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Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production

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ABSTRACT **Estradiol is known to exert a protective effect against the development of atherosclerosis, but the mechanism by which this protection is mediated is unclear. Since animal studies strongly suggest that production of endothelium-derived relaxing factor is enhanced by estradiol, we have examined the effect of estrogens on nitric oxide (NO) synthase (NOS) activity, protein, and mRNA in cultured bovine aortic endothelial cells. In reporter cells rich in guanylate cyclase, it has been observed that long-term treatment (≥24 hr) with ethinylestradiol (EE_2) dose-dependently increased guanylate cyclase-activating factor activity in the conditioned medium of endothelial cells. However, conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline by endothelial cell homogenate or quantification of nitrite and nitrate released by intact cells in the conditioned medium did not reveal any change in NOS activity induced by EE_2 treatment. Similarly, Western and Northern blot analyses did not reveal any change in the endothelial NOS protein and mRNA content in response to EE_2. However, EE_2 dose- and time-dependently decreased superoxide anion production in the conditioned medium of endothelial cells with an EC_{50} value (0.1 nM) close to that which increased guanylate cyclase-activating factor activity (0.5 nM). Both of these effects were completely prevented by the antiestrogens tamoxifen and RU54876. Thus, endothelium exposure to estrogens appears to induce a receptor-mediated antioxidant effect that enhances the biological activity of endothelium-derived NO. These effects could account at least in part for the vascular protective properties of these hormones.**

The incidence of cardiovascular disease, the leading cause of mortality in western societies, is higher in men than in premenopausal women but increases in postmenopausal women. An abundance of epidemiological data supports a role for estrogens in this atheroprotective effect, prompting recommendations for their widespread use in postmenopausal replacement therapy (1, 2). However, the mechanism whereby this protection is mediated remains obscure. It is traditionally thought to be due to potentially favorable changes in blood lipids and lipoproteins (1), but a number of human (3) as well as animal studies strongly suggest a direct effect on the vascular system (4–6) and more specifically that basal endothelium-derived relaxing factor is enhanced in estradiol-treated females compared with oophorectomized controls (7–11).

The endothelium-derived relaxing factor has been identified as nitric oxide (NO) or a closely related compound derived from the amino acid L-arginine, able to induce stimulation of the soluble guanylate cyclase enzyme contained in vascular smooth muscle cells (12–14). The endothelial NO synthase (NOS), which catalyses the generation of NO together with L-citrulline from an unusual five electron oxidation of one nitrogen of the L-arginine guanidino group, has been purified (15, 16) and its cDNA sequence determined (17–20). Endothelial NOS is a complex enzyme whose activity requires several cofactors [NADPH, FAD, flavine adenine mononucleotide, and tetrahydrobiopterin] and depends on calmodulin and calcium. Upon acute stimulation by numerous hormones (acetylcholine, bradykinin, and others) that increase intracellular calcium, the enzyme is phosphorylated and translocates from membrane to cytosol, although the precise effect of these two changes is presently unknown (21) and NO release is stimulated. In contrast with the large number of stimuli which regulate endothelial NOS activity acutely, only a few factors are known to regulate its gene expression. Exercise training (22) and mechanical stimuli such as shear stress (19) and cell proliferation (23) increase endothelial NOS mRNA and protein, whereas tumor necrosis factor α decreases endothelial NOS mRNA posttranscriptionally (24).

Because the molecular mechanisms of the endothelium-derived relaxing factor-enhanced activity observed in estradiol-treated animals has not yet been precisely elucidated, we sought to determine the effects of estradiol on NO production and simultaneously on NOS mRNA, protein, and activity in a well-characterized culture system of endothelial cells.

MATERIALS AND METHODS

Cell Culture and Materials. Bovine aortic endothelial cells (BAEC) were obtained and grown as described (25, 26) in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated charcoal-treated newborn calf serum at 37°C and 1 ng of basic fibroblast growth factor per ml in 10% CO_2-containing humidified atmosphere. The cells used in this study were between the 5th and 15th passage. Several measures were taken to avoid artifacts of cell culture on BAEC phenotype due to proliferation, as reported (23). All passages were made using a splitting ratio of 1:4. Confluency was determined by visual inspection of the cells when >95% of the cells were in contact with adjacent cells. Under our culture conditions, the cells invariably reached confluency 2 days after passage, and all the experiments were done in BAEC 3 days after confluency (100,000 cells/cm^2). For estrogen stimulation, the cells were treated with ethinylestradiol (EE_2) instead of estradiol to prevent metabolism of the steroid hormone (26).

All reagents were purchased from Sigma except when specified. RU54876 was obtained from Roussel–UCLAF (27), and tamoxifen was from ICI. Protein concentrations were deter-

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Abbreviations: NO, nitric oxide; NOS, NO synthase; BAEC, bovine aortic endothelial cells; EE_2, ethinylestradiol; SOD, superoxide dismutase.

mined using the Bio-Rad Coomassie brilliant blue G-250 method, with bovine serum albumin as standard.

Measurement of NO Bioactivity Using RFL6 Reporter Cells. NO bioactivity was measured as the concentration of guanylate-cyclase stimulating activity produced in BAEC supernatant following the method described by Ishii *et al.* (28). BAEC were cultured in 6-well plates (900,000 cells per well). Briefly, after the culture medium had been removed, BAEC were washed twice with 2 ml of Locke's buffer (154.0 mM NaCl/5.6 mM KCl/2.0 mM $CaCl_2$/1.0 mM $MgCl_2$/3.6 mM $NaHCO_3$/5.6 mM glucose/10.0 mM Hepes, pH 7.4) and equilibrated for 20 min in 1 ml of the same buffer at 37°C. Five minutes before sampling or stimulation, superoxide dismutase (SOD, 100 units/ml) was added to the incubation medium. In some experiments, cells were simultaneously treated with the calcium ionophore A23187 (calcimycin, 1 μM) for 2 min. Conditioned medium (600 μl) was then transferred to RFL6 incubation dishes. The RFL6 cells themselves had been cultured to confluency in 6-well plates (200,000 cells per well), washed as described above in Locke's buffer, and then equilibrated for 20 min in 1 ml of the same buffer containing 0.3 mM 3-isobutyl-1-methylxanthine at 37°C. Five minutes before transfer of the BAEC conditioned medium, SOD was also added to the RFL6 incubation.

After 3 min, the RFL6 medium was removed, ice-cold 4 M KOH solution and then 50 mM sodium acetate buffer (pH 4.0) were added, and the samples were stored frozen (−80°C). cGMP levels in RFL6 cells were determined by enzyme immunoassay after acetylation (Cayman Chemicals, Ann Arbor, MI). All measurements of a single experiment were made in the same enzyme immunoassay serum with an intra-assay coefficient of variability of 10%.

Measurement of NOS Activity by Conversion of L-[U-^{14}C]-Arginine to L-[U-^{14}C]Citrulline. NOS activity was evaluated by conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline as described (21). BAEC from 60-cm^2 dishes were washed three times with cold phosphate-buffered saline (PBS), scraped with a rubber policeman, collected in centrifuge tubes, and spun at 500 × *g* for 6 min. The cells were then homogenized with a Dounce homogenizer in 120 μl of homogenization buffer (50.0 mM Tris·HCl/0.1 mM EDTA/0.1 mM EGTA, pH 7.5/1 μM pepstatin A/2 μM leupeptin/1 μM bestatin/1 mM phenylmethylsulfonyl fluoride/0.1% 2-mercaptoethanol). The homogenates (500–600 μg total protein) were then assayed for NOS activity. Each sample (100 μl) was incubated in a buffer (50.0 mM Tris·HCl/0.1 mM EDTA/0.1 mM EGTA, pH 7.5) containing the cofactors (100 nM calmodulin/2.5 mM $CaCl_2$/1 mM NADPH/3 μM tetrahydrobiopterin) and the substrate {100 μM L-arginine/L-[U-^{14}C]arginine (0.2 μCi; 1 Ci = 37 GBq; specific activity, 55 Ci/mmol; Amersham} for 15 min at 37°C. The mixture also contained 1 mM L-citrulline to minimize any conversion of the formed L-[U-^{14}C]citrulline back to L-[U-^{14}C]arginine (29). After the incubation period, the reaction was quenched by addition of 1 ml of stop buffer (20 mM Hepes/2 mM EDTA/2 mM EGTA, pH 5.5). The reaction mixture was applied to a 1-ml column containing Dowex AG 50WX-8 resin (Na^+ form; Bio-Rad) that had been preequilibrated with the stop buffer. L-[U-^{14}C]Citrulline was eluted twice with 0.5 ml of stop buffer, and the radioactivity was determined by liquid scintillation counting.

Measurement of NOS Activity by Quantification of NO Release. The production of NO was evaluated by measuring nitrite (NO_2^-) and nitrate (NO_3^-), the stable degradation products of NO, as described (30). BAEC, grown in 6-well dishes, were washed gently three times with modified Krebs/Hepes buffer, pH 7.4 (99.00 mM NaCl/4.69 mM KCl/1.87 mM KCl/1.87 mM $CaCl_2$/1.20 mM $MgSO_4$/25.00 mM $NaHCO_3$/1.20 mM K_2HPO_4/20.00 mM Hepes/11.10 mM D-glucose), and then incubated in 1 ml of the Krebs/Hepes buffer with or without 1 μM A23187 at 37°C for 1 hr. Two hundred microliters of the buffer was then injected into a reflux chamber containing Vanadium III dissolved in HCl heated to >85°C to reduce both nitrite and nitrate stoichiometrically to NO. The released NO was purged with a stream of nitrogen gas directed via a vacuum into the reaction chamber of a chemiluminescence NO analyzer (Cosma, Igny, France). The chemiluminescence analyzer was calibrated daily using nitrate standards. The amounts of nitrogen oxides released were normalized to the protein content in the respective culture dish.

Western Blot Analysis. BAEC were split in 20-cm^2 dishes and protein homogenates prepared as described above for measurement of NOS activity. Protein was size-fractionated electrophoretically (31) using SDS/7.5% polyacrylamide gel and transferred to nitrocellulose membranes blocked with 5% casein Tris (15 mM)-buffered saline solution (pH 7.6)/0.1% Tween (TBS-T) at room temperature. The membranes were incubated with a 1:2000 dilution of a monoclonal antibody against a peptide from the sequence of human endothelial NOS crossreacting with the bovine enzyme (Transduction Laboratories, Lexington, KY), washed four times with TBS-T, then incubated with a sheep antimouse secondary antibody conjugated to horseradish peroxidase (Amersham) and washed again. Signals were detected using the ECL detection system (Amersham) and autoradiography films (Hyperfilm ECL; Amersham). To quantify the NOS protein content, serial dilutions (4, 6, 9, and 12 μg of protein per lane) were analyzed. The films were then scanned using a densitometer, and a graph of peak area plotted against protein concentration.

RNA Isolation, Northern Blot Analysis, and Hybridization. BAEC, grown in 60-cm^2 dishes, were washed twice with 10 ml PBS and then lysed in guanidinium isothiocyanate. Total RNA was isolated using phenol extraction according to Chomczynski and Sacchi (32). The RNA (10 and 20 μg) was size-fractionated on a 1% agarose/3% formaldehyde gel and subsequently transferred to a nitrocellulose membrane. Hybridizations were performed overnight using a [^{32}P]dCTP (Amersham) labeled, random primed, 2.1-kb cDNA fragment of bovine endothelial NOS obtained by *Sst* I (GIBCO/BRL) digestion of the full-length bovine NOS × NOS cDNA (19). The hybridization solution contained 50% formamide, 100 μg of sheared salmon sperm DNA per ml in 5× standard saline citrate (SSC), 5× Denhardt's solution, 10% dextran sulfate, and 1% SDS at 42°C. The membranes were then washed twice with 2× SSC/1% SDS for 30 min at 55°C, and subsequently once with 0.2× SSC/0.1% SDS for 30 min at 55°C. In all studies, the nitrocellulose membranes were stripped and rehybridized with a β-actin cDNA as a control.

Measurement of Superoxide Anion Production. Superoxide production was measured as the SOD inhibitable reduction of cytochrome *c* (33–35). BAEC (400,000 cells/well) were preincubated with Hanks' balanced salt solution (HBSS) for 15 min at 37°C, washed once with HBSS, and incubated with 1 ml HBSS containing 1 mg of cytochrome *c* per ml with or without SOD (200 units/ml) in humidified air on a shaking table. Cytochrome *c* reduction was determined at zero time to obtain basal values, and after the indicated time, the absorbance of the medium was read spectrophotometrically at 550 nm against a distilled water blank. Reduction of cytochrome *c* in the presence of SOD was substracted from the values without SOD: the proportion of superoxide specific reduction of cytochrome *c* (i.e., SOD inhibitable) was between 30% to 50% according to the experiments. The OD differences between comparable wells with or without SOD were converted to equivalent superoxide anion release by using the molecular extinction coefficient for cytochrome *c* of 21 $mM^{-1} \cdot cm^{-1}$. The superoxide anion production was linear during the time period of the experiments (90 min) and expressed in nmol per mg of protein per hour.

Statistical Analysis. The data are expressed as mean ± SD. Comparisons of data between different groups were made by ANOVA and a Scheffe's post-hoc test used when differences were indicated.

RESULTS

Effect of EE_2 on NO Bioactivity Using RFL6 Reporter Cells. We first studied the effect of EE_2 on NO expressed as the concentration of guanylate cyclase-stimulating activity produced by BAEC. As shown in Fig. 1*A*, treatment for 24–48 hr with increasing concentration of EE_2 from 0.1 nM to 0.01 μM induced guanylate cyclase-stimulating activity in the conditioned medium of BAEC as estimated by the increase in cGMP formation in RFL6 cells. The peak response was obtained at 1 nM and the EC_{50} value for this hormone was evaluated at 0.5 nM. We did not detect any effect when the duration of the treatment was 12 hr or less (data not shown). 17α-Estradiol (0.01 μM) was ineffective. As shown in Fig. 1*B*, the guanylate cyclase-stimulating activity in the conditioned medium of BAEC treated for 2 min with 1 μM of the calcium ionophore A23187 increased dramatically both in untreated cells and in cells treated for 2 days with 1 nM EE_2. Interestingly, the 2-fold increase in EE_2-treated cells compared to untreated cells was in the same range than the increase measured in basal conditions. cGMP levels in RFL6 cells were not affected by A23187 (not shown). Finally the increase in guanylate cyclase-stimulating activity induced by A23187 in EE_2-treated cells could be prevented by the antiestrogens tamoxifen (1 μM) or RU54876 (0.05 μM).

Effect of EE_2 on NOS Activity. We examined the NOS activity by measuring conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline from EE_2-treated cells (0.01 μM for 48 hr) and control endothelial cell homogenates. The NOS activity in homogenates of EE_2-treated cells was not significantly different from control cells (10.3 ± 1.8 and 10.1 ± 1.6 pmol/mg of protein per min, respectively; n = 5 in each group; representative of two experiments). The conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline from both EE_2-treated cells and control cells was abolished by removal of NADPH and inhibited >90% by addition of 30 μM N^{ω}-methyl-L arginine (not shown).

We then assessed the effect of EE_2 on NOS activity in intact BAEC (Fig. 2). The basal release of nitrite and nitrate and the stimulated release by 1 μM A23187 were not significantly different in EE_2-treated cells and control endothelial cells.

Effect of EE_2 on NOS Protein and mRNA Content in BAEC. Scanning densitometry of Western blot analysis was used to quantify the NOS protein content from serial dilutions of control and EE_2-treated (0.01 μM EE_2 for 48 hr) cell homogenates (Fig. 3*A*). This approach did not reveal any change in the EE_2-treated cells compared with the controls.

Fig. 3*B* shows a representative Northern blot analysis of NOS mRNA levels from BAEC treated with 0.01 μM EE_2 for 48 hr. The size of the NOS transcript was similar (4.2 kb). The amount of NOS transcripts was similar in EE_2-treated cells compared with control cells, as were the amounts of β-actin transcripts or of 18S and 28S rRNA abundance.

Effect of EE_2 on Superoxide Anion Production. We then studied the effect of EE_2 on superoxide anion production of BAEC. As shown in Fig. 4, the superoxide anion production of BAEC cells was linear and was stimulated about 3-fold by 1 μM A23187, but when BAEC were pretreated with EE_2 (0.01 μM for 48 hr), this stimulation was strongly inhibited. As shown in Fig. 5*A*, treatment with increasing concentrations of EE_2 from 0.01 nM to 0.01 μM for 48 hr decreased superoxide anion production in the conditioned medium of BAEC in a dose-dependent manner. The peak response was obtained between 1 nM and 0.01 μM and the EC_{50} value was evaluated at 0.1 nM. EE_2 treatment at 0.01 μM induced a time-dependent decrease in superoxide anion production, the onset of the effect beginning after 16 hr exposure (Fig. 5*B*). Treatment with 17α-estradiol (0.01 μM) was ineffective. Finally, the inhibition of superoxide anion production promoted by 1 nM EE_2 (6.9 ± 2.2) vs. control (11.7 ± 2.4 nmol/mg of protein per hr) was completely prevented by the antiestrogens 1 μM tamoxifen (12.3 ± 3.1 nmol/mg of protein per hr; n = 2) or 0.05 μM RU54876 (10.2 ± 2.8, n = 3).

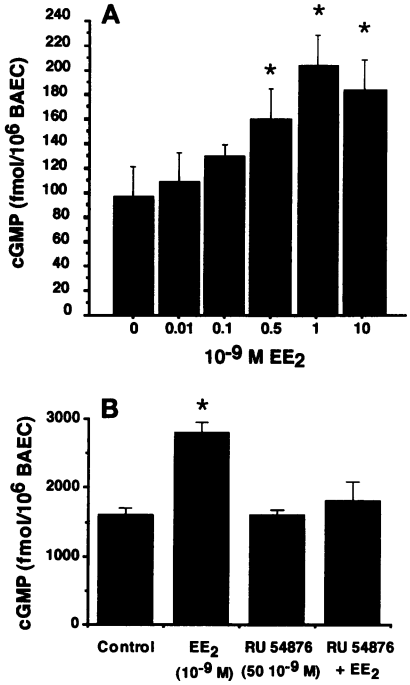

FIG. 1. Effect of EE_2 on the NO bioactivity produced by BAEC. (*A*) Dose-response relationship using varying concentrations of EE_2 treatment for 48 hr. NO bioactivity was measured as the guanylate cyclase-stimulating activity using RFL6 reporter cells as described in ref. 28. Results are given as mean ± SD of cGMP generated in triplicate measurements and are representative of three separate experiments. Similar data were obtained after 24- and 72-hr treatments (two experiments). (*B*) NO bioactivity induced by the calcium ionophore A23187 (1 μM for 2 min) in the supernatant of untreated BAEC (Control), or cells treated for 48 hr with EE_2 (1 nM), RU54876 (0.05 μM), or EE_2 plus RU54876. The data are mean ± SD of triplicate measurements and representative of two experiments. Similar results were obtained using another estrogen antagonist, tamoxifen (1 μM). *, $P < 0.01$.

DISCUSSION

In the present experiments we sought to examine the effect of EE_2 on NO production in endothelium. From the amount of cGMP induced in the RFL6 reporter cells, we first found that chronically EE_2-treated BAEC released more biologically active NO in their culture medium than control cells, both under basal and stimulated conditions. The EC_{50} value for this

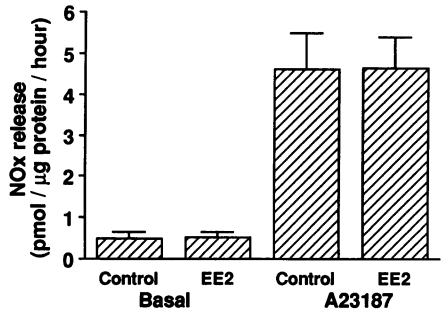

FIG. 2. Release of nitrogen oxides (NOx) from control or EE_2-treated (0.01 μM for 48 hr) BAEC without (basal) or stimulated with 1 μM A23187 at 37°C for 1 hr. NOx [nitrite (NO_2^-) and nitrate (NO_3^-)] was stoichiometrically reduced to NO· and subsequently quantified by chemiluminescence NO analyzer. Data are the average of triplicate incubations ± SD and representative of three experiments.

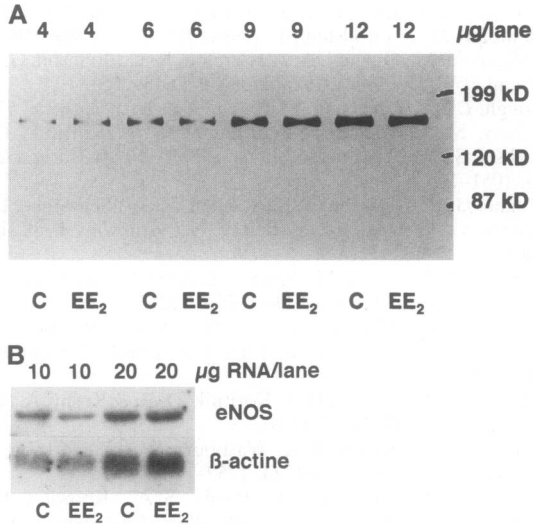

FIG. 3. (*A*) Characterization of endothelial NOS protein by Western blot analysis. Serial dilutions (4–12 μg/lane) of control and EE_2-treated (0.01 μM for 48 hr) cell homogenates were electrophoretically separated by PAGE, transferred to nitrocellulose membranes, and probed with a monoclonal antipeptide antibody specific for endothelial NOS. (*B*) Northern blot analysis of endothelial NOS mRNA in control and EE_2-treated BAEC. Total RNA (10 and 20 μg per lane) was electrophoretically separated in an agarose formaldehyde gel, transferred to nitrocellulose membranes, and probed with the bovine endothelial NOS cDNA labeled with [α-^{32}P]dCTP by random priming. Reprobing with ^{32}P-labeled β-actin demonstrated that equal amounts of RNA were analyzed for control (C) and EE_2-treated cells. The results are representative of three experiments.

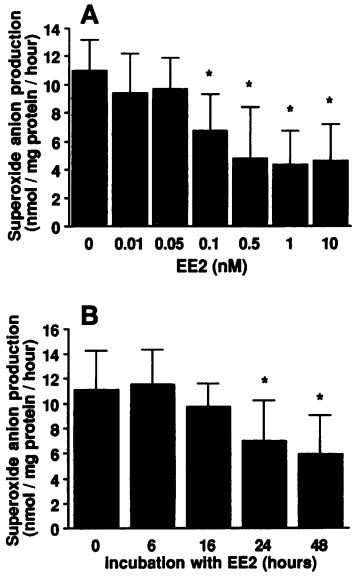

FIG. 5. Effect of EE_2 on superoxide anion production by BAEC. (*A*) Dose-response relationships using varying concentrations of EE_2 for 48 hr. The results are given as mean ± SD of the rate of production calculated from the duplicate measurements of the three time points (30, 60, and 90 min) and are representative of four experiments. *, $P < 0.05$ vs. control. (*B*) Time course analysis of EE_2 (0.01 μM) effect on superoxide anion production of BAEC. The results are given as mean ± SD of the rate of production calculated from the duplicate measurements of the three time points (30, 60, and 90 min) and are representative of four experiments. *, $P < 0.05$ vs. 0 hr.

effect was 0.5 nM EE_2, the required duration of EE_2 exposure was between 12 and 24 hr, and the effect could be inhibited by EE_2 antagonists. These data suggested a traditional mechanism of estrogen action involving the functional estrogen receptors that have been characterized in this population of cells (26, 36). Recently, the promoter regions of the human and bovine endothelial NOS genes have been sequenced, and several half-palindromes of the estrogen-responsive element sequence have been recognized (37, 38) suggesting regulation at the level of gene expression. We then explored the endothelial NOS activity, protein, and mRNA.

Two different approaches were used to study NOS activity: conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline by cell homogenates and measurement of the stable degradation products of NO, nitrogen oxides NO_2^- and NO_3^-, released in the supernatant of cultured endothelial cells under both basal

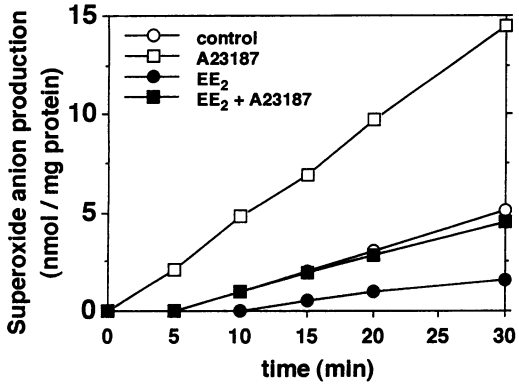

FIG. 4. Superoxide anion production from control or EE_2-treated (0.01 μM for 48 hr) BAEC without or with 1 μM calcium ionophore A23187. The superoxide anion production was measured as described. The results are given as mean of duplicate measurements for each point and are representative of three experiments.

conditions and in the presence of the calcium ionophore A23187. Neither of these approaches revealed any significant change in NOS activity after EE_2 exposure. While both of these assays reflect NOS activity, they provide different yet complementary information. Measurement of nitrogen oxides release reflects the intracellular activity of NOS and may be influenced by the presence and amount of cofactors necessary for NOS function. For example, it has recently been suggested that intracellular levels of biopterin can be increased by cytokines in the endothelium and in macrophages and thus may increase NO production (39). In contrast, measurement of arginine/citrulline conversion by cell homogenates assesses the "maximal" activity of the NOS when all of the known required cofactors are added in excess. Both assays may also reflect changes in activity due to enzyme phosphorylation (21). In the present experiments, neither of these approaches revealed any change in NOS activity after EE_2 exposure. These observations were in perfect agreement with the measurements of endothelial NOS mRNA and protein concentrations by Northern and Western blot analysis (Fig. 3). While Western blot analysis is only semiquantitative, the approach employed in the present studies allowed comparison of the detected signals using serial dilutions of proteins from control and EE_2-treated BAEC homogenates. No difference was detected in the level of endothelial NOS immunoreactive protein. No difference was found either in endothelial NOS mRNA abundance in control and EE_2-treated BAEC. These series of results have also been confirmed by using a quantitative competitive reverse transcriptase–PCR assay (40) where again no difference could be measured in the number of copies of endothelial NOS mRNA per microgram of total RNA from control or EE_2-treated cells even after transient transfections of BAEC with the human estrogen receptor (ref. 26; F.B. and S.C., unpublished data). They agree with observations by others (41), but appear at variance with those reported by Weiner *et al.* (11), Goetz *et al.* (42), and Hishikawa *et al.* (43),

where an upregulation of endothelial NOS gene expression was observed in the pregnant guinea pig, in the pregnant rat, and in human endothelial cells, respectively. At the present time, no clear explanation can be given for these discrepancies. Although no information was given by Hishikawa *et al.* (43) on mRNA levels, the dose-response curve of 17β-estradiol effect on NO production and endothelial NOS protein in human aortic endothelial cells, apparently grown in culture media from which steroids had not been removed, suggested a nonsaturable phenomenon completely different to the saturable effect that we observed. The *in vivo* observations made by Weiner *et al.* (11) and Goetz *et al.* (42) draw the attention on the participation of other regulatory factors of endocrine or paracrine nature but yet of undefined origin. Indeed, the influence of additional factors other than estrogens has already been proposed by Goetz *et al.* (42) to explain the upregulation of endothelial NOS gene expression induced by pregnancy. Further studies will be necessary to characterize this factor(s).

Because the increased amount of guanylate cyclase stimulating activity released by EE_2-treated BAEC could not be explained by an increase in NOS activity, other mechanisms were explored. It has been acknowledged very early on that superoxide anion inactivates endothelium-derived relaxing factor (44, 45) and that endothelium generates substantial amounts of superoxide anion, although the mechanisms of production have not been extensively characterized (46, 47). We therefore investigated superoxide anion production in BAEC in response to EE_2. We found that EE_2 inhibited dramatically superoxide anion production in a time- and dose-dependent manner. The EC_{50} of EE_2 for inhibition of superoxide anion production was similar to its EC_{50} for stimulation of guanylate cyclase-stimulating activity production, and again this effect could be inhibited by EE_2 antagonists. Furthermore, the endothelial system that generates superoxide anion was responsive to increases in intracellular calcium, as indicated by its stimulation in response to the calcium ionophore A23187; this response was also inhibited by pretreatment of BAEC with EE_2. Altogether these data suggest that the increase in guanylate cyclase-stimulating activity in EE_2-treated BAEC could be the consequence of an inhibition of basal as well as stimulated production of superoxide anion, a free radical able to react rapidly with NO leading to its inactivation and the associated generation of poorly defined reactive intermediates. It is suggested from the RFL6 reporter cell experiments that the NO superoxide anion interaction in BAEC occurs intracellularly since exogenous SOD, even added in large amounts, does not enter the cell (48), but apparently protected NO released in the supernatant from further degradation.

In conclusion, we have been able to demonstrate that EE_2 prevents NO degradation by decreasing superoxide anion production and BAEC cultures should constitute a good model for further studies on the characterization of the molecular target(s) involved in this effect. Although EE_2 did not influence NO production in these cultured endothelial cells, such an effect may also contribute *in vivo* to the increased release of biologically active NO. This increased NO bioactivity may promote vasodilation, may inhibit proliferation of the adjacent vascular smooth muscle, and may inhibit platelet aggregation (14) as well as the inflammatory reaction induced by cytokines (49, 50). In addition, the decrease in superoxide anion production should confer the estrogens with antioxidant properties (51). All these effects could contribute to the atheroprotective properties of estrogen.

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