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Estradiol increases rat aorta endothelium-derived relaxing factor (EDRF) activity without changes in endothelial NO synthase gene expression: possible role of decreased endothelium-derived superoxide anion production

M.A. Barbacanne[a], J. Rami[a], J.B. Michel[b], J.P. Souchard[c], M. Philippe[b], J.P. Besombes[a], F. Bayard[a], J.F. Arnal[a,*]

[a] *INSERM U397 et Laboratoire de Physiologie, Institut Louis Bugnard, CHU Rangueil, 31054 Toulouse, France*
[b] *INSERM U460, CHU Xavier Bichat, 75870 Paris, France*
[c] *Laboratoire de Synthèse, Physico-Chimie et Radiobiologie, Université Paul Sabatier, 31062 Toulouse, France*

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Abstract

Objectives: Estradiol is known to exert a protective effect against atherosclerosis, but the mechanism(s) whereby this protection is mediated is/are unclear. However, estradiol-treated castrated animals exhibit increased activity of endothelium-derived relaxing factor (EDRF), which could contribute to vasculoprotection. In the present work, we investigated the molecular mechanism(s) of the enhancement of EDRF activity in the thoracic aorta of oophorectomized female rats given 17β-estradiol (E_2, 2 or 40 μg/kg/day) compared to those given a placebo. **Methods and Results:** The abundance in the thoracic aorta of NO synthase I, II and III mRNA (using RT-PCR) and of NO synthase I, II and III immunoreactive protein (using Western blotting) was unaltered by E_2. NO synthase activity (based on arginine/citrulline conversion) in thoracic aorta homogenates did not differ significantly among the three groups, suggesting that NO production was not enhanced by E_2. In contrast, lucigenin-enhanced chemiluminescence of aorta from the E_2 group was decreased compared to that of the placebo group. Desendothelialization and exogenously added superoxide dismutase suggested that this difference was due to a decrease in extracellular endothelium-derived production of superoxide anion ($O_2^{-\cdot}$). Experiments in cultured bovine aortic endothelial cells confirmed a decreased extracellular production of $O_2^{-\cdot}$ in response to ethinylestradiol (1 nM) using both lucigenin-enhanced chemiluminescence and ESR spectroscopy. Luminol-enhanced chemiluminescence revealed that ethinylestradiol-treated cultured endothelial cells generated less peroxynitrite (the byproduct of $NO^{\cdot}$ and $O_2^{-\cdot}$ interaction) than control cells. **Conclusion:** Estradiol increases rat aorta EDRF activity in the absence of changes in endothelial NO synthase gene expression. The decreased endothelium-derived generation of $O_2^{-\cdot}$ in response to estrogens could account for enhanced EDRF–NO bioactivity and decreased peroxynitrite release. All of these effects could contribute to the vascular protective properties of estrogens.

Keywords: Nitric oxide synthase; Endothelial cell; Estrogens; Superoxide anion

1. Introduction

The incidence of cardiovascular disease, the leading cause of mortality in western societies, is higher in men

Abbreviations: BAECs, bovine aortic endothelial cells; CS, calf serum; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; E_2, 17β-estradiol; EE_2, ethinylestradiol; NO, nitric oxide; PBS, phosphate-buffered saline; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavine adenine dinucleotide; FMN, flavine adenine mononucleotide; $O_2^{-\cdot}$, superoxide anion; SOD, superoxide dismutase

*Corresponding author. Tel.: +33-5-61-32-21-47; Fax: +33-5-61-32-21-41.

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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 by which this protection is mediated remains obscure. It is traditionally thought to be due to potentially favourable changes in blood lipids and lipoproteins [1] but a number of studies in humans [3] as well as animals [4–6] strongly suggest a direct effect on the vascular system.

Endothelium-derived relaxing factor (EDRF), identified as nitric oxide (NO), is a free radical messenger that is able to induce relaxation of the vascular smooth muscle cells [7–9]. The endothelial NO synthase, or NO synthase III, which converts L-arginine to L-citrulline and NO, has been purified and its cDNA sequence determined [9–11]. Endothelial NO synthase is a complex enzyme whose activity requires several cofactors (NADPH, FAD, FMN, tetrahydrobiopterin) and depends on calmodulin and calcium. NO synthase III activity is stimulated by numerous agonists (acetylcholine, bradykinin, etc.) or by a mechanical stimulus (shear stress). Increased shear stress also appears to be the major determinant of NO synthase III gene expression in vitro and in vivo [12,13]. Even if NO or a closely related compound accounts for EDRF, NO can be inactivated by superoxide anion ($O_2^{-\cdot}$), which thereby influences EDRF activity by decreasing the half-life of NO [14,15].

EDRF is enhanced in 17β-estradiol (E_2)-treated females compared to oophorectomized controls [16–20]. Weiner et al. [21] and Goetz et al. [22] reported that pharmacological doses of E_2 induced an upregulation of NO synthase III mRNA abundance in guinea pig skeletal muscle and rat aorta, respectively. However, we found that exposure of cultured bovine aortic endothelial cells (BAECs) to physiological doses of estrogens did not alter NO synthase III gene expression, but induced a receptor-mediated antioxidant effect that enhanced the biological activity of endothelium-derived NO [23]. To further elucidate the molecular mechanisms by which estrogens enhance EDRF activity in vivo, we gave oophorectomized female rats either placebo, or low or high doses of E_2 and studied the thoracic aorta for (1) EDRF activity, evaluated from the relaxation of precontracted aorta; (2) NO synthase III mRNA and protein abundance, as well as the gene expression of NO synthase I or II; (3) NO synthase activity, by arginine–citrulline conversion assay; (4) $O_2^{-\cdot}$ production using lucigenin-enhanced chemiluminescence and, finally, (5) the effect of ethinylestradiol (EE_2) in vitro on lucigenin- and luminol-enhanced chemiluminescence and on electron spin resonance (ESR) spectroscopy using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as spin trap using the BAEC culture model.

2. Methods

2.1. Animal preparation, cell culture and materials

All experimental protocols were performed in accordance with the recommendations of the French Accreditation of Laboratory Animal Care. A total of 110 female Sprague-Dawley rats (weighing 160–180 g; Iffa-Credo) were anaesthetized for bilateral ovariectomy. Ten days later, the castrated animals were administered either E_2 (2 or 40 μg/kg/day) or the vehicle (propanediol) subcutaneously for five days. The rats were then sacrificed with an overdose of pentobarbital. The uterus was removed and weighed. The thoracic aorta was removed, cleaned of excess adventitial tissue, and care was taken not to injure the endothelium.

BAECs were obtained and grown as described previously [23,24] in phenol red-free Dulbecco's Modified Eagle medium supplemented with 10% heat-inactivated charcoal-treated newborn calf serum at 37°C in culture dishes (20 cm^2) and in a 10% CO_2-containing humidified atmosphere. The cells used in this study were between the fifth and fifteenth passage. Several measures were taken to avoid artifacts of cell culture on BAEC phenotype due to proliferation, as previously reported [25]. 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 two days after passage with 1 ng/ml basic fibroblast growth factor (bFGF). All of the experiments were done in BAECs four days after confluency (100 000 cells/cm^2). The cells for estrogen stimulation were treated with EE_2 instead of estradiol, to prevent metabolism of the steroid hormone [26].

All reagents were purchased from Sigma (St. Louis, MO, USA), except when specified. Protein concentrations were determined using the Bio-Rad Coommasie Brilliant Blue G-250 method, with bovine serum albumin as standard.

2.2. Isolated vascular ring experiments

Ring segments (3 mm) of rat thoracic aorta were suspended in individual organ chambers filled with Krebs buffer (20 ml) with the following millimolar composition: NaCl 118.3, KCl 4.69, $CaCl_2$ 1.25, $MgSO_4$ 1.17, K_2HPO_4 1.18, $NaHCO_3$ 25.0 and glucose 11.1, pH 7.40. The solution was aerated continuously with 95% O_2–5% CO_2 and maintained at 37°C. Care was taken not to injure the endothelium during ring preparation. Tension was recorded with a linear force transducer. The resting tension was gradually increased to 1.5 g over a period of 1 h, and the ring segments were exposed to 80 mM KCl until the

optimal tension for generating force during isometric contraction was reached. The vessels were left at this resting tension throughout the remainder of the study. The vessels were then precontracted with L-phenylephrine (0.5 mM). When a stable contraction plateau had been reached, the rings were exposed cumulatively to either acetylcholine (ACh, 1 nM–3 μM) or sodium nitroprusside (SNP, 1 nM–3 μM).

2.3. Measurement of NO synthase activity by conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline

Thoracic aorta were washed three times with cold phosphate-buffered saline (PBS) and homogenized with a Dounce homogenizer in 500 μl of homogenization buffer (50 mM Tris–HCl, 0.1 mM EDTA, 0.1 mM EGTA, pH 7.5, containing 0.1% CHAPS, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride and 0.1% β-mercaptoethanol). The homogenates (500–600 μg total protein) were then assayed for NO synthase activity. Each sample (100 μl) was incubated in 50 mM Tris-HCl buffer, pH 7.5, containing the cofactors 100 nM calmodulin, 2.5 mM $CaCl_2$, 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 10 μM tetrahydrobiopterin, 1 mM dithiotreitol and the substrate, 1 μCi L-[U-^{14}C]arginine (specific activity, 296 mCi/mmol) for 15 min at 37°C. After the incubation period, the reaction was quenched by the addition of 1 ml of stop buffer (20 mM Hepes, 2 mM EDTA and 0.2 mM EGTA, pH 3). The reaction mix 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.

2.4. Western blotting analysis

Protein homogenates were prepared as described above for measurement of NO synthase activity. Protein was size-fractionated electrophoretically using a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose membranes that were blocked with 5% casein Tris-buffered saline solution, pH 7.6, containing 0.1% Tween (TBS-T) at room temperature. The membranes were incubated with a 1:2000 (v/v) dilution of a monoclonal antibody against a peptide from the sequence of human endothelial NO synthase that cross-reacted with the rat and the bovine enzymes (Transduction Laboratories, Lexington, KY), washed four times with TBS-T, then incubated with a sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham) and washed again. Signals were detected using the ECL detection system (Amersham) and autoradiography films (Hyperfilm TM ECL, Amersham). Two dilutions (25 and 50 μg of protein per lane) were analyzed to quantify the NO synthase protein content. The films were then scanned using a densitometer, and a graph of peak area against protein concentration was plotted. A similar approach was used to detect the neuronal and inducible NO synthase, with specific antibodies purchased from Transduction Laboratories.

2.5. RNA isolation and RT-PCR amplification

2.5.1. Reverse transcription

As previously described [13], total RNA was prepared from aorta using Trizol solution (Gibco-BRL) and a polytron homogenizer. A 100-ng amount of total RNA were reverse transcribed (final volume, 18 μl) with M-MLV reverse transcriptase (8 U/μl, 1 h at 37°C) (Gibco-BRL) in the presence of 1 μg of oligo d(T) [12–18].

2.5.2. PCR reaction

Part of the reverse transcription solution (3 μl) was mixed with the PCR mix (1× Taq-buffer containing 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Gibco-BRL), 0.1 mmol/l dNTP (Pharmacia), 8 pmol of sense and antisense primers and 400 000 cpm of a ^{33}P-radiolabelled mix of both primers) and subjected to 4 min of initial denaturation at 94°C; 35, 32 or 28 (for NO synthase I, II and III, respectively) cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C; then 1 min at 62°C and 10 min at 72°C.

Primers for NO synthase I included 5′-CTGGCTCAACAGAATACAGGCT-3′ (sense) and 5′-GCAGTGTACAGCTCTCTGAAGA-3′ (antisense), which amplify a 293 bp mRNA region. Primers for NO synthase II included 5′-TGCTTTGTGCGGAGTGTCAGT-3′ (sense) and 5′-CGGACCATCTCCT GCATTTCT-3′ (antisense), which amplify a 227 bp mRNA region. Primers for NO synthase III included 5′-TTCCGGCTGCCACCTGATCCTAA-3′ (sense) and 5′-AACATGTGTCCTTGCTCGAGGCA-3′ (antisense), which amplify a 340 bp mRNA region. NO synthase mRNA expression was calculated by normalizing NO synthase mRNA to GAPDH mRNA. Primers for GAPDH included 5′-GTGAAGGTCGGAGTCAACG-3′ (sense) and 5′-GGTGAAGACGC CAGTGGACTC-3′ (antisense), which amplify a 299 bp mRNA region. The annealing temperature for GAPDH primers was 55°C, and the PCR was performed for 27 cycles.

The primers were chosen to encompass several introns in order to avoid amplification of contaminating genomic DNA. A negative control was used for each set of samples to check the reverse transcription and the PCR amplification reagents for any contamination. PCR amplification was verified to be exponential and the product was proportional to the input.

The PCR products were separated on an 8% acrylamide-*N-N′*-(1,2-dihydroxyethylene)-bis-acrylamide (DHEBA) (29:1) gel in a 1× TBE buffer using a miniprotean II cell apparatus (Biorad). DHEBA was obtained from ICN Biochemicals. After ethidium bromide staining, the bands

corresponding to the amplified fragments were excised, dissolved for 2 h at 50°C in 750 μl of 25 mmol/l periodic acid and the radioactivity was counted in a β scintillation counter. On the negative control lane, gel slices corresponding to the position of these bands were excised, counted and used as background.

2.6. Lucigenin- and luminol-enhanced chemiluminescence

Classical luminometers are useful tools for assessing $O_2^{-\cdot}$ production from cultured cells, but are not sensitive enough to detect lucigenin-enhanced chemiluminescence elicited by aortic segments. We therefore followed a different approach that has been described previously [27,28]. Lucigenin chemiluminescence was detected using a scintillation counter (Packard Tri-Carb 2100 TR) in out-of coincidence mode. The descending thoracic aorta was isolated and removed, taking care not to damage the endothelium. Segments (1 cm) of thoracic aorta, freed of adventicia, were incubated with Krebs–Hepes buffer maintained at 37°C for 30 min and then gently transferred to scintillation vials containing 250 μM lucigenin and other additions (final volume of 2 ml). Counts were obtained at 1-min intervals at room temperature for 15 min. The background, determined from vials containing all components with the exception of the aortic segment, was subtracted from the values. Lucigenin-enhanced chemiluminescence with aortic rings was also measured in the presence of an inactivator of $O_2^{-\cdot}$, superoxide dismutase (SOD, 150 U/ml), to assess extracellular $O_2^{-\cdot}$ production. To assess endothelial $O_2^{-\cdot}$ production, the endothelium was removed, by gently rubbing the inner surface of the vessel with the closed tips of a thin forceps, and incubated with Krebs–Hepes buffer maintained at 37°C for 30 min. Lucigenin-enhanced chemiluminescence was then assessed from these desendothelialized vessels.

The production of reactive oxygen intermediates from BAECs was measured by chemiluminescence in the presence of lucigenin or luminol. The medium was removed from the 20 cm^2 Petri dishes and the cells were washed three times with Hanks' Balanced Salt Solution (HBSS; 0.14 g/l $CaCl_2$, 0.40 g/l KCl, 0.06 g/l KH_2PO_4, 0.0977 g/l $MgSO_4$, 8 g/l NaCl, 0.048 g/l Na_2HPO_4 and 1 g/l glucose). BAECs (about $2 \cdot 10^6$ cells) were then scraped off using a rubber policeman in 400 μl of HBSS and transferred into a luminometer cuvette. The big cellular aggregates were then dissociated by gentle pipetting. Luminol (66 μM final) or lucigenin (100 μM final) was then added. The cuvette was put in a thermostatically (37°C) controlled 1251 LKB luminometer, as described previously. Chemiluminescence was triggered with bradykinin (100 nM final) or the calcium ionophore A23187 (10 μM) (600 μl final volume in the cuvette) and continuously monitored for 10 min. The basal chemiluminescence (obtained before stimulating the cells) was automatically subtracted, and the peak and the area under the curve (expressed in mV/s) were calculated using a Hewlett Packard 85 computer.

2.7. ESR measurements

BAECs cultured in a 25-cm^2 flask were washed with PBS, and then incubated with a mix containing 150 mM DMPO, 1 g/l glucose, 0.2 g/l $CaCl_2$, 0.0059 g/l diethylenetriaminepentaacetic acid (DTPA), 0.15 g/l NaCl, 0.37 g/l KCl in sodium phosphate buffer (2.35 g/l NaH_2PO_4/7.61 g/l Na_2HPO_4, pH 7.4) and A23187 for 15 min. The supernatant was then transferred to a flat quartz cell that was inserted in a TM 110 Bruker cavity. ESR spectra were recorded at room temperature with an ER 200 D Bruker spectrometer by starting a 3-min scan 5 min after the end of the incubation with the cells. The ESR spectrometer was operated at 9.66 GHz with high frequency at 100 kHz, a modulation amplitude of 1 Gauss, a time constant of 0.5 s, a microwave power of 10 mW, field: mid range at 3500 Gauss and scan range 200 Gauss. The intensity of the ESR signal was calculated by adding the height of the four peaks, and was expressed in arbitrary units.

2.8. Statistical analysis

The data were expressed as mean±standard error. Comparisons of data between two groups were made using the unpaired *t*-test. Comparisons of data between different groups were made by ANOVA and a Sheffe's post-hoc test was used when differences were indicated. p values <0.05 were considered to be significant.

3. Results

3.1. Effect of E_2 on uterus weight and endothelium-dependent relaxation (EDRF)

Compared to placebo-treated ovariectomized rats, uterus weight was increased 1.8- and 3.4-fold in rats given 2 and 40 μg E_2/kg/day respectively (Table 1), indicating a dose-dependent effect of E_2 on this target sexual organ. The vascular effect of E_2 was studied on thoracic aorta contraction and relaxation. Neither of the doses of E_2 significantly altered the contraction in response to the α1-adrenergic agonist phenylephrine (Table 1). When the vessels were precontracted to 70% of the maximal contraction, E_2 dose-dependently increased EDRF activity, which was estimated from the relaxation in response to acetylcholine. The EC_{50} of the low dose E_2-treated group was significantly decreased in comparison to the placebo group ($p=0.03$), but was borderline ($p=0.08$) in comparison to the high dose E_2-treated group (Fig. 1 Table 1). There was no significant difference in relaxation between the three groups in response to the endothelium-independent vasodilator sodium nitroprusside, although a trend towards an

Table 1
Uterus weight, contraction of aortic rings in response to phenylephrine (PE), endothelium-dependent relaxation of aortic rings in response to acetylcholine (ACh), endothelium-independent relaxation of aortic rings in response to sodium nitroprusside (SNP) from placebo, 2 or 40 μg/kg/day of 17β-estradiol (E_2)-treated rats

	Placebo $n=12$	E_2 (2 μg/kg/day) $n=10$	E_2 (40 μg/kg/day) $n=8$	ANOVA p value
Uterus (mg)	115±4	202±23[b]	393±63[c]	0.0001
PE EC_{50} (10^{-8} M)	10.2±4.3	7.2±2.5	8.0±1.3	NS (0.77)
PE maximum contraction (g)	3.92±0.34	4.29±0.23	3.73±0.25	NS (0.27)
Ach (EC_{50}, 10^{-8} M)	8.01±1.18	5.06±1.13[a]	2.77±0.50[b]	0.003
ACh (% maximum relaxation)	79±5	85±4	93±4	NS (0.07)
SNP (EC_{50}, 10^{-8} M)	2.22±0.38	2.65±0.61	1.10±0.60	NS (0.09)
SNP (% maximum relaxation)	113±3	110±3	104±1	NS (0.29)

[a] $p<0.05$ vs. placebo.
[b] $p<0.01$ vs. placebo.
[c] $p<0.001$ vs. placebo;
NS: not significant.

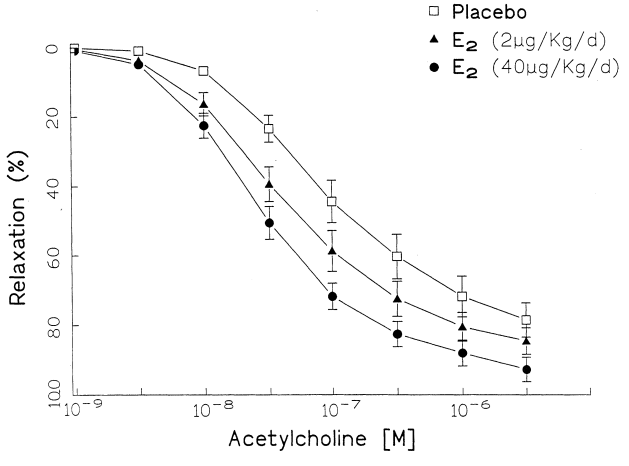

Fig. 1. Effect of E_2 on endothelium-dependent relaxation in response to acetylcholine. Rat thoracic aorta were contracted with phenylephrine, and acetylcholine was then added in a cumulative fashion. The ANOVA with repeated measures revealed that the dose–response curves of both doses of E_2 were statistically shifted leftwards in relation to the placebo, whereas E_2 (2 μg/kg/day) and E_2 (40 μg/kg/day) did not differ significantly.

increased EC_{50} was observed in the group that received the high E_2 dose (Table 1).

3.2. Effect of E_2 on NO synthase I, II and III mRNA and protein abundance in thoracic aorta

We studied the effect of E_2 on the mRNA abundance of NO synthases I, II and III in thoracic aorta using RT-PCR. GAPDH coamplification was used as a control. As shown in Table 2, we did not find any difference in aorta mRNA abundance of the three NO synthases in response to E_2. We then evaluated the effect of E_2 on NO synthase III protein abundance using a monoclonal antibody raised against a sequence of human NO synthase III. A Western blot was quantified by scanning densitometry. The NO synthase III protein was quantified and did not reveal any change in NO synthase III immunoreactivity in response to either the low or high E_2 dose (Fig. 2). We also examined the presence of NO synthase I and II protein abundance using monoclonal antibodies raised against sequences of human NO synthase I and II, respectively, but could not detect

Table 2
NO synthase III, I and II mRNA abundance and NO synthase activity (arginine/citrulline conversion) in thoracic aorta from rats treated with placebo or 17β-estradiol (E_2, 2 or 40 μg/kg/day)

	Placebo $n=8$	E_2 (2 μg/kg/day) $n=8$	E_2 (40 μg/kg/day) $n=8$	ANOVA (p value)
NOS III/GAPDH mRNA	1.1±0.2	1.0±0.3	1.1±0.2	NS ($p=0.94$)
NOS I/GAPDH mRNA	2.0±0.2	1.8±0.4	2.0±0.2	NS ($p=0.86$)
NOSII/GAPDH mRNA	1.3±0.2	1.2±0.3	1.0±0.2	NS ($p=0.63$)
NOS activity (fmol citrulline/μg protein/min)	1.67±0.17	1.79±0.30	1.83±0.22	NS ($p=0.88$)

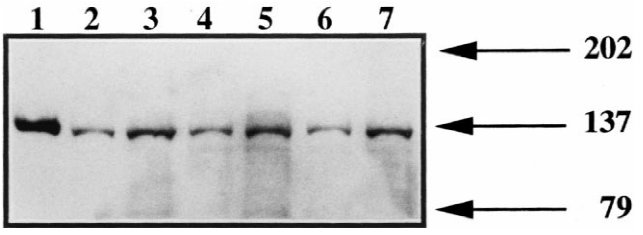

Fig. 2. Characterisation of endothelial NO synthase III protein by Western blotting analysis. Serial dilutions (25 μg in lanes 2, 4 and 6, and 50 μg in lanes 3, 5 and 7) of placebo (lanes 2 and 3), E_2, 2 μg/kg/day (lanes 4 and 5) and E_2, 40 μg/kg/day (lanes 6 and 7)-treated aorta homogenates were electrophoretically separated by polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes and probed with a monoclonal anti-peptide antibody that was specific for NO synthase III. Lane 1 corresponds to 5 μg of BAEC homogenates that were used as a standard. Scanning densitometry did not reveal any change in NO synthase III immunoreactivity in response to either the low or the high dose of E_2. The results are representative of three separate experiments.

any signal, which suggests that the abundance of the immunoreactive protein was below the threshold of detection (not shown).

3.3. Effect of E_2 on NO synthase activity in thoracic aorta

We then examined NO synthase activity by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline in thoracic aorta homogenates from E_2-treated (2 or 40 μg E_2/kg/day) and placebo-treated rats. The NO synthase activity in homogenates of E_2-treated thoracic aorta was not significantly different from that of the placebo control (Table 2). The conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline from both EE_2-treated and control cells was abolished by removal of calcium and addition of 1 mM EGTA, and inhibited >90% by the addition of 30 μM L-NAME, an inhibitor of NO synthase (not shown).

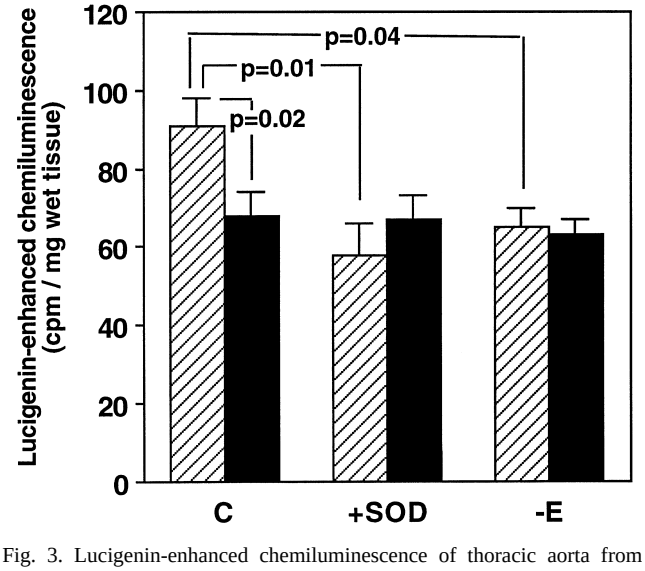

Fig. 3. Lucigenin-enhanced chemiluminescence of thoracic aorta from castrated rats treated with E_2 (40 μg E_2/kg day) (black bars, $n=12$) or with the placebo (hatched bars, $n=11$). Intact thoracic aorta were compared to intact vessels with extracellularly added SOD and to desendothelialized (−E) vessels.

3.4. Effect of E_2 on lucigenin-enhanced chemiluminescence from thoracic aorta

Lucigenin-enhanced chemiluminescence was used to evaluate the $O_2^{\cdot-}$ generation of thoracic aorta from castrated rats that were treated or not with E_2 (40 μg E_2/kg/day). As shown in Fig. 3, intact thoracic aorta from placebo castrated rats generated more $O_2^{\cdot-}$ than that of castrated rats receiving E_2 ($p=0.02$). In placebo castrated rats, the addition of SOD to intact thoracic aorta significantly decreased lucigenin-enhanced chemiluminescence ($p=0.01$), whereas the removal of endothelium significantly lowered the signal ($p=0.04$). In contrast, neither the addition of SOD to intact thoracic aorta nor endothelium abrasion influenced lucigenin-enhanced chemiluminescence in castrated rats that received E_2. Thus, in vivo E_2 treatment appeared to decrease the extracellular $O_2^{\cdot-}$ production of aortic endothelium.

3.5. Effect of EE_2 on lucigenin- and luminol-enhanced chemiluminescence from cultured BAECs

To further evaluate the endothelial generation of $O_2^{\cdot-}$, we first used lucigenin-enhanced chemiluminescence from cultured BAECs (Fig. 4A). The $O_2^{\cdot-}$ production of BAECs stimulated by 10^{-5} M A23187 was decreased about two-fold when the cells were pretreated with EE_2 (10^{-9} M for 48 h). Similarly, bradykinin-stimulated production of $O_2^{\cdot-}$ was inhibited to a similar extent after EE_2 pretreatment. L-NAME (30 μM) did not alter the signal of untreated or EE_2-treated BAECs whatever the stimulus. SOD (150 U/ml) inhibited 64 and 45% of the A23187-stimulated signal of untreated and EE_2-treated BAECs, respectively. SOD inhibited the bradykinin-stimulated signal to a similar extent. The inhibition of $O_2^{\cdot-}$ production promoted by 10^{-9} M EE_2 was completely prevented by the antiestrogen RU54876 (5×10^{-8} M) (not shown).

The chemical nature of the reactive oxygen intermediates detected by luminol-enhanced chemiluminescence in stimulated BAECs was investigated using SOD (an inactivator of $O_2^{\cdot-}$) and L-NAME (an inhibitor of NO synthase activity). The signal of both control and EE_2-treated BAECs was found to be dependent on the enhanced production of both $NO^{\cdot}$ and $O_2^{\cdot-}$, leading to the conclusion that peroxynitrite ($ONOO^-$) is the oxidant species responsible for the activation of luminol. The luminol-enhanced chemiluminescence of BAECs stimulated by 10^{-5} M A23187 was decreased about two-fold when the cells were pretreated with EE_2 (10^{-9} M for 48 h) (Fig. 4B). Luminol-enhanced chemiluminescence in response to bradykinin

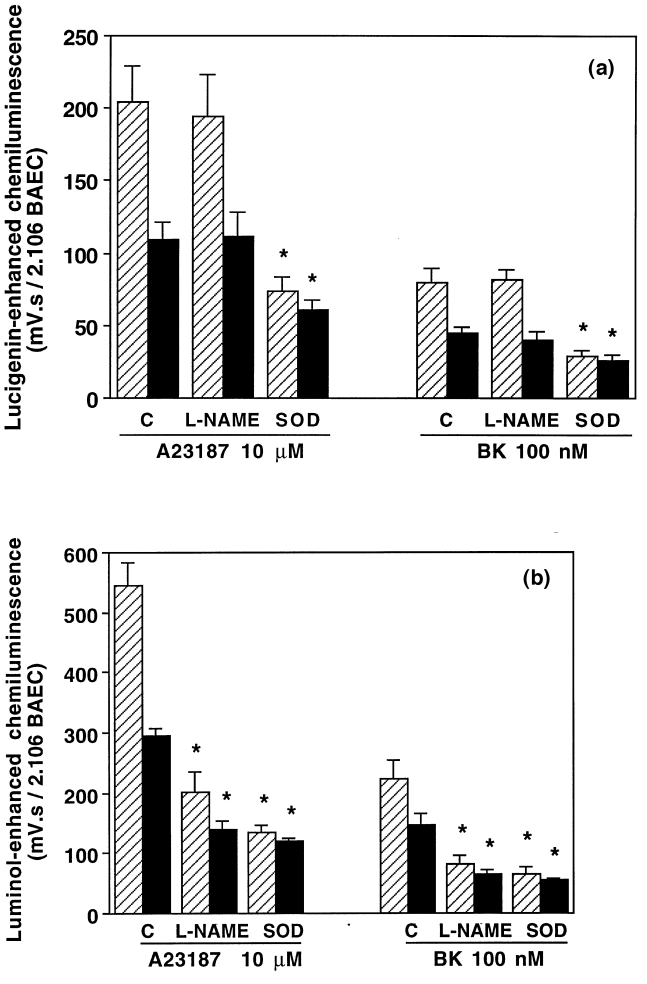

Fig. 4. Effect of EE_2 on lucigenin (A)- or luminol (B)-enhanced chemiluminescence in BAECs. The superoxide anion production of BAECs pretreated with EE_2 (10^{-9} M for 48 h, black bars) or with the solvent (hatched bars) was stimulated by 10^{-5} M A23187 or with 10^{-7} M bradykinin. BAECs were either preincubated or not for 30 min with 30 μM L-NAME or with 150 U/ml SOD, and then coincubated with the same concentration for stimulation. The results are given as mean±SEM from six replicate measurements obtained in two separate experiments. *$p<0.01$ vs. respective control.

was inhibited to a similar extent after EE_2 pretreatment (Fig. 4B). The inhibition of peroxynitrite production promoted by EE_2 (10^{-9} M) was completely prevented by the antiestrogen RU54876 (5×10^{-8} M) (not shown).

3.6. Effect of EE_2 on ESR signals detected using DMPO as spin trap from cultured BAECs

The ESR signal given by unstimulated (basal) cells was less than two-fold that of the baseline (Fig. 1C). After 15 minutes incubation of DMPO with control BAECs stimulated by the calcium ionophore A23187 (10 μM), a typical ESR signal was obtained, resulting from the DMPO-OH adduct (Fig. 5A). This adduct could result from hydroxyl radical trapping by DMPO in the extracellular medium. However, it is well known that the DMPO-OOH adduct,

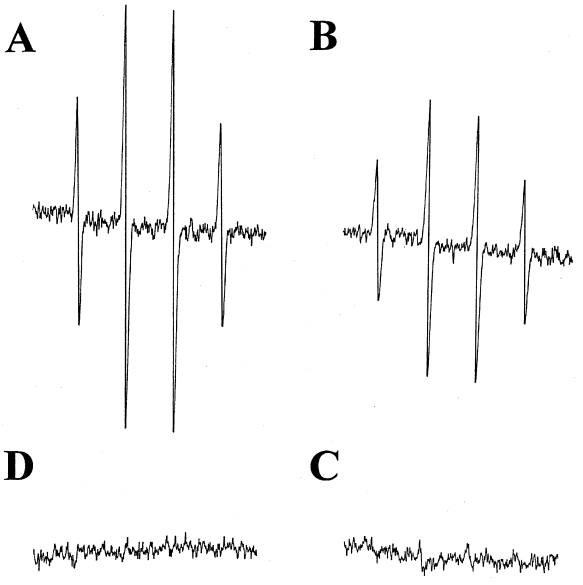

Fig. 5. Effect of EE_2 on ESR signals detected using DMPO as spin trap from cultured BAECs. The cells (2.5×10^6), which were treated (B) or not (A) with 10^{-9} M EE_2 for 48 h, were incubated in the presence of the spin trap DMPO and stimulated with 10 μM A23187 for 15 min. In parallel experiments, control (D) and EE_2-treated (not shown) cells were coincubated with A23187 and 50 U/ml SOD. The ESR signal given by unstimulated cells (C) was less than two-fold that of the baseline. Data are representative of duplicate results from three different experiments.

resulting from the trapping of $O_2^{-\cdot}$, decomposes rapidly into a more stable adduct: DMPO-OH [29]. To identify the reactive oxygen intermediate released by the BAECs and initially trapped by DMPO, the effects of SOD and catalase were tested as previously reported [30,31]. When 50 U/ml SOD was coincubated, the ESR signal was completely suppressed (Fig. 5D). In contrast, coincubation of DMPO, catalase (2000 U/ml) and A23187 (10 μM) did not alter the morphology or the amplitude of the ESR signal given by BAECs (not shown). These data demonstrate that the ESR adduct DMPO-OH detected in the supernatant of stimulated BAECs originated from the trapping of extracellular $O_2^{-\cdot}$. The ESR signal elicited by BAECs stimulated by 10^{-5} M A23187 was decreased by 38±4% when the cells were pretreated with 10^{-9} M EE_2 for 48 h ($p<0.01$ vs. control) (Fig. 5). Altogether, these results demonstrate that the extracellular production of $O_2^{-\cdot}$ in BAECs is decreased in response to EE_2 (1 nM).

4. Discussion

Our aim in the above-described experiments was to examine the mechanism(s) by which E_2 enhances EDRF activity in vivo. We first found that the EDRF activity was increased in thoracic aorta from rats that had received a low or high dose of E_2. This enhanced EDRF activity occurred in the absence of any change in NO synthase III

mRNA or protein abundance, or NO synthase activity, strongly suggesting that it was not due to increased NO production. We then explored the production of $O_2^{-\cdot}$, which is an important mechanism of NO inactivation. In vivo, E_2 treatment decreased the lucigenin-enhanced chemiluminescence of thoracic aorta, demonstrating that estrogen treatment decreased $O_2^{-\cdot}$ production. In vitro experiments using cultured BAECs confirmed that estrogen treatment not only decreased $O_2^{-\cdot}$ production, but also ONOO− production. To the best of our knowledge, this is the first time that the enhancement of EDRF activity is promoted by a decrease in $O_2^{-\cdot}$ production mediated by a physiological stimulus (i.e. E_2) in vivo has been described.

Pregnancy increases EDRF activity in uterine arteries of guinea pigs [32], rats [33], sheep [34] and women [35]. The increased EDRF activity in uterine arteries was shown, at least in part, to be due to increased NO production in pregnant guinea pigs [21] and in pregnant sheep [34]. Pregnancy was also reported to increase EDRF activity and/or NO production in systemic arteries of rat aorta [22,36] and of rat mesenteric artery [36]. The promoter regions of the human and bovine endothelial NO synthase 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. However, their implication in NO synthase III gene expression is controversial [39,40], and other (indirect) mechanisms, such as increased shear stress due to hemodynamic changes, could contribute to increased NO synthase III gene expression in pregnancy [12,13]. In contrast to these numerous reports of increased NO synthase III gene expression and/or activity in pregnancy, only one study reported increased NO synthase III mRNA abundance in systemic arteries after E_2 treatment [22]. It is noteworthy that a very high pharmacological dose was used in this study (1000 μg/kg/day, i.e. 25-fold higher than the high dose used in the present study). The reason for the discrepancy between this previous work [22] and our results is unclear, although a very high pharmacological dose can elicit non-saturable phenomena. The absence of difference in NO synthase III mRNA abundance in placebo and E_2-treated vessels was confirmed at the level of the immunoreactive protein. While Western blot analysis is only semiquantitative, the approach employed in the present study allowed comparison of the detected signals using two dilutions of proteins from placebo and E_2-treated thoracic aorta homogenates. We also investigated the NO synthase I and II mRNA and protein abundance in response to E_2, in particular, because NO synthase I (neuronal) was reported to be increased in the cerebellum under estrogens [21] and because NO synthase II (inducible) was reported to be increased in uterus during pregnancy [41]. However, in response to E_2, the abundance of NO synthase I and II mRNA remained unchanged and proteins remained undetectable in the aorta. Finally, the absence of an effect of E_2 on NO synthase activity, estimated from the arginine/citrulline conversion by thoracic aorta homogenates, was in agreement with the unaltered abundance of NO synthase III protein (Table 2 Fig. 2). Altogether, these data demonstrate that the enhanced EDRF activity observed in aorta from E_2-treated rats is not due to an increase in NO production.

$O_2^{-\cdot}$ was recognized at an early stage to inactivate EDRF, i.e. NO bioactivity [14,15]. Endothelium generates substantial amounts of $O_2^{-\cdot}$, although the mechanisms of production [NAD(P)H oxidase, xanthine oxidase, metabolism of arachidonic acid, etc.] have not been extensively characterized [42,43]. Moreover, we previously reported that EE_2 did not enhance the expression of NO synthase in BAECs, but increased the release of bioactive NO by inhibiting $O_2^{-\cdot}$ production (evaluated by the technique of cytochrome *c* reduction) [23]. We therefore sought to assess $O_2^{-\cdot}$ production in thoracic aorta from rats that had or had not received E_2. Three techniques are available for the assessment of $O_2^{-\cdot}$ production: ESR with spin trap such as DMPO, ferricytochrome *c* reduction and lucigenin-enhanced chemiluminescence. This latter technique is the only one that is sensitive enough to detect the tiny amounts of $O_2^{-\cdot}$ generated by a rat thoracic aorta [27,28,43,44] (and unpublished data).

In the first set of experiments, we found that lucigenin-enhanced chemiluminescence of thoracic aorta from castrated rats treated with E_2 was decreased by about 25% compared to that of untreated rats. This difference could be attributed to decreased extracellular $O_2^{-\cdot}$ production in the endothelium in response to E_2 because (1) the removal of the endothelium lowered $O_2^{-\cdot}$ production in untreated rats but not in E_2-treated rats or (2) the addition of SOD similarly lowered $O_2^{-\cdot}$ production in untreated intact thoracic aorta but not in E_2-treated aorta. In the second set of experiments, lucigenin-enhanced chemiluminescence elicited by EE_2-treated BAECs was decreased by about two-fold compared to untreated cells. This confirmed that estrogens inhibit endothelial $O_2^{-\cdot}$ production, in agreement with the present in vivo data and with a previous in vitro study (cultured BAECs) based on ferricytochrome *c* reduction [23]. Exogenous SOD inhibited three-quarters of the lucigenin-enhanced chemiluminescence from cultured BAECs. As SOD does not penetrate into the cells, three-quarters of the lucigenin-enhanced chemiluminescence probably corresponded to extracellular $O_2^{-\cdot}$ production by cultured BAECs. In contrast, only one-third of the lucigenin-enhanced chemiluminescence of thoracic aorta was inhibited by added SOD. One interpretation could be that only one-third of the signal corresponds to extracellular $O_2^{-\cdot}$ production in thoracic aorta. However, two other explanations would seem more likely. Firstly, as the aortic wall is a dense structure, extracellularly applied SOD will not gain access efficiently to the medial smooth muscle cells. Secondly, the lucigenin-enhanced chemiluminescence from smooth muscle cells could arise from an intracellular source. In any case, it should be emphasized

that the lucigenin-enhanced chemiluminescence from desendothelialized vessels was uninfluenced by E_2. Finally, the $O_2^{-\cdot}$ production of cultured rat aortic smooth muscle cells was also unaltered by E_2 (unpublished data).

However, although lucigenin luminescence has been widely used to assess $O_2^{-\cdot}$ production [7,28,43,44], it may not be a useful tool under certain in vitro conditions [45–47]. This is why we also used ESR spectroscopy using DMPO as spin trap to detect $O_2^{-\cdot}$ production in another set of experiments. The appropriate controls revealed that DMPO allowed the trapping of endothelium-derived $O_2^{-\cdot}$. EE_2-treated BAECs generated about two-fold less $O_2^{-\cdot}$ production than control BAECs, in agreement with the results provided by lucigenin-elicited luminescence. Thus, as ESR spectroscopy appears, at least in endothelium, to validate the use of lucigenin as a detector of $O_2^{-\cdot}$, we can conclude from the present data that E_2 decreases extracellular $O_2^{-\cdot}$ production in endothelium both in vitro and in vivo.

$O_2^{-\cdot}$ is known to react rapidly with NO in solutions, the rate constant, *k*, being 6.7×10^9 M^{-1} s^{-1}, which is about three times higher than that of SOD-catalysed dismutation (2×10^9 M^{-1} s^{-1}) [47], leading to the reciprocal inactivation of both reactive species. Thus, the vascular $O_2^{-\cdot}$ concentration may be influenced by the NO concentration. In the present study, the inhibition of NO synthase by L-NAME had no significant influence on lucigenin-enhanced chemiluminescence, either in untreated or EE_2-treated BAECs. Thus, in agreement with previous works [44,48,49], endogenous NO does not appear to regulate the $O_2^{-\cdot}$ responsible for lucigenin-enhanced chemiluminescence, perhaps because the production of $O_2^{-\cdot}$ largely exceeds that of NO. However, luminol-enhanced chemiluminescence was inhibited both by SOD and L-NAME, suggesting that the signal was elicited by the product of interaction of $O_2^{-\cdot}$ and NO, i.e. peroxynitrite, as previously demonstrated [50]. The decrease in luminol-enhanced chemiluminescence of EE_2-treated BAECs is likely to be the direct consequence of decreased $O_2^{-\cdot}$ production.

In conclusion, we have been able to demonstrate that E_2 increases rat aorta EDRF activity in the absence of changes in endothelial NO synthase gene expression. We also showed that E_2 decreases $O_2^{-\cdot}$ generation in endothelium in vivo, probably accounting for the enhanced EDRF activity. In vessels exposed to estrogens, the stimulation of NO production thus appears to be more efficient (higher NO bioactivity) and potentially less deleterious (lower generation of peroxynitrite). Many beneficial effects could arise from this antioxidant mechanism. This increased NO bioactivity may promote vasodilation, inhibit proliferation of the adjacent vascular smooth muscle, and may inhibit platelet aggregation [9] as well as the inflammatory reaction induced by cytokines [51,52]. In addition, $O_2^{-\cdot}$ has been implicated in the oxidation of low density lipoproteins (LDLs), and the decrease in $O_2^{-\cdot}$ production should confer the estrogens with antioxidant properties [53–55]. Finally, the decreased generation of peroxynitrite, another species involved in the atherosclerotic process, could help to protect the vessel wall [56,57]. All of these effects could contribute to the atheroprotective properties of estrogen.

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