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17β-Estradiol Decreases Nitric Oxide Synthase II Synthesis in Vascular Smooth Muscle Cells*

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ABSTRACT

Several studies have provided evidence for a direct effect of 17β-estradiol on vessel wall *via* interaction with the constitutively expressed nitric oxide synthase (NOS) by endothelium. The aim of the present study was to investigate the effect of 17β-estradiol on inducible NOS (NOS II) in primary culture of smooth muscle cells (SMC) from rat aorta. We here prove that 17β-estradiol decreases the content and activity of NOS II in SMC. This effect appears to be the consequence of ER activation, because: 1) ERα and ERβ are expressed in rat aorta SMC grown in culture; 2) low concentrations of hormone modulate NOS II activity; 3) the specific ERα antagonist ICI182,780 completely blocks 17β-estradiol effect. On the other hand, progesterone is deprived of any effect on NOS II content or activity, proving the specificity of 17β-estradiol effect. In addition, we show that 17β-estradiol can counteract the increase in NOS II activity following cytokine treatment. The observation could indicate a novel mechanism for the protective effects exerted by these hormones in cardiovascular diseases and atherosclerosis in particular. (*Endocrinology* **140:** 2004–2009, 1999)

THE LOW INCIDENCE of vascular events in premenopausal women as well as the rapid increase in angina, myocardial infarction, and stroke in women following menopause are well recognized. Several explanations for these epidemiological observations have been proposed (1, 2). It is well documented that women on estrogen replacement therapy have a 40–50% reduction in the risk of coronary artery disease (3, 4). While a substantial portion of this effect has been attributed to estrogen-mediated increase in high-density lipoprotein-cholesterol (5–7), the alterations in lipid profile account for only about 50% of the cardiovascular benefit observed in estrogen-treated women, suggesting that other mechanisms may be involved (8). Several studies provide evidence for a direct effect of estrogen on the vessel wall mediated through specific intracellular receptors acting as ligand-activated transcription factors (9, 10). A number of animal studies have demonstrated 17β-estradiol specific binding to vascular cells, supporting the hypothesis that vascular tissue is estrogen sensitive (11, 12). Recently, the presence of a functional estrogen receptor-α (ERα) has been demonstrated both in vascular smooth muscle and in endothelial cells (13–15). It is now known that the two ERs so far described (named ERα and ERβ) (16) are hormone-dependent transcription factors that modulate the transcription of selected genes by binding to consensus regulatory sequences, referred to as EREs (estrogen responsive elements) located in the promoter of the target genes (17).

Recent studies indicate that 17β-estradiol up-regulates nitric oxide synthase (NOS) III messenger RNA (mRNA) in cultured endothelial cells (18–20), and this may represent a possible mechanism for the protective role of estrogens on the vessel wall. NO is the most potent endogenous vasodilator. In the vessel wall, its synthesis is regulated by two major types of NOS: a constitutive form (cNOS, NOS III), normally expressed in endothelial cells, and an inducible form (iNOS, NOS II), mostly expressed in smooth muscle cells after exposure to inflammatory stimuli (21–23). Excessive NOS II production induced by proinflammatory stimuli (cytokines and lipopolysaccharides) has been associated with atherosclerosis, whereas reduced NO synthesis due to impaired NOS III activity may be responsible for hypertension and vascular pathologies (21, 24).

The aim of the present study was to investigate the effects of 17β-estradiol and progesterone on NOS II protein content and activity in primary cultures of smooth muscle cells from rat aorta. The results here presented demonstrate that 17β-estradiol, but not progesterone, decreases NOS II protein content and activity, suggesting that estrogens may contribute to an anti-inflammatory action in these cells.

Materials and Methods

Materials

The steroids 17β-estradiol, 17α-estradiol and progesterone, as well as the antiestrogen tamoxifen were obtained from Sigma Chemical Co. (Milano, Italy). The antiestrogen ICI 182,780 was kindly provided by Zeneca Pharmaceuticals (Macclesfield, UK). Antirabbit NOS II polyclonal antibody was purchased from Transduction Laboratories, Inc. (Lexington, KY). Oligodeoxynucleotides were synthesized by Pharmacia Biotech (Cambridge, UK).

Cell cultures

Smooth muscle cells (SMC) were obtained from the intimal-medial layers of aorta of male Sprague Dawley rats (Charles River Laboratories, Inc., Calco, Italy) (200–250 g), according to Ross (25). Cells were grown

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in monolayers in medium 199 (M199) supplemented with 10% (vol/vol) FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 2.2 g/liter $NaHCO_3$ (referred to as "M199 + FCS") and were incubated at 37 C in a humidified atmosphere with 5% CO_2. SMC were characterized for growth and morphology and by immunostaining with monoclonal antibodies recognizing the actin α-isoform typically expressed by SMC (26). For the experiments here described, cells grown at passages 2–6 were seeded into either 60-mm Petri dishes (2×10^5 cells/dish) or 24-well plates (5×10^4 cells/well). Cells were grown in M199 + FCS for 48 h; then, the medium was replaced with phenol red-free M199 with 10% FCS (wM199 + FCS). At 16–24 h before the hormonal treatment, cells were incubated with phenol red-free M199 without serum (wM199). Pharmacological agents were added for 24 h, as specified in each figure legend. Typically, the treatments were done with confluent cells.

RT-PCR

Total cell RNA was isolated from rat uterus, used as a positive control for ER mRNA expression, and VSMC cells with the Bio/RNA-X Cell kit (Bio/Gene, Kimbolton Cambridge, UK), using 1 ml of RNA-X reagent for 1 g of tissue or for 10×10^6 cells. 1 μg of total RNA was denatured at 68 C with 10 pmol oligo-d$(T)_{12-18}$ (Perkin Elmer, Milan, Italy) and reverse-transcribed using MuMLV reverse transcriptase (HT Biotechnology Ltd., Cambridge, UK) as previously described (27). One-tenth of the complementary DNA (cDNA) reaction was amplified containing 2.5 U of DynaZyme-DNA polymerase (Finenzyme OY, Espo, Finland), the buffer provided by Finenzyme, 0.2 mM dNTP and 100 pmol PCR primers in 100 μl final volume. Amplification of ER-α mRNA was obtained as previously described (28). Briefly, the region of 254 bp, from nucleotide (nt) 530 to nt 784, was amplified using as primers ERα-1a (5′-AGCGT-GTCGCCGAGTCCG-3′) and ERα-1β (5′-AGCACAGTAGCGAGTCTC-3′). After denaturing at 94 C for 5 min, PCR amplification was performed for 30 cycles (94 C for 15 sec, 48 C for 20 sec, and 72 C for 30 sec) followed by a final extension step (72 C for 3 min). The duration and temperature of the PCR cycles were experimentally optimized to fall into the exponential phase of the amplification (28). At the 30th cycle, the reaction was terminated by addition of 50 μl of DNA dye (50% glycerol and 0.25% wt/vol xylene cyanol). Fifteen microliters of the incubation mixture were then loaded onto a 3% agarose gel, together with the appropriate molecular weight markers. The bands corresponding to the amplified DNA were then visualized by ethidium bromide staining.

For amplification of the rat ER-β, a region of 262 bp, from nt 39 to nt 301, was amplified by primers ERβ-1α (5′-TTCCCGGCAGCACCAG-TAACC-3′) and ERβ-1β (5′-TCCCTCTTTGCGTTTGGACTA-3′). After denaturing at 94 C for 5 min, PCR amplification was performed for 40 cycles (94 C for 1 min, 50 C for 1 min, and 72 C for 1 min). A subsequent Southern blot and hybridization with a rat ER-β probe was done to prove the specificity of the signal. No amplification product was detected in samples in which either the RT or the cDNA were omitted.

Southern blot

Probe preparation. Plasmid pCMV5-ratER-β (a gift from Jan-Ake Gustaffson) was used as template for the preparation of nonradioactively labeled DNA probe by means of PCR; probe corresponded to nt 39 to nt 301 of the rat ER-β. PCR reaction mix contained 15 ng plasmid DNA, 0.8 mM ERβ-1α and ERβ-1β primers, 0.35 mM Dig-11 dUTP (Boehringer Mannheim, Mannheim, Germany), 0.65 mM dTTP and 1 mM of each dATP, dCTP dGTP, and 2 U *Taq* DNA polymerase (Perkin Elmer) in 20 μl final volume. PCR reaction profile was as follows: 95 C for 5 min, then 30 cycles at 92 C for 1 min, 50 C for 1 min and 72 C for 1 min. The 262-bp amplification product was purified on a 1% agarose gel in Tris-acetate EDTA.

Electrophoresis, blotting, and hybridization. Ten microliters of PCR mix were loaded on 2% agarose gel in Tris-borate EDTA and subjected to electrophoresis performed at 100 V. The gel was denatured at room temperature for 30 min in 0.5 M NaOH and 1 M NaCl and neutralized in 0.5 M Tris, pH 7.7, 1.5 M NaCl, and 1 mM EDTA for 15 min at room temperature. DNA was transferred onto nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Milan, Italy) by capillarity blotting o/n in 20 × SSC (3 M NaCl, 0.3 M Na_3 citrate·$2H_2O$) and then fixed to the membrane by UV irradiation and baking at 80 C for 48 h. After two subsequent prehybridizations of 1 h at 65 C in "pre 1" solution (250 mM Na-posphate buffer pH 7.2, 7% SDS, 1% BSA and 1 mM EDTA) and "pre2" solution (5 × SSC, 50% formamide, 0.2% SDS, 1% Sarcosyl and Blocking reagent (Boehringer Mannheim) dissolved in 0.01 M Maleic acid, 0.015 M NaCl), heat-denatured Dig-11dUTP-labeled probe was annealed at 42 C o/n and washed at room temperature twice in 5 × SSC for 30 min, once in 1 × SSC containing 0.1% SDS for 30 min and finally, twice in 0.1 × SSC with 0.1% SDS for 15 min. The subsequent enhanced chemiluminescent reaction was performed as specified by the manufacturers (Boehringer Mannheim).

Western blot

Confluent cells were incubated for 24 h either with a cytokine cocktail [interleukin-1β (IL-1β) 10 ng/ml, interferon γ (INF-γ) 10 ng/ml, tumor necrosis factor (TNF)-α 25 ng/ml and lipopolysaccharides (LPS) 10 μg/ml] as positive control and/or with steroid and nonsteroid ligands (17β-estradiol, progesterone, 17α-estradiol, tamoxifen, and ICI 182,780) as described in the figure legends. The incubation was ended by addition of sample buffer (0.125 M Tris-HCl pH 6.8, 40 mM EDTA, 4.6% SDS, 20% glycerol). Protein concentration was measured according to Lowry's method with BSA as a standard (29) and twenty-eight-microgram proteins were separated on SDS-denaturating PAGE using discontinuous gradient gels (10–5%) (30). Proteins were electrophoretically transferred to nitrocellulose membranes. Immunoblotting was performed with the polyclonal antirabbit NOS II antibody (1:1000 dilution in BSA 5%, Transduction Laboratories, Inc.) and with the secondary peroxidase conjugated goat antirabbit antibody (1:2000 dilution in BSA 5%, Bio-Rad Laboratories, Inc.), which was then revealed by enhanced chemiluminescence (ECL, Amersham). Antibody specificity was assayed by testing extracts from cytokine-stimulated rat peritoneal macrophages. To compare the NOS II content in the experimental samples, scanning densitometry was performed; care was taken to ensure that the OD of all the bands considered were in the range of linearity previously assessed.

Nitrite assay

Confluent cells were stimulated for 24 h with the cytokines cocktail in the presence or absence of either 17β-estradiol or progesterone at different concentrations. Nitrite production was determined in the cell culture supernatants by the Griess' reaction (31). Briefly, 200 μl of the supernatant were placed in each well of a 96-well plate. Subsequently, 20 μl of 6.5 M hydrochloric acid and 20 μl of 37.5 mM sulfanilic acid were added. After incubation for 10 min at room temperature, 20 μl of 12.5 mM *N*(1-napthyl)ethylendiamine was added. Thirty minutes later, the OD was read at 550 nm and compared with a standard curve which showed a linearity range between 0.045 and 2.0 μmol/mg protein. Protein content was determined on cell extracts by the Bradford's method (32) and used to normalize the nitrite values.

Statistical analysis

For NOS II protein accumulation, values are expressed as densitometric units. Data are obtained from three different experiments, and each value represents the mean ± SD. In experiments in which NOS II activity was measured, statistical analysis was performed with one-way ANOVA, followed by Fisher's test. Each value represents the mean ± SEM of three different experiments (run in triplicate).

Results

17β-estradiol removal causes an increase in the NOS II content in SMC

As reported by several authors, the NOS II protein content is barely detectable by Western blot analysis in SMC grown in medium containing phenol red (M199 + FCS; see Fig. 1A, lane 1). However, these cells express a high concentration of NOS II protein in response to inflammatory stimuli, such as 24 h treatment with the cytokine cocktail (10 ng/ml interleukin-1β, 10 ng/ml interferon γ, 25 ng/ml tumor necrosis factor α, and 10 μg/ml lipopolysaccharides), which leads to an increase in NOS immunoreactivity of about 25–70 fold

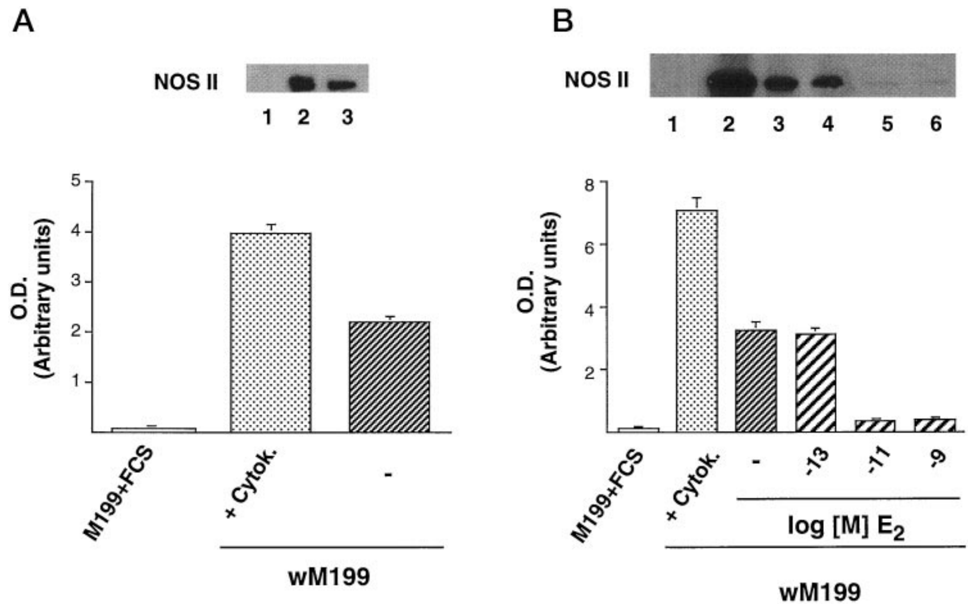

FIG. 1. Estrogen decreases NOS II protein content in SMC. A, Vascular SMC were grown for 6 days in complete medium (M199 + FCS, lane 1) or in phenol red-free medium for 5 days and then deprived of FCS for 24 h (wM199, lanes 2 and 3). Cells were stimulated for 24 h with cytokine-cocktail (+ cytok., lane 2). B, SMC were grown as in A (lanes 1–3) or in the presence of increasing concentrations of 17β-estradiol (10^{-13}–10^{-9} M) (lanes 4–6). Equal amounts of proteins were loaded in each lane (28 μg). Quantitation of NOS II content was performed by scanning densitometry of the 130-kDa band following staining with specific polyclonal anti-NOS II antibody using a peroxidase linked secondary antibody. A Western analysis representative of a single experiment and the graph corresponding to means ± SD of three independent experiments are shown.

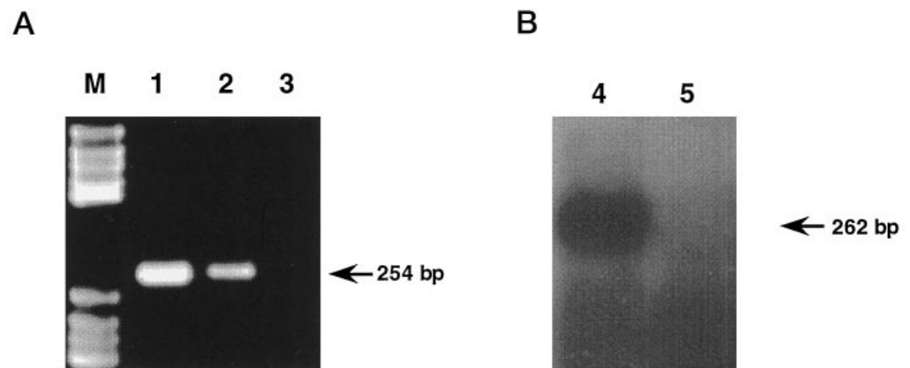

FIG. 2. Expression of ER isoforms mRNA in rat SMC. Total RNA extracted from rat uterus (lane 1) and from SMC at 4th (lane 2, 4 and 5) or 9th passage (lane 3) was assayed for RT-PCR in the presence (lanes 1–4) or absence (lanes 5) of reverse transcriptase. Bands of the expected molecular weight (A, 254 bp for ER-α; B, 262 bp for ER-β) were observed after separation of the amplified product on agarose gel (A) and subsequent Southern blot analysis (B).

with respect to controls. Interestingly, when SMC were grown in the absence of phenol red and serum (wM199), NOS II content was significantly increased (of about 50%) with respect to cytokine-stimulated cells (Fig. 1B, compare lanes 2 and 3). To verify whether this increase was due to the well described estrogen-like activity of phenol red, we tested the effect of estrogen addition. Fig. 1B shows that the presence of 17β-estradiol at concentration as low as 0.01 nM is sufficient to reduce the NOS II protein content to the levels measured in complete medium (M199 + FCS). The effect is maintained at 1 nM 17β-estradiol.

This observation suggests that estradiol may control NOS II synthesis in SMC at concentrations compatible with ER activation.

Analysis of estrogen receptor mRNA content in SMC

To prove that the SMC grown *in vitro* were expressing the estrogen receptor isoforms α and β, total RNA from cells at the 4th and 9th passage of growth was retrotranscribed and amplified by PCR with specific primer sets. Fig. 2A shows that cells at the 4th passage express ERα mRNA (lane 2), while, at the conditions used in this assay, no amplification product was observed from cells grown at the 9th passage (lane 3). In another series of experiments, we could demonstrate that in SMC grown *in vitro* the expression of ERα decreases progressively with time in culture and, by the 8th passage, it falls below the detection limits of our assay. As a consequence, in our experiments we used only cells between the 2nd and 6th passage in culture (data not shown).

In the case of ER-β, to ensure that the amplification product corresponded to the ER-β sequence, a Southern blot analysis of the specific PCR amplification product was performed. Figure 2B shows the autoradiography of RT-PCR and Southern blot for ER-β; a specific amplification signal of the expected size of 262 bp is detected in SMC cDNA (lane 4), whereas a control sample, in which the reverse transcriptase was omitted from the cDNA reaction, did not show any amplification product, suggesting that no DNA contamination was present in any step of the experimental procedure (lane 5).

NOS II content in SMC is controlled by the state of activation of ER

To verify whether activation of the ER was mediating the hormone-dependent decrease in NOS II content previously described, we investigated the effect of two antagonists of ER, ICI 182,780 and tamoxifen, which block the estrogen-induced transcriptional activity of the receptor. Figure 3

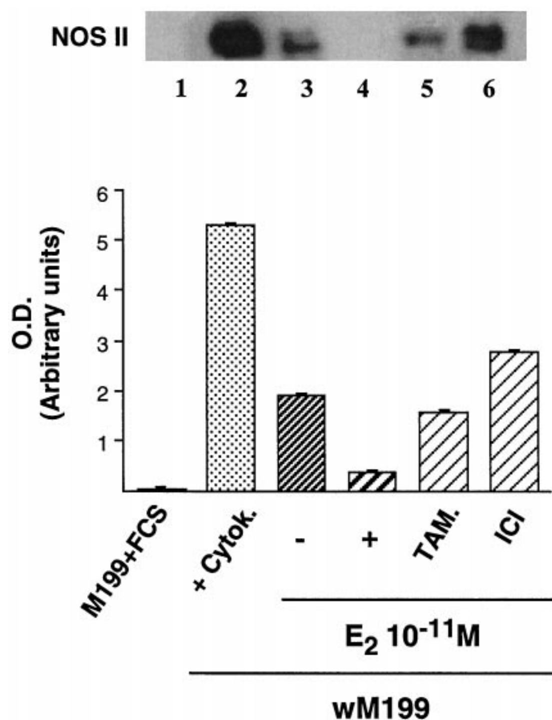

FIG. 3. Tamoxifen and ICI 182,780 blockade of estrogen-induced decrease of NOS II protein content. Cells were treated with 10^{-11} M 17β-estradiol alone or in the presence of 10^{-8} M tamoxifen (TAM, lane 5) or 10^{-8} M ICI 182,780 (ICI, lane 6) for 24 h. Lanes 1 and 2 represent unstimulated and cytokine-stimulated cells, respectively. The Western analysis is representative of a single experiment while the graph reports the means ± SD of three independent experiments are shown.

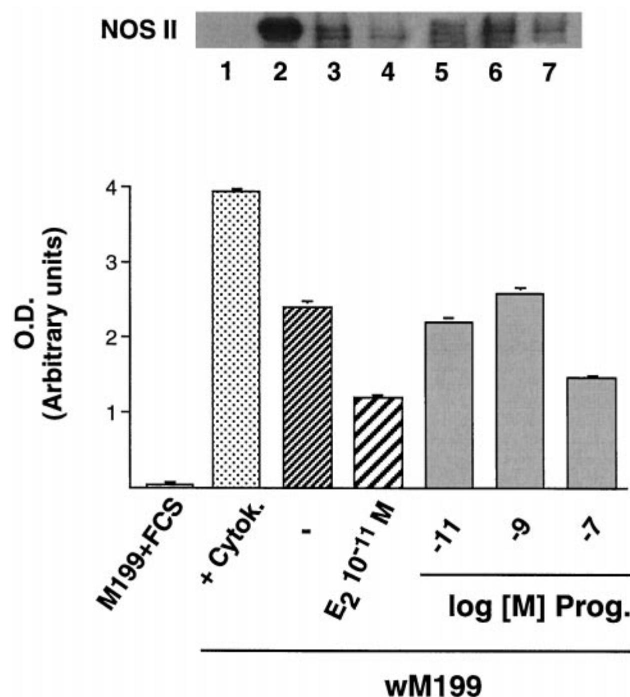

FIG. 4. Progesterone does not affect NOS II protein content in SMC. Control (lane 1), cytokine-stimulated (lane 2) and unstimulated cells (lane 3) grown in wM199 were treated as in Fig. 1. Treatment with 17β-estradiol 10^{-11} M (lane 4) or with increasing concentrations of progesterone (from 10^{-11}–10^{-9} M, lanes 5–7) was carried out for 24 h. A representative Western analysis and graph corresponding to means ± SD of three independent experiments are shown.

shows that in cells grown in medium without estrogen (phenol red-free medium, wM199) NOS II protein immunoreactivity is relatively high, and treatment with 0.01 nM 17β-estradiol reduces its levels of about 90%. A dose of 10 nM tamoxifen was able to block 17β-estradiol effect, leading NOS II protein content to a level very close to that of cells grown in wM199 alone. Interestingly, in the presence of ICI 182,780 the levels of NOS II protein were even higher than in wM199 alone. This observation is not reconcilable with a blockade of the effect of estrogen-like compounds, which might still be present in wM199, because in all of our experiments we failed to see any effect of ICI 182,780 or tamoxifen alone (data not shown). The double band recognized by NOS II antibody, in samples where NOS is present at high concentrations, could result from the recognition of heterogeneous NOS subunits, as reported by other authors (33).

To evaluate the specificity of estrogen action, we also studied the effects on NOS II protein of another sex steroid, progesterone. Unlike 17β-estradiol, progesterone did not affect NOS II levels assessed by immunoreactivity when used at concentrations sufficient to fully activate its cognate receptor (10^{-9} M). A decrease in NOS II accumulation, however, was observed at supraphysiological progesterone concentrations (10^{-7} M) (Fig. 4).

Effects of 17β-estradiol and progesterone on nitrite production and NOS II synthesis

The supernatants of SMC were then assayed to investigate whether the estrogen-induced changes in NOS II protein reflected also changes in nitrite accumulation. As shown in Fig. 5, when nitrites were measured in unstimulated cells, the treatment with estradiol did not have any significant consequence; we believe that this is principally due to the lack of sensitivity of the methodology used. In fact, we did not detect any difference in NO production even in cells grown in M199 + FCS or in wM199, whereas the NOS enzyme levels were significantly different (see results shown in Fig. 1, lanes 1 and 3). Estradiol, however, at concentrations as low as 0.01 nM, significantly diminished the nitrites in the medium of cytokine-stimulated cells. This observation clearly suggests that estradiol significantly reduces the extent of cytokine-induced production of NO. Consistent with our previously data, progesterone was capable of decreasing the nitrite content in the medium only at pharmacological concentrations.

We further investigated whether 17β-estradiol had any effect on NOS II level in cytokine-stimulated cells. Figure 6 shows that estradiol at nanomolar concentrations significantly reduced the cytokine-induction of NOS II protein, thus confirming the results obtained with the nitrite assay.

Discussion

The results reported in this paper prove that estradiol, by interacting with its cognate receptor, reduces the accumulation and activity of NOS II in rat SMC. The finding that ER-α and ER-β are both expressed in these cells indicate that the effect here reported might be due to the activation of either receptors. Previous studies proved that estradiol increases NOS III mRNA and activity in vascular endothelial

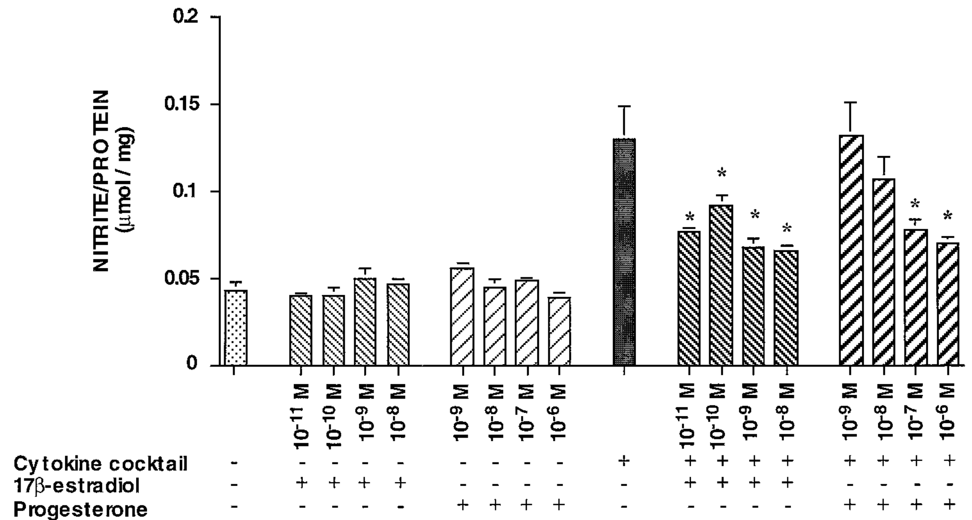

FIG. 5. Dose-response effect of 17β-estradiol and progesterone on cytokine-induced nitrite production. Vascular SMC were assayed in the presence of vehicle (−) or the cytokines cocktail (+) for 24 h and the hormonal effects were assessed by adding increasing concentrations of 17β-estradiol (10^{-11}–10^{-8} M) and progesterone (10^{-9}–10^{-6} M) at the same time as cytokines. Nitrite production was measured in the cell culture supernatants by the Griess' reaction. Data represent the mean ± SEM of three experiments, each run in triplicate. All comparison were made against cytokine-stimulated cells (*, $P < 0.05$: ANOVA followed by Fisher's test for multiple comparison).

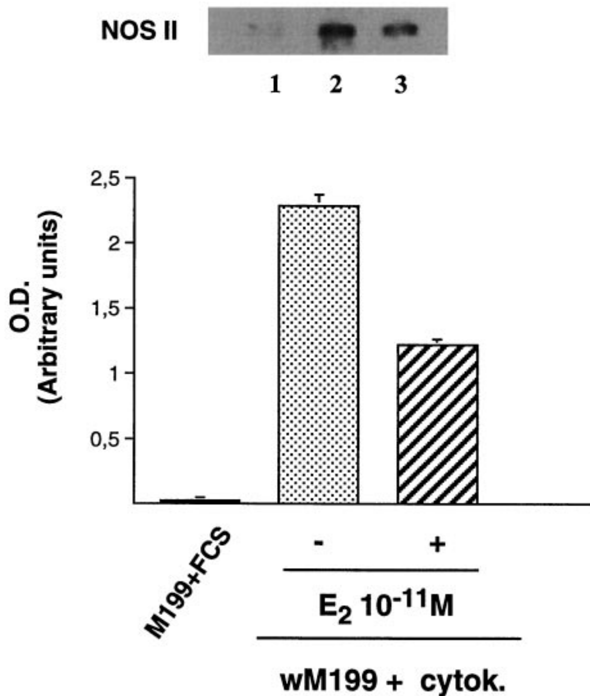

FIG. 6. 17β-estradiol decreases NOS II content in cytokine-stimulated SMC. Vascular SMC were grown in M199 + FCS (lane 1) or in wM199 + cytokine cocktail in the absence (lane 2) or presence of 10^{-11} M 17β-estradiol (lane 3) for 24 h. Data in the graph correspond to means ± SD of three independent experiments.

cells (19, 20) and in a variety of other tissues (34). In the present study, for the first time in a well defined cell system, we provide evidence of an effect of estradiol on NOS II. These results are in agreement with *in vivo* studies by Kauser *et al.* (35). On the other hand, *ex vivo* studies by Binko (36) and Kauser (37), have shown contrasting results in NOS II protein following estradiol treatment of endothelium-denuded aortic rings. The apparent discrepancies might be related to the different protocols of NOS II stimulation and hormone concentration used. However, it is worth to underline that in the above studies the hormonal effect on NOS II protein levels were evaluated in the presence of endothelial cells. Because it is well known that these latter cells are sensitive to estrogen action, their initial presence in the preparation could influence the SMC response.

Unlike the other forms of NOS so far described (NOS I and NOS III) (23), NOS II is considered as the high throughput path for NO production, which is reserved for mechanisms involving host defense, like infection and inflammation. The production of NOS II by SMC may play a role in the formation of the atherosclerotic plaque dependent on sustained inflammatory reactions occurring within the vessel wall. The estrogen-mediated prevention of cytokine-induced production of NO and NOS II by SMC shown in Figs. 5 and 6 suggests the potential for an antiinflammatory role played by this hormone, which might be of great interest also with regard to a possible protective role exerted by estrogen in other pathologies, such as multiple sclerosis (38) and Alzheimer's disease (39–41), in which immune responses play a relevant role. Interestingly, recent studies carried out in macrophages, and our unpublished observations in microglial cells, demonstrated that progesterone (42) and estradiol (43) decrease NO production following cytokine stimulation.

The question as to how estrogen negatively regulates NOS II is still open. It is known that NOS II can be transcriptionally regulated, at least in rodent cells (23). Studies with IL-1β in rat vascular smooth muscle cells (44) and with IL-1β or cAMP-elevating agents (45) in rat glomerular cells prove that NOS II transcriptional control is not restricted to a single cell type or regulator. The complexity of NOS II promoter suggests that a substantial number of factors may participate in its regulation. The fact that we did not identify any consensus binding site for the ER does not eliminate the possibility of a direct interaction between ER and NOS II promoter. By interacting with other factors, such as AP-1 or NF-kB components, estrogen could directly influence NOS II mRNA synthesis (46). It has been shown that NF-κB-mediated induction of the IL-6 promoter, which does not contain any ERE consensus sequence, can be blocked by estrogen through the interaction of the ER with members of the NF-κB family of transcription factors (47–49).

On the other hand, our study does not exclude the possibility of a posttranscriptional control of NOS II mRNA or

protein; several reports proved that NOS II can be regulated by mRNA destabilization or by posttranslational mechanisms also in rat SMC (50).

In conclusion, our study might suggest that estradiol exerts an antiinflammatory activity in rat SMC through a decreased NO production. This effect is not observed with progesterone. Thus, the negative regulation of NOS II enzyme may represent another mechanism associated with the beneficial effects of estrogen in cardiovascular disorders.

Acknowledgments

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References

1. **Barret-Connor E, Bush TL** 1991 Estrogen and coronary heart disease in women. J Am Med Assoc 265:1861–1867
2. **Wenger NK, Speroff L, Packard B** 1993 Cardiovascular health and disease in women. N Engl J Med 329:247–256
3. **Stampfer MJ, Colditz GA, Willett WC, Manson JE, Rosner B, Speizer FE, Hennekens CH** 1991 Postmenopausal estrogen therapy and cardiovascular disease: ten year follow-up from the nurses health study. N Engl J Med 325:756–762
4. **Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, Ernster VL, Cummings SR** 1992 Hormone therapy to prevent disease and prolong life in postmenopausal women. Ann Intern Med 117:1016–1037
5. **Bush TL** 1990 The epidemiology of cardiovascular disease in postmenopausal women. Ann NY Acad Sci 592:263–271
6. **Gruchow HW, Anderson AJ, Barboriak JJ, Sobocinski KA** 1988 Postmenopausal use of estrogen and occlusion of coronary arteries. Am Heart J 115:954–963
7. **Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnikar V, Sacks FM** 1991 Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. N Engl J Med 325:1196–1204
8. **Bush TL, Barret-Connor E, Cowan LD, Criqui MH, Wallace RB, Suchindran CM, Tyroler HA, Rifkind BM** 1987 Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. Circulation 75:1102–1109
9. **Farhat MY, Lavigne MC, Ramwell PW** 1996 The vascular protective effects of estrogen. FASEB J 10:615–624
10. **Mendelsohn ME, Karas RH** 1994 Estrogen and the blood vessel wall. Curr Opin Cardiol 9:619–626
11. **Colbburn P, Buonassisi V** 1978 Estrogen-binding sites in endothelial cell cultures. Science 201:817–819
12. **Nakao J, Chang WC, Murota SI, Orimo H** 1981 Estradiol-binding sites in rat aortic smooth muscle cells in culture. Atherosclerosis 38:75–80
13. **Karas RH, Patterson BL, Mendelsohn ME** 1994 Human vascular smooth muscle cells contain functional estrogen receptor. Circulation 89:1943–1950
14. **Orimo A, Inoue S, Ouchi Y, Orimo H** 1995 Vascular smooth muscle cells possess estrogen receptor and respond to estrogen. Ann NY Acad Sci 758:592–594
15. **Venkov CD, Rankin AB, Vaughan DE** 1996 Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. Circulation 94:727–733
16. **Kuiper GGJM, Gustafsson J-Å** 1997 The novel estrogen receptor-β subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. FEBS Lett 410:87–90
17. **Beato M** 1989 Gene regulation by steroid hormones. Cell 56:335–344
18. **Kauser K, Rubanyi GM** 1994 Gender differences in bioassayable endothelium-derived nitric oxide release from isolated rat aortae. Am J Physiol 267:H2311–H2317
19. **Hayashi T, Fukuto JM, Ignarro LJ, Chaudhuri G** 1995 Gender differences in atherosclerosis: possible role of nitric oxide. J Cardiovasc Pharmacol 22:65–68
20. **Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa T, Hidaka H, Iguchi A** 1995 Estrogen increases endothelial nitric oxide by a receptor-mediated system. Biochem Biophys Res Commun 195:435–441
21. **Cooke JP, Dzau VJ** 1997 Nitric oxide synthase: role in the genesis of cardiovascular disease. Annu Rev Med 48:489–509
22. **Förstermann U, Nakane M, Tracey WR, Pollock JS** 1993 Isoforms of nitric oxide synthase: functions in the cardiovascular system. Eur Heart J [Suppl I] 14:10–15
23. **Förstermann U, Kleinert H** 1995 Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Arch Pharmacol 352:351–364
24. **Buttery LDK, Springall DR, Chester AH, Evans TJ, Standfield N, Parums DV, Yacoub MH, Polak JM** 1996 Inducible nitric oxide synthase is present within human atherosclerotic lesion and promotes the formation and activity of peroxinitrite. Lab Invest 75:77–185
25. **Ross R** 1971 The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibres. J Cell Biol 50:172–186
26. **Skalli O, Ropraz P, Trezclak A, Benzonana G, Gillessen D, Gabbiani G** 1986 A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. J Cell Biol 103:2787–2796
27. **Santagati S, Garnier M, Carlo P, Violani E, Picotti GB, Maggi A** 1997 Quantitation of low abundance mRNAs in glial cells using different polymerase chain reaction (PCR)-based methods. Brain Research Protocols 1:217–223
28. **Santagati S, Bettini E, Asdente M, Muramatsu M, Maggi A** 1993 Theoretical considerations for the application of competitive polymerase chain reaction to the quantitation of a low abundance mRNA: estrogen receptor. Biochem Pharmacol 46:1797–1803
29. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
30. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685
31. **Robbins RA, Springall BR, Worren JB, Kwon OJ, Buttery LDK, Wilson AJ, Adcock IM, Riveros-Moreno B, Moncada S, Polack J, Barnes PJ** 1994 Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. Biochem Biophys Res Commun 198:835–843
32. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248–254
33. **Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF** 1991 Purification and characterisation of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc Natl Acad Sci USA 88:7773–7777
34. **Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S** 1994 Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc Natl Acad Sci USA 91:5212–5216
35. **Kauser K, Sonnenberg D, Tse J, Rubanyi GM** 1997 17beta-estradiol attenuates endotoxin-induced excessive nitric oxide production in ovariectomized rats in vivo. Am J Physiol 273:H506–H509
36. **Binko J, Majewski H** 1998 17β-estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS. Am J Physiol 274:H853–H859
37. **Kauser K, Sonnenberg D, Diel P, Rubanyi GM** 1998 Effect of 17beta-oestradiol on cytokine-induced nitric oxide production in rat isolated aorta Br J Pharmacol 123:1089–1096
38. **Sandyc R** 1996 Estrogen's impact on cognitive functions in multiple sclerosis. Int J Neurosci 86:23–31
39. **Simpkin JV, Green PS, Gridley KE** 1997 A fundamental role for oestrogens in cognition and neuroprotection. In: Brioni JD, Decker MV (eds) Pharmacological Treatment of Alzheimer's Disease: Molecular and Neurobiological Foundations. John Wiley & Sons, New York
40. **Honjo H, Tanaka K, Kashiwagi T, Urabe M, Okada H, Hayashi M, Hayashi K** 1995 Senile dementia of Alzheimer's type and oestrogens. Horm Metab Res 27:204–207
41. **Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R** 1996 Effect of oestrogens during menopause on risk and age at onset of Alzheimer's disease. Lancet 348:429–433
42. **Miller l, Alley EW, Murphy WJ, Russell SW, Hunt JS** 1996 Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. J Leukoc Biol 59:442–450
43. **Hayashi T, Yamada K, Esaki T, Muto E, Chaudhuri G, Iguchi A** 1998 Physiological concentrations of 17beta-estradiol inhibit the synthesis of nitric oxide synthase in macrophages via a receptor-mediated system. J Cardiovasc Pharmacol 31:292–298
44. **Perrella MA, Yoshizumi M, Fen Z, Tsai JC, Hsieh CM, Kourembanas S, Lee ME** 1994 Transforming growth factor-beta 1, but not dexamethasone, downregulates nitric-oxide synthase mRNA after its induction by interleukin-1 beta in rat smooth muscle cells. J Biol Chem 269:14595–14600
45. **Kunz D, Mühl H, Walker G, Pfeilschifter** 1994 Two distinct signalling pathways trigger the expression of inducible nitric oxide synthase in rat renal mesangial cells. Proc Natl Acad Sci USA 91:5387–5391
46. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
47. **Xie Q, Kashiwabara Y, Nathan C** 1994 Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 269:4705–4708
48. **Kleinert H, Euchenhofer C, Ihrig-Biedert I, Förstermann U** 1996 In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF-κB. J Biol Chem 271:6039–6044
49. **Kurebayashi S, Miyashita Y, Hirose T, Kasayama S, Akira S, Kishimoto T** 1997 Characterisation of mechanisms of interleukin-6 gene repression by estrogen receptor. J Steroid Biochem Mol Biol 60:11–17
50. **Nathan C, Xie Q-W** 1994 Regulation of biosynthesis of nitric oxide. J Biol Chem 269:13725–13728