Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells

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Received 1 February 1995

Abstract We have examined the effects of sex hormones on calcium-dependent NO production and protein levels of NO synthase in cultured human aortic endothelial cells, which were treated with various doses of 17β-estradiol and testosterone for 8–48 h. Treatment with 17β-estradiol enhanced calcium-dependent NO production, but testosterone had exerted no effect. Western blot using monoclonal anti-human endothelial NO synthase antibody clarified that increased NO production by 17β-estradiol treatment was accompanied by increased NO synthase protein. Our results provide evidence that human endothelial NO synthase can be regulated by estrogens.

Key words: Nitric oxide; Human endothelial cell; Estrogen; NO synthase; Testosterone; Atherosclerosis

1. Introduction

Nitric oxide (NO) is a cellular messenger with numerous biological functions, including regulation of vascular tone, cellular signaling in the brain, and killing of pathogens in nonspecific immune responses. NO is derived from the guanidino nitrogen atom of L-arginine through a reaction catalyzed by NO synthase (NOS) (EC 1.14.13.39). NOS was originally divided into two functional classes based on sensitivity to calcium, but differences in properties and differential inhibitor/substrate specificity suggest that there are three major forms of NOS. This has recently been confirmed by the cloning and characterization of three distinct NOS cDNAs [1–3]. This permits the definitive classification of the gene products so far characterized in this way as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) [4].

Many epidemiological studies have demonstrated that there is a gender difference in the morbidity associated with hypertension. In some animal experiments, administration of estradiol has been reported to increase endothelium-dependent relaxation [5,6], and female rabbit aorta has been reported to release a higher basal level of NO than that of male [7]. These results suggest that eNOS might be regulated by sex hormones. But the effect of estrogen on eNOS mRNA level is controversial in bovine [8] and porcine [9] endothelial cells. Recently, Weiner et al. reported that calcium-dependent NOS was induced during pregnancy in guinea pigs [10]. The aim of this study is to clarify, whether, at physiological levels, sex hormones modulate NO production and eNOS protein in human aortic endothelial cells. We tested this hypothesis in cultured human aortic endothelial cells by measuring calcium-dependent NO production and level of eNOS protein in both presence and absence of sex hormones.

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Abbreviations: NO, nitric oxide; NOS, NO synthase; cNOS, constitutive NOS; iNOS, inducible NOS; nNOS, neuronal NOS; HAEC, human aortic endothelial cells; LNMMA, N^G-monomethyl-L-arginine.

2. Materials and methods

2.1. Cell Culture

Human aortic endothelial cells (HAEC) were obtained from Krabo (Osaka, Japan). Cells were seeded onto a collagen-coated 24-multiwell plate or 35 mm dish (Corning, NY, USA), at an initial density of 1×10^5 cells/cm^2, containing 500 μl or 2 ml of Endomedia (Immuno Bio Laboratory, Gunma, Japan), 10% fetal calf serum (Mitsubishi-kasei, Tokyo, Japan). Cells were used between passages 2 and 4.

2.2. Measurement of NO production

After treatment, each well was washed 3 times with 1 ml of Hanks' solution and then incubated at 37°C for 30 min, before stimulation with 1 μM ionomycin. The ionomycin-stimulated NO production from HAEC was measured as the levels of nitrite/nitrate, oxidized products of nitric oxide, using an automated system TCI-NOX 1000 (Tokyo Kasei Kogyo Co., Tokyo, Japan) based on the Griess reaction [11]. Nitrite/nitrate values at time 0, just after ionomycin stimulation, were subtracted from cumulative values, during the 30 min ionomycin stimulation period, obtained from the same wells.

2.3. Western blot

eNOS protein was analyzed by Western blot with anti-human eNOS antibody (Transduction Laboratory, Kentucky, USA). The antibody was used at 250 times dilution. This antibody is specific for human eNOS: the Transduction Laboratory confirmed that it does not cross-react with nNOS and iNOS. The confluent cells in 35 mm dishes (1×10^6 cells/dish) were washed with Hank's solution and then lysed in 200 μl of solution containing 10% glycerol, 2.3% SDS, Tris-HCl (pH 6.8) 62.5 mM, 0.01% Bromophenol blue and 5% mercaptoethanol. The lysate was then heated at 100°C for 5 min. Whole cell lysates which contained 50 μg of protein were subjected to SDS-PAGE (7.5% gradient). The separated proteins were electrophoretically transferred to membranes, then incubated with anti-human eNOS antibody for 1 h as previously described [12].

2.4. Statistical analysis

The data are expressed as the means and S.E.M.s in the text and figures. Statistical significance was assessed by *t*-test, and a *P* value less than 0.05 was considered significant.

3. Results

Without stimulation, NO production was below 50 pmol/h/10^5 cells. In acute experiments, HAEC were stimulated with ionomycin along with 17β-estradiol (2×10^4 pg/ml) or testosterone (2×10^4 pg/ml). Neither hormone showed significant effects on ionomycin-stimulated NO production (Fig. 1A, time 0).

SSDI 0014-5793(95)00124-7

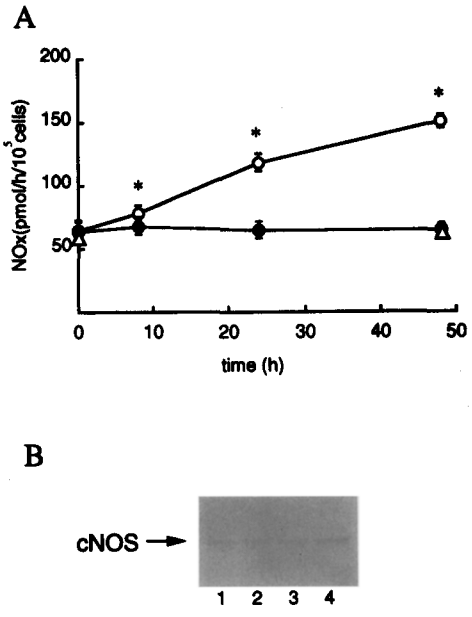

Fig. 1. Time courses of 17β-estradiol and testosterone effects on calcium-dependent NO production (A) and human endothelial cNOS protein (B). (A) Cells were treated with either 2×10^4 pg/ml of 17β-estradiol or testosterone for the indicated times. After sex hormone treatments, NO production was stimulated by ionomycin (1 μM) and accumulated nitrite and nitrate were measured. Nitrite and nitrate values at time 0 were subtracted from cumulative values after ionomycin stimulation for 30 min obtained from the same wells. Closed circles indicate no treatment. Open circles indicate treatment with 2×10^4 pg/ml of 17β-estradiol. Open triangles indicate treatment with 2×10^4 pg/ml of testosterone. Means ± S.E.M. are shown ($n = 6$). *$P < 0.05$ compared with the no treatment value at time 0. (B) eNOS protein was analyzed by Western blot with the anti-human eNOS antibody (lanes 1–4, 50 mg of protein per lane). Data are representative of 4 experiments. Lane 1, control; lane 2, 2×10^4 pg/ml of 17β-estradiol for 12 h; lane 3, 24 h; lane 4, 48 h.

Next, we examined the long term effects of treatment with 17β-estradiol or testosterone on ionomycin-stimulated NO production. Pretreatment for more than 8 h with 17β-estradiol significantly increased the ionomycin-stimulated NO production, while testosterone showed no effect even at 48 h (Fig. 1A). Western blot using anti-human eNOS antibody also clarified that pretreatment with 17β-estradiol increased eNOS protein in a time dependent manner (Fig. 1B).

Fig. 2A shows the dose response curve of 17β-estradiol on ionomycin-stimulated NO production. Above 200 pg/ml, 17β-estradiol significantly increased calcium-dependent NO production in a dose dependent manner. The results of Western blot (Fig. 2B) also support the notion that 17β-estradiol increased NO production by increases in eNOS protein.

To clarify the mechanism of induction of eNOS, HAEC were pretreated with tamoxifen (1 μM) for 4 h. The 17β-estradiol (2×10^4 pg/ml) was then added to the medium and the culture was continued for another 48 h. As a negative control, L-N^G-monomethyl-arginine (LNMMA) was added just before ionomycin stimulation. LNMMA almost completely inhibited ionomycin-stimulated NO production both in control and 17β-estradiol treatment. Tamoxifen itself showed no effect on ionomycin-stimulated NO production, but showed significant inhibition on estrogen-induced increase in ionomycin-stimulated NO production. (Fig. 3). Subtracting the value of 17β-estradiol treatment from no treatment, the inhibitory effect of tamoxifen was calculated to be about the half that of the estrogen-induced increase in ionomycin-stimulated NO production.

4. Discussion

Our studies demonstrate for the first time that eNOS increases in response to 17β-estradiol in human aortic endothelial cells. Moreover, physiological concentrations of 17β-estradiol, which range from 2 to 2×10^4 pg/ml [13], cause this induction. The highest concentration occurs during pregnancy, reaching approximately 2×10^4 pg/ml. This suggests that a protective effect on the cardiovascular system, exerted by 17β-estradiol supplementation, is related to eNOS induction in vivo. Our results may also explain why pre-menopausal women are less prone than men to coronary artery disease and why there is an increased prevalence of cardiovascular disease in post-menopausal women.

Human eNOS has been clarified at the gene level, and some regulatory sequences including a shear stress responsive element, heavy meal responsive elements, and an AP-2 binding site have been reported in the 5′ flanking region of the human eNOS gene [14]. However, in human eNOS, the existence of an estrogen responsive element (5′-CAGGTCAGAGTGACCTG-3′) [15] remains controversial. Two left half palindromic sites of an estrogen responsive element (GGTCA) exist at −1,347 and −444 and four right half sites (TGACC) exist at −1,198, −774, −463 and −310, but the complete sequence has yet to be

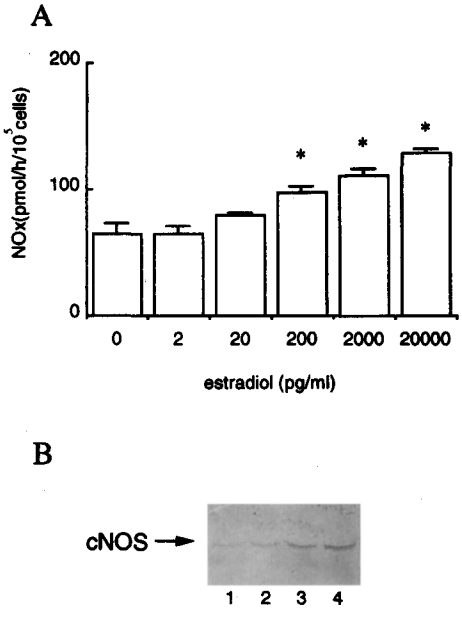

Fig. 2. Dose–response curves of 17β-estradiol effects on calcium-dependent NO production (A) and increased eNOS protein (B). (A) Cells were treated with various doses of 17β-estradiol for 48 h. After 17β-estradiol treatment, NO production was stimulated with ionomycin (1 μM) and accumulated nitrite and nitrate were measured. Nitrite and nitrate values at time 0 were subtracted from cumulative values after ionomycin stimulation for 30 min obtained from the same wells. Means ± S.E.M. are shown ($n = 6$). *$P<0.05$ compared with the value at time 0. (B) eNOS protein was analyzed by Western blot with the anti-human eNOS antibody (lanes 1–4, 50 μg of protein per lane). Data are representative of 4 experiments. Lane 1 indicates control; lane 2, 2×10^2 pg/ml of 17β-estradiol for 48 h; lane 3, 2×10^3; lane 4, 2×10^4.

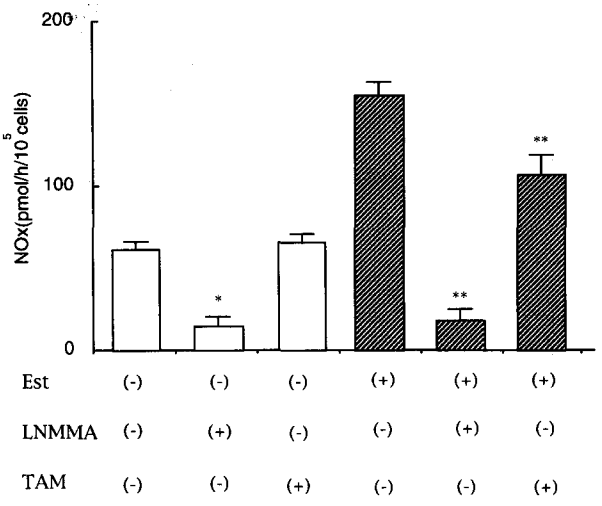

Fig. 3. Effects of N^G-monomethyl-L-arginine and tamoxifen on estradiol-induced NO production. NOx was measured as the nitrite and nitrate production resulting from stimulation with 1 μM of ionomycin in each case. Est indicates treatment with 17β-estradiol (2×10^4 pg/ml) for 48 h. LNMMA indicates treatment with 500 μM of LNMMA just before stimulated by ionomycin. TAM indicates treatment with 1 μM tamoxifen for 48 h. When cells were treated with both tamoxifen and 17β-estradiol, tamoxifen was added 4 h before addition of 17β-estradiol. Means ± S.E.M. are shown ($n = 6$). *$P < 0.05$ compared with the value of no treatment cells. **$P < 0.05$ compared with the value of 17β-estradiol treated cells. Abbreviations are: LNMMA, N^G-monomethyl-L-arginine; TAM, tamoxifen.

clarified. Recently Rosseli et al. reported that estrogen supplementation increased NO production in vivo, based on the results of a two year clinical study [16]. Taken together with our data, these results [10,16] suggest that the partial sequences may have functional roles as responsive elements in eNOS.

We used tamoxifen to investigate the roles of estrogen receptors. As compared to LNMMA, the tamoxifen inhibition was weaker though still significant. This may be explained by tamoxifen being a partial agonist of estrogen receptors. We cannot exclude the possibility that 17β-estradiol exerts its effect by binding to its receptors, or, by modulating the calcium-calmodulin system in a manner similar to that of nNOS [17].

eNOS plays important roles in maintaining homeostasis of the cardiovascular system. It is, therefore, important to clarify the regulation of eNOS, but it has not been thoroughly investigated. Cytokines have been reported to down-regulate mRNA of eNOS [18,19], but to augment NO production [18,19]. There are also discrepancies among reports about the effects of estrogen on mRNA levels of eNOS between bovine and porcine endothelial cells [8,9]. To clarify these discrepancies, it was necessary to examine the effect of estrogen on protein levels of eNOS in human endothelial cells. Herein, we have demonstrated that estrogen up-regulates eNOS proteins in human aortic endothelial cells and our results also support the report about pregnant guinea pigs [10]. These findings encourage further studies of sex hormones for therapeutic intervention in NO-mediated changes in vascular tone in man.

Acknowledgements: This work was supported in part by Special Grant-in-Aid for Innovative Collaborative Research Projects from Keio University.

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