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Estrogen Affects Vascular Tone Differently According to Vasoactive Substances in Ovariectomized Sprague-Dawley Rat

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Abstract

The favorable effects of estrogen on cardiovascular diseases can be explained by several mechanisms such as changes in serum lipid profiles and thrombogenecity. Estrogen also affects the vascular tone, but there has been no report in which the effect of estrogen was tested comprehensively for several vasoactive substances, especially after long-term administration. Two weeks after bilateral ovariectomy in 8-week old female Sprague-Dawley rats, placebo or 17 β-estradiol (E_2) pellets (0.5 mg; released over 3 weeks) were implanted subcutaneously. Two weeks after pellet implantation, organ chamber experiments were performed using aortae. Compared with control, E_2-treated vessels showed impaired endothelium-dependent relaxation to acetylcholine. E_2 enhanced the contraction to norepinephrine and U46619 and had no effect on endothelin-1-induced contraction. In contrast, the contraction to angiotensin (AT)-II was inhibited by E_2. Northern blot analysis for AT_1 receptor expression using cultured aortic smooth muscle cells showed no difference between control and E_2-treated cells, suggesting that AT_1 receptor downregulation is not the likely mechanism. These results suggest that E_2 affects the vascular tone variably according to vasoactive substances.

Key Words: Estrogen, rat, aorta, ovariectomy, vascular tone, endothelium-dependent relaxation, norepinephrine, angiotensin II, U46619, endothelin-1

INTRODUCTION

The incidence of coronary artery disease is increased in postmenopausal women and epidemiologic studies have suggested that hormone replacement therapy may prevent its occurrence.[1,2] Estrogen protects the cardiovascular system by several mechanisms. First, estrogen has a beneficial effect on serum lipid profile; low-density lipoprotein (LDL) is decreased and high-density lipoprotein is increased by estrogen.[3] In addition, estrogen has an antioxidant effect preventing the oxidation of LDL which is more atherogenic than native LDL.[4] Serum fibrinogen[5,6] and plasminogen activator inhibitor-1[7] are also decreased by estrogen, thus reducing thrombogenecity. Interestingly, estrogen also affects the vascular tone. Previous reports suggested that endothelial function is improved by estrogen, probably mediated by increased release of nitric oxide.[8-15] Estrogen also has a direct relaxant effect on vascular smooth muscle independent of the endothelium.[16-18] However, the effect of estrogen is variable according to experimental settings. For example, estrogen increases the contractile response to norepinephrine in the rat aorta.[19] In addition, the contractile response to thromboxane analogue U46619 is decreased in the coronary artery of a guinea pig[20] but increased in the pulmonary vessel of a rat.[21] Thus, the effect of estrogen on vascular tone seems to vary according to the animals, vascular beds, and vasoactive substances used for experiments, and possibly the treatment period as well. We investigated the effect of estrogen on the responses of blood vessel to various vasoactive substances in ovariectomized rats. To specifically determine the chronic effect of estrogen, we used subcutaneously implanted estrogen pellets which were released over three weeks.

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MATERIALS AND METHODS

Materials

Female Sprague-Dawley rats (8 weeks old) were anesthetized intramuscularly with ketamine (4.25 mg/kg) and xylazine (0.3 mg/kg), and both ovaries were removed in prone position. Two weeks after ovariectomy, the rats were divided into a placebo-(control) group and a 17 β-estradiol (E_2)-treated group. In the E_2 group, 0.5 mg E_2 pellets (3-week release form) were implanted subcutaneously with a 10-gauge trochar. Corresponding placebo pellets were implanted in control rats. Two weeks after pellet implantation, the rats were anesthetized in the same way. Venous samples were obtained from the femoral vein for measurement of serum E_2 and the thoracic aortae were obtained for organ chamber study.

Methods

Organ chamber study: The thoracic aortae were kept at 4°C until experiment in modified Krebs-Ringer bicarbonate solution (control solution; NaCl 118.0 mM, KCl 4.7 mM, $CaCO_3$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 23.0 mM, EDTA 0.026 mM, glucose 11.1 mM). The vessels were cleaned of adherent tissue and cut into rings 3 mm in length. The vascular rings were suspended in the organ chamber between two stainless steel stirrups and isometric tension was measured via a force transducer by physiograph. The vessels were stretched in multiple steps until optimal preload (2 gm) was reached. After waiting 30 minutes for the vessels to stabilize, contraction with 60 mM KCl solution followed by washing with control solution was repeated two times. For contractile responses (below), contraction with 60 mM was repeated once again. The last KCl contraction was used as a standard to normalize the contractile responses for each concentration of agonists.

Endothelium-dependent relaxation to acetylcholine (ACh); After the tension returned to baseline, the vessels were precontracted with norepinephrine (NE; 10^{-7}M) and cumulative concentrations ($10^{-9}-3\times10^{-6}$ M) of ACh were added.

Contractile responses to NE, U46619 and endothelin (ET)-1; When the tension returned to baseline after the last KCl contraction, cumulative concentrations of NE ($10^{-9}-10^{-5}$ M), U46619 ($10^{-9}-3\times10^{-6}$ M) and ET-1 ($10^{-10}-10^{-7}$ M) were added to the quiescent vessels.

Contractile response to angiotensin (AT) II; When the tension returned to baseline after the last KCl contraction, a single concentration of AT II (10^{-7} M) was added into the quiescent vessels. Cumulative concentration-contraction responses could not be performed due to the easy desensitization of vessels to AT II.

Aortic smooth muscle cell culture: Vascular smooth muscle cells were isolated from rat thoracic aortae by enzymatic dissociation and characterized as described previously.[22] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Cells from passage 11 were made quiescent by incubation with serum-free DMEM for 24 hours, and then treated with 1 μM E_2 or vehicle (100% ethanol). Cells were harvested at 0, 3, 6, 12 and 24 hours after incubation with E_2 or vehicle.

Northern blot analysis for AT_1 receptor expression: After recovery from cultured rat aortic smooth muscle cells by acid guanidinium thiocyanate- phenol-chloroform,[23] total RNA (10 μg) was size- fractionated on 1.0% agarose/formaldehyde gel and transferred overnight to nylon membranes (Boehringer-Mannheim). The membranes were hybridized with rat AT_1 receptor cDNA probe labelled by random priming with [α-^{32}P] dCTP. Hybridized membranes were processed using the FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Film Co, Tokyo, Japan).

Measurement of serum E_2: Radioimmunoassay kits were used to measure serum E_2 levels.

Drugs

E_2 pellets were obtained from Innovative Research of America (Saratosa, Fa, USA). NE, ACh, AT II, U46619 and ET-1 were obtained from Sigma (St Louis, Mo, USA). ET-1 was dissolved in 0.1% bovine serum albumin and other reagents were dissolved in distilled water and stored at −20°C until used.

Statistical analysis

For endothelium-dependent relaxation response,

maximal relaxation and pIC_{50} (negative log molar concentration of ACh which relaxed the vessel to 50% of the contraction by NE) were obtained. For contractile responses, the area under the curve (AUC; in arbitrary unit), maximal contraction (percent of the contraction to 60 mM KCl) and pD_2 (negative log molar concentration which provoked 50% of the contraction to 60 mM KCl) were calculated. All data were expressed as mean±SEM. Mann-Whitney U- test was used to compare control and E_2-treated groups. $p<0.05$ was considered to be significant.

RESULTS

Organ chamber experiment

Endothelium-dependent relaxation to ACh (Fig. 1): Compared with control group, endothelium-dependent relaxation response was impaired in the E_2 group (control vs E_2; maximal relaxation; 100.0±3.8% vs 87.2±1.9%; pIC_{50}; 7.7±0.1 vs 7.2±0.1; n=6; $p<0.05$).

Contractile response to NE (Fig. 2): Contraction to NE was enhanced in the E_2 group compared with control (control vs E_2; AUC; 231±7 vs 347±20; maximal contraction; 141.8±3.8% vs 186.5±11.4%; pD_2; 7.19±0.06 vs 7.79±0.05; n=6; $p<0.05$).

Contractile response to AT II (Fig. 3): Contraction to AT II was inhibited in the E_2 group compared with control (control vs E_2; 25.3±1.8% vs 10.7±1.5%; n=5; $p<0.05$).

Contractile response to U46619 (Fig. 4): Maximal contraction was enhanced in the E_2 group compared with control (control vs E_2; 149.2±6.0% vs 208.2±19.8%; n=6; $p<0.05$). AUC and pD_2 were not significantly different between the two groups (control vs E_2; AUC; 359±20 vs 464±50; pD_2; 8.15±0.07 vs 8.12±0.12).

Contractile response to ET-1 (Fig. 5): No significant difference was observed for all parameters tested (control vs E_2; AUC; 188±8 vs 209±18; maximal contraction; 151.7±6.7% vs 178.3±9.7%; pD_2; 8.37±0.03 vs 8.37±0.05).

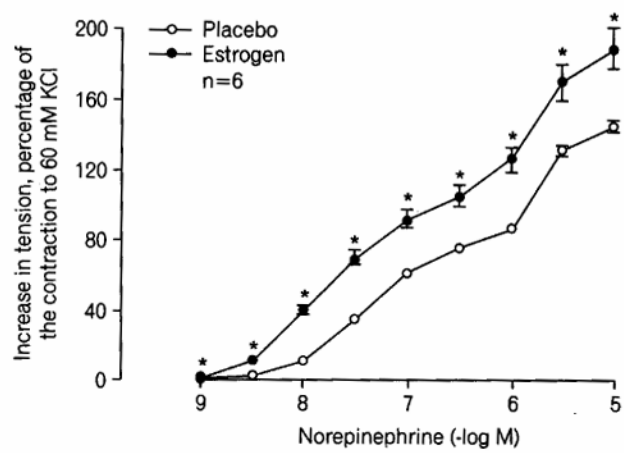

*Fig. 2. Effect of estrogen (17β-estradiol) on the contraction to norepinephrine. Data are expressed as mean±SEM. * $p<0.05$ between control and E_2-treated vessels.*

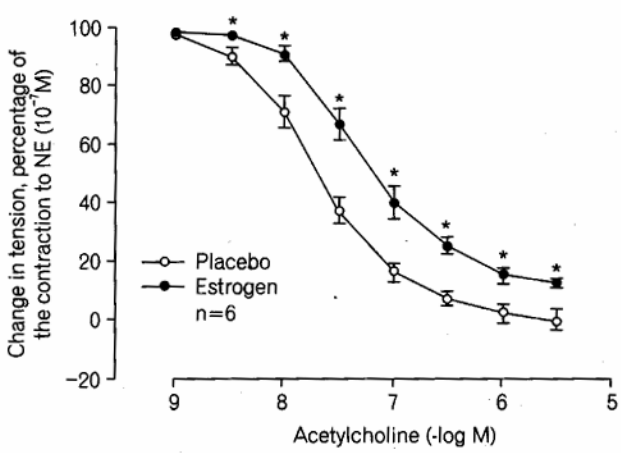

*Fig. 1. Effect of estrogen (17β-estradiol) on endothelium-dependent relaxation to acetylcholine. Data are expressed as mean±SEM. * $p<0.05$ between control and E_2-treated vessels.*

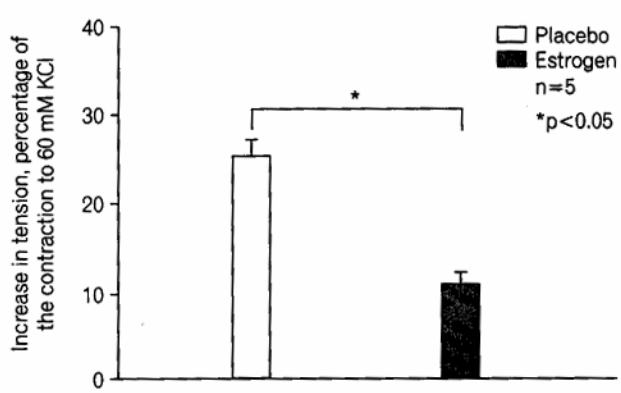

Fig. 3. Effect of estrogen (17β-estradiol) on the contraction to angiotensin II. Data are expressed as mean±SEM.

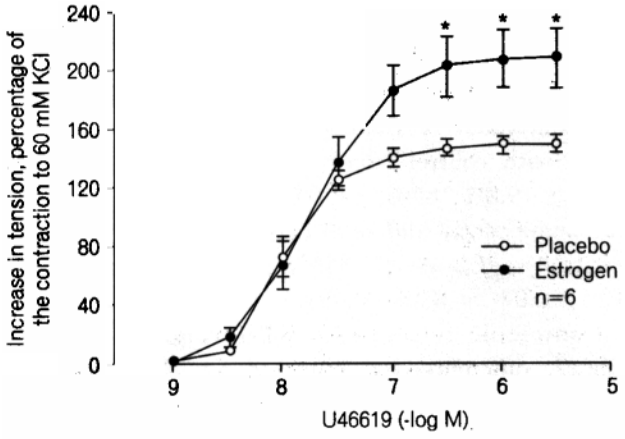

*Fig. 4. Effect of estrogen (17 β-estradiol) on the contraction to U46619. Data are expressed as mean ±SEM. * $p<0.05$ between control and E_2-treated vessels.*

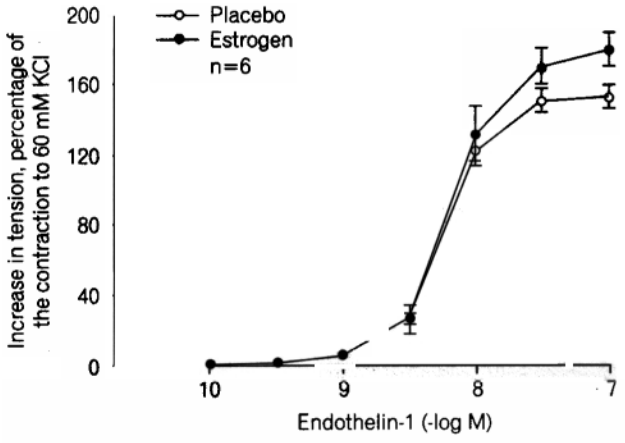

Fig. 5. Effect of estrogen (17 β-estradiol) on the contraction to endothelin-1. Data are expressed as mean ±SEM.

Northern blot analysis

The expression of AT_1 receptor mRNA did not differ between the vehicle- and E_2-treated aortic smooth muscle cells (Fig. 6).

Serum E_2 concentrations

Serum E_2 concentration was about six times higher in the E_2 group compared with control (131.9±39.4 vs 22.3±2.5 pg/ml; n=6; $p<0.05$).

DISCUSSION

Estrogen replacement therapy is considered to prevent not only osteoporosis, but also cardiovascular disease. Estrogen seems to protect the cardiovascular system by several mechanisms. In addition to beneficial effects on serum lipid profile and thrombogenecity,[3-7] extracelluar matrix synthesis is also inhibited by estrogen,[24] preventing the progression of atherosclerosis. Estrogen also stimulates angiogenesis,[25] probably contributing to the development of new collateral circulation in the heart.

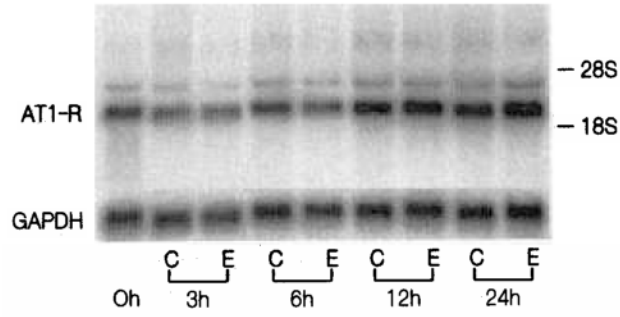

Fig. 6. Northern blot analysis for angiotensin AT_1 receptor expression. Rat aortic smooth muscle cells were treated with E_2 (1 μM; E) or vehicle (ethanol; C) and harvested at each time point. Total RNA (10 μg) was size-fractionated on 1% agarose/formaldehyde gel, transferred to nylon membranes and hybridization was done using [α-^{32}P] dCTP labelled rat AT_1 receptor cDNA.

In this study, we investigated the effect of E_2 on the endothelial and vascular smooth muscle function of the rat. For the latter, the contractile responses of aortae to various important vasoactive substances were examined. Serum E_2 levels changed appropriately by ovariectomy and E_2 replacement.

Endothelium-dependent relaxation to ACh was impaired in E_2-treated rats in our study. This unexpected finding is in contrast to several previous reports, which suggested a beneficial effect of estrogen on endothelial function.[4,8-15,26-31] However, some other reports showed that endothelium-dependent relaxation to ACh was not affected by estrogen.[32-35] Our result is contrary to earlier studies in that ACh-induced relaxation was even less effective in E_2-treated vessels. Although the mechanism is unclear, the preserved endothelial function in our study, despite ovariectomy, may have caused the different result from other reports. Although we waited for two weeks after ovariectomy before E_2 replacement, it may not have been long enough for endothelial dysfunction to develop. Exogenous estrogen may affect normal endothelium differently compared to

dysfunctional endothelium. For example, in one study where ovariectomy was not performed, estrogen administration did impair endothelium-dependent relaxation to ACh of rat aorta.[36] Thus, the possibility cannot be dismissed that a different result may be obtained if E_2 is replaced after endothelial dysfunction develops fully. Indeed, studies performed in hypercholesterolemic swine[4] or postmenopausal women[29] (where endothelial function was likely to be impaired) showed a beneficial effect of estrogen on endothelial function. Thus, further experiments using blood vessels obtained several months after ovariectomy and/or from older rats seem to be justified. The other possible explanation of our result is that the release of endothelium-derived contracting factor (EDCF; e.g. cyclooxygenase product) provoked by E_2 may have superceded that of endothelium-derived relaxing factor (EDRF; e.g. nitric oxide), since E_2 has been reported to stimulate not only the release of EDRF, but also EDCF.[32]

The contractile responses to various agonists were tested in our study. First, contraction to NE was enhanced in E_2-treated rats. Similar results have been presented by others,[16,19] but not all.[37] Although not investigated in our study, inhibition by E_2 of the neuronal reuptake of NE [32] or increased alpha-adrenergic receptor affinity[38] may be plausible explanations.

In contrast to NE, the contractile response to AT II was inhibited by E_2 treatment. To see if this effect was associated with AT receptor downregulation, we performed Northern blot analysis for cultured rat aortic smooth muscle cells using rat AT_1 receptor cDNA probe. However, we were unable to see any difference between vehicle- and E_2-treated smooth muscle cells. Thus, it seems less likely that E_2 treatment affects the expression of AT_1 receptor. Recently, Nickenig et al reported that ovariectomy increased the contractile response of rat aorta to AT II compared to sham-operated rats, the meaning of which is similar to our results.[39] However, they showed an increased AT_1 receptor mRNA expression by ovariectomy which was normalized by estrogen replacement. They also showed that estrogen downregulated AT_1 mRNA expression in cultured vascular smooth muscle cells, suggesting that E_2 controlled the AT_1 transcription. The reason for the discrepancy between Nickenig's study and ours is not clear. There are additional reports which also showed estrogen-induced inhibition of the contractile response to AT II.[19,40] Interestingly, Carriere et al showed that chronic estradiol treatment decreased angiotensin II receptor density in the anterior pituitary gland and adrenal cortex, but not in the mesenteric artery.[40] Thus, the reason for E_2-induced change in the contractile response to AT II remains uncertain. The possibility also exists that the intracelluar signal transduction of AT II may be affected by E_2.

The maximal contractile response to U46619 (but not sensitivity) was enhanced by E_2 treatment. U46619 is a thromboxane analog and thromboxane A_2 is associated with local vasoconstriction (vasospasm) rather than systemic vascular resistance. Although only the maximal contraction at high concentrations was affected, it may not be neglected if activated platelets release enough thromboxane at a specific location in the vessel. Farhat et al showed that pulmonary vasoconstriction to U46619 was enhanced after administration of E_2 for one week in the rat.[21] They suggested that it might be mediated by the release of cyclooxygenase product since indomethacin partially inhibited this phenomenon. The mechanism may be other than receptor upregulation since estrogen did not increase thromboxane A_2 receptors in cultured vascular smooth muscle cells in one study.[41]

In contrast to NE, U46619 and angiotensin II, the contractile response to endothelin-1 was not significantly affected by E_2 treatment.

The overall effect of E_2 on systemic vascular resistance is uncertain according to this study. However, in our recent study using spontaneous hypertensive rats, blood pressure did not change significantly following 60 days' E_2 administration (data not shown). Thus, the variable effects of E_2 to several vasoactive substances may negate one another without changing systemic blood pressure. This may be the reason why postmenopausal estrogen replacement therapy did not affect blood pressure of hypertensive patients.[42]

In conclusion, 2 weeks' E_2 treatment in ovariectomized young Sprague-Dawely rats did not improve, but impaired endothelium-dependent relaxation to ACh. However, further experiments seem to be required since endothelial function was not impaired in our study, even in placebo-treated ovariectomized rats. The contraction to NE and U46619 was enhanced, but AT II-induced contraction was inhibited by E_2, suggesting that E_2 affects vascular smooth muscle fun-

ction differently according to the vasoactive substances.

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