifferentiated THP-1 cells. We confirmed that this method is adequate for measuring NADPH oxidase activity on the ground of low activity with cytosolic fraction (100,000 × g supernatant, lane 4) substitute for membrane fraction and reaction without NADPH (lane 5).

As shown in Fig. 2, physiological concentrations of 17β-estradiol (10⁻⁸ and 10⁻¹⁰ M) inhibited NADPH oxidase activity stimulated by TNF-α plus IFN-γ (10 ng/ml) in PMA-differentiated THP-1 cells, but 10⁻⁶ M 17β-estradiol did not inhibit activity and 17β-estradiol (10⁻⁸ and 10⁻¹⁰ M) did not inhibit NADPH oxidase activity in the absence of TNF-α plus IFN-γ (data not shown). The estrogen receptor antagonist ICI 182,780 abolished the inhibition of NADPH oxidase activity by 17β-estradiol. The stereoisomer of 17β-estradiol (17α-estradiol), testosterone and progesterone were also tested but did not inhibit NADPH oxidase activity (Fig. 3). These results indicate that inhibition of the NADPH oxidase activity is specific to the action of 17β-estradiol, and is mediated through the estrogen receptor.

Considering that 17β-estradiol was shown to inhibit NADPH oxidase activity, we examined the expression of two components of NADPH oxidase, p47phox and p67phox. We examined p47phox and p67phox protein expression in the effects of differentiation and cytokine treatment. As shown in Fig. 4, p47phox protein expression is enhanced 2.7-fold by PMA-induced differentiation, 3.8-fold by the presence of TNF-α, and 6.8-fold by the presence of TNF-α plus IFN-γ in comparison with nondifferentiated THP-1 cells. In contrast, p67phox protein expression was stimulated by differentiation, but not further enhanced by TNF-α or TNF-α plus IFN-γ.

As shown in Fig. 5, 17β-estradiol (10⁻⁸ and 10⁻¹⁰ M) significantly attenuates the up-regulation of p47phox protein expression caused by TNF-α plus IFN-γ. In correlation with inhibition of NADPH oxidase activity, 10⁻⁶ M 17β-estradiol did not attenuate p47phox expression. Moreover, 17β-estradiol did not affect p67phox protein expression.

In order to determine whether the effect of 17β-estradiol on p47phox expression is correlated with changes in p47phox mRNA expression, we performed RT-PCR analysis (Fig. 6). The p47phox mRNA levels were up-regulated by differentiation, and further up-regulated by TNF-α plus IFN-γ in comparison to undifferentiated cells (data not shown). As with protein expression, 17β-estradiol (10⁻⁸ and 10⁻¹⁰ M)

Fig. 6. The effect of 17β-estradiol on p47phox mRNA expression in THP-1 cells. 1: 6-h incubation of TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) in PMA-differentiated THP-1 cells; 2, 3 and 4: in the presence of 10⁻⁸M (lane 2), 10⁻⁹M (lane 3) and 10⁻¹⁰M (lane 4) 17β-estradiol. p47phox mRNA band intensities were calculated with β-actin band intensities and compared with TNF-α (10 ng/ml) plus IFN-γ (100 U/ml) induced THP-1 cells (lane 1). Data represent the mean ± S.E. of three separate experiments (*P < 0.05 vs. lane 1).
revealed that shifted band intensities were decreased with an excess of cold oligonucleotide competitor in the binding assay (lane 6). The arrow indicates the specific DNA–protein complex.

We detected p47(phox)-mediated NF-κB nuclear translocation in THP-1 cells. 1: no stimulation; 2: 1.5-h incubation of TNF-α (10 ng/ml) plus IFN-γ (100 U/ml); 3, 4 and 5: in the presence of 10^-8 M (lane 3), 10^-9 M (lane 4) and 10^-10 M (lane 5) 17β-estradiol. Specific DNA-binding complexes of NF-κB were identified using 100-fold excess of cold oligonucleotide competitor in the binding assay (lane 6). The arrow indicates the specific DNA–protein complex.

We investigated whether 17β-estradiol inhibits NADPH oxidase activity in the presence of TNF-α plus IFN-γ, whose expression has been reported in atherosclerotic lesions [18]. We have shown that, as one of the anti-atherosclerotic effects of estrogen through the role of NO, the effects of estrogen on the stimulation of eNOS expression [19] and the prevention of eNOS mRNA destabilization by TNF-α will cause an increase in the release of nitric oxide from endothelial cells [20]. This study shows that the effects of 17β-estradiol on the inhibition of NADPH oxidase activity prolong the NO half-life, and may thus partially protect against the development of atherosclerosis.

It is expected that the production of ONOO− and nitrotyrosine is inhibited through the inhibition of NADPH oxidase activity by 17β-estradiol. However, we could not detect a decrease in nitrotyrosine in MnSOD caused by 17β-estradiol using the immunoprecipitation technique because we could not detect any accumulation of NO stimulated by TNF-α plus IFN-γ. This finding was supported by the fact that there was no inducible NO metabolites stimulated by lipopolysaccharide (LPS) plus IFN-γ, although the inducible nitric oxide synthase (iNOS) mRNA expression was detected in human monocytes [21]. Recently it has been suggested that a nitrite-dependent peroxidase reaction rather than ONOO− may cause the production of nitrotyrosine in activated macrophages, but this reaction has not been shown in THP-1 cells [22].

In our study of the effects of 17β-estradiol on the inhibition of NADPH oxidase activity, we focused on the regulation of p47(phox) and p67(phox) expression. Our results show that TNF-α plus IFN-γ induces p47(phox) protein and mRNA expression, but not p67(phox). THP-1 cells have well characterized TNF-α and IFN-γ receptors. These data are in agreement with previous reports that have shown that p47(phox) expression is induced in cytokine-differentiated THP-1 cells through the regulation of transcription [23] and that p47(phox) expression is up-regulated by IFN-γ in purified monocytes from chronic granulomatous disease patients [24]. Additionally, these monocytes did not up-

In order to clarify that 17β-estradiol inhibits p47(phox) expression resulting from an inhibition of NF-κB activation, we examined an EMSA. As shown in Fig. 7, EMSA revealed that shifted band intensities were decreased with 10^-8 M (lane 4) and 10^-10 M 17β-estradiol (lane 5) treatment compared with TNF-α plus IFN-γ (lane 2). These data confirm that 17β-estradiol inhibits p47(phox) expression through the inhibition of NF-κB activation induced by TNF-α plus IFN-γ.

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4. Discussion

Premenopausal women have a lower risk of cardiovascular disease than age-matched males. Postmenopausal women exhibit a significant increase in risk due to the loss of circulating estrogen [11]. It has been suggested that estrogen possesses anti-atherosclerotic effects. We have observed that a physiological concentration of 17β-estradiol retards the progression of rabbit atherosclerosis induced by high-cholesterol diets and balloon catheter injury [12]. However, the cellular and molecular mechanisms by which 17β-estradiol has its effect are still unclear. On the other hand, it has been shown that O2 production was increased in an atherosclerotic aorta induced by a high cholesterol diet. NADPH oxidase appears to be an important source of O2 in vascular vessels, as has been suggested by experiments on the NADPH oxidase knockout mouse [13] or by using novel competitive inhibitor of NADPH oxidase [14]. We detected p47(phox)-positive staining in macrophages of rabbit atherosclerotic lesions (unpublished data). The purpose of this study was to examine the effects of 17β-

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regulate of p67<sup>phox</sup> expression. With regard to the regulation of transcription, we have shown that 17β-estradiol inhibits the iNOS expression that is stimulated by LPS plus IFN-γ in the J774 mouse macrophage cell line [25].

The data presented here show that 17β-estradiol inhibits NF-κB activation caused by TNF-α plus IFN-γ. In 1996, Caulin-Glaser et al. [26] reported that 17β-estradiol inhibited the endothelial cell adhesion molecule expression (E-selectin, ICAM-1 and VCAM-1) induced by interleukin-1 in endothelial cells. This report suggested that 17β-estradiol can modulate NF-κB nuclear translocation, DNA binding or transactivation. Recently, 17β-estradiol was reported to inhibit NF-κB activation by increasing NF-κB p105 levels [27]. We are still uncertain that up-regulation of p105 levels is involved in inhibition of p47<sup>phox</sup> expression by 17β-estradiol.

Wagner et al. [28] recently reported that 17β-estradiol inhibits constitutive NADPH oxidase (gp91<sup>phox</sup>) expression in human endothelial cells. NADPH oxidase is the principal source of O<sub>2</sub> in endothelial cells [29] and plays a critical role in the development of atherosclerosis [30]. It is possible that 17β-estradiol also inhibits inducible gp91<sup>phox</sup> expression. However, this remains to be investigated.

In the present study, we have demonstrated that 17β-estradiol inhibits NADPH oxidase activity induced by TNF-α plus IFN-γ in PMA-differentiated THP-1 cells, at least in part by regulation of p47<sup>phox</sup> expression through the NF-κB inhibition. This effect was specific to 17β-estradiol and the estrogen receptor-mediated system. This mechanism of estrogen action may be clinically relevant to postmenopausal women patients suffering from cardiovascular disease.

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