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Estrogen Attenuates Endothelin-1 Production by Bovine Endothelial Cells via Estrogen Receptor

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To investigate the mechanism underlying the antiatherogenic effect of estrogen, we examined the role of estrogen in the regulation of endothelin-1 (ET-1) production by cultured bovine carotid arterial endothelial cells. Treatment of the cells with 17β-estradiol dose dependently inhibited gene expression and peptide secretion of ET-1. This inhibitory effect of 17β-estradiol was more marked when ET-1 production was stimulated by transforming growth factor-β1. Simultaneous addition of ICI 182,780, a pure estrogen receptor antagonist, completely blocked the effect of 17β-estradiol, indicating that 17β-estradiol acted via estrogen receptor. These results suggest that reduced ET-1 production by endothelial cells may be involved in the vasoprotective effect of estrogen.

Estrogen replacement therapy has been shown to reduce the risk of atherosclerotic diseases in postmenopausal women by ≈50% (1, 2). Studies using animal models also indicate that estrogen has the protective effects against atherosclerosis (3–5). Direct vasoprotective actions (3, 4) as well as lipid-altering properties (5, 6) are noted as the mechanisms underlying the antiatherogenic effects of estrogen. Estrogen is reported to possess both endothelium-dependent (7) and -independent (8) vasodilator actions. Regulation of nitric oxide production (9), regulation of prostacyclin production (10) and a calcium-antagonistic effect (8) may be involved in these effects. In addition, the existence of estrogen receptor (ER) in vascular endothelial cells (ECs) (11) and vascular smooth muscle cells (VSMCs) (12) suggests the estrogen receptor-mediated pathway of estrogen action.

Endothelin-1 (ET-1), a vasoconstrictor peptide, is also a potent mitogen for VSMCs (13). Plasma and tissue ET-1 levels are elevated in atherosclerotic diseases (14), and an endothelin receptor antagonist suppresses neointimal formation in rat (15), suggesting that ET-1 contributes to the development of atherosclerosis. On the other hand, the possible role of estrogen in the regulation of ET-1 production has been reported. Plasma ET-1 level is higher in men than in women (16, 17), and is decreased in male-to-female transsexuals (17). Moreover, we recently demonstrated that endogenous and exogenous estrogen reduced plasma ET-1 concentrations by regulating the production in rats (18). In this previous study, we could not elucidate the involvement of ECs in the estrogen-related regulation of ET-1 production although ECs may be the main source of ET-1 (19) and play a critical role in the development of atherosclerosis. Furthermore, no direct evidence has been reported concerning the question whether estrogen could regulate ET-1 production by ECs.

Thus, to answer this question, we examined the effect of estrogen on the secretion of ET-1 in the culture supernatant and the expression of ET-1 mRNA using cultured bovine ECs.

MATERIALS AND METHODS

Cell culture. ECs were isolated from bovine carotid artery by scraping according to the method described previously (20). These cells were identified as ECs by their "cobble-stone" appearance and by the immunohistochemical detection of factor VIII (Immunohistochemical staining kit; Biomeda, Foster City, CA). Moreover, the cells were positively stained with the monoclonal anti-estrogen receptor antibody (12) (data not shown). ECs were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. ECs of 5th to 7th passage were used in the experiments.

Enzyme-linked immunosorbent assay (ELISA) of ET-1 in the culture supernatant. ECs were seeded in 24-well multiplates at a density of 2.5×10^4 cells/well and grown to confluence. Then the medium was replaced with phenol red-free DMEM (Sigma) containing 17β-estradiol (E2, Sigma) or vehicle (0.1% ethanol). Twenty four hours later, porcine transforming growth factor β1 (TGF-β1, 0–2.0 ng/ml; R & D Systems, Minneapolis, MN) was added, and after incubation for another 24 h the culture supernatant was collected to measure ET-1. The cells were lysed with 0.5N NaOH and used to determine protein concentration. ET-1 concentrations in the culture supernatant were assayed using a sensitive sandwich ELISA kit (YSE-7720; Yamasa Shoyu, Chiba, Japan) (21). The range of measurement was 25-400 pg/ml, and the intraassay coefficient of varia-

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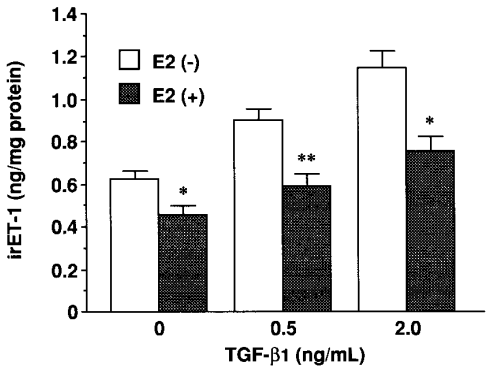

FIG. 1. Effect of 17β-estradiol (E2) on the secretion of endothelin-1 (ET-1) by cultured endothelial cells (ECs). ECs were pretreated with (E2 (+)) or without (E2 (−)) 10 nM E2 for 24 h. Transforming growth factor-β1 (TGF-β1; 0, 0.5, 2.0 ng/ml) was then added and the cells were incubated for another 24 h. Immunoreactive ET-1 (irET-1) in the culture supernatant was measured and normalized by cellular protein. Values are expressed as mean ± SEM (n = 4). *, **$p < 0.05$, 0.01 vs E2(−).

tion was 3.4–6.2%. The cross-reactivities with ET-2, ET-3, and big ET-1 were 100, 1, and 0%, respectively. The diluted culture supernatant was applied to the kit, then ET-1 concentrations were normalized by cellular protein concentrations which were determined using Bio-Rad protein assay kit (Bio-Rad).

Northern blot analysis of ET-1 mRNA. ECs were seeded in 10 cm-culture dishes and grown to confluence. The medium was then replaced with phenol red-free DMEM containing E2 or vehicle. Twenty four hours later, TGF-β1 (0 or 2.0 ng/ml) was added to the dishes. RNA was extracted by the guanidinium thiocyanate method (22) after the incubation for 4 h, because the previous report (23) and our preliminary experiment showed the peak of ET-1 mRNA expression at this time point. Total RNA (20 μg) was electrophoresed on 1% agarose gel, blotted onto nylon membrane (Hybond-N+, Amersham) and hybridized with ^{32}P-labeled rat ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments (kindly provided by Dr. Koji Maemura and Dr. Hiroki Kurihara, Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo) for 16 h. Labeling was performed using [α-^{32}P]dCTP (Amersham) and Rediprime DNA labeling system (Amersham).

Statistics. The values in the text and figures are expressed as mean ± SEM. The data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Newman-Keuls' test was performed to isolate the difference between the groups. A p value less than 0.05 was considered to be significant.

RESULTS

As shown in Fig. 1, immunoreactive ET-1 secreted in the culture supernatant was decreased by pretreatment of ECs with 10 nM E2 for 24 h. The expression of ET-1 mRNA was also decreased by E2 pretreatment (Fig. 2). Although E2 inhibited both basal and induced production of ET-1, the effect was more marked when ET-1 production was stimulated by TGF-β1 (Fig. 1 and Fig. 2). We examined the time-related effect of E2 but no significant effect was observed when ECs were pretreated with 10 nM E2 for up to 8 h (data not shown). Thus, in the following experiments, we pretreated ECs with E2 for 24 h and subsequently stimulated ET-1 production by TGF-β1. Figure 3 shows the dose-dependent effect of E2 on ET-1 secretion (Fig. 3A) and ET-1 mRNA expression (Fig. 3B); 100 nM E2 inhibited ET-1 secretion by 30% and ET-1 mRNA expression by 60%.

We further investigated the cellular mechanism of the E2 effect. 17α-Estradiol (10 nM), an inactive E2 stereoisomer, or progesterone (10 nM) had no significant effect on ET-1 mRNA expression (data not shown). We examined the involvement of nitric oxide because it is reported that E2 induces nitric oxide production (24) and nitric oxide attenuates ET-1 production (25) by ECs. As shown in Fig. 4A, simultaneous addition of 10 μM N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase, with E2 had no influence on the E2 effect on ET-1 mRNA. We also examined the involvement of prostacyclin but the addition of 10 μM indomethacin did not influence the E2 effect (data not shown). We next examined whether estrogen receptor mediated the effect of E2 on ET-1 expression. As shown in Fig. 4B, ICI 182,780 (ZENECA Pharmaceuticals), a pure estrogen receptor antagonist (26), dose dependently and completely blocked the E2 effect although ICI 182,780 alone had no effect on ET-1 mRNA expression (data not shown), indicating that E2 acted via estrogen receptor.

DISCUSSION

In the present study, we demonstrated that E2 decreased both the secretion and the gene expression of ET-1 by bovine ECs. We also showed that this inhibitory effect of E2 was mediated by estrogen receptor. We recently reported that estrogen reduced not only the

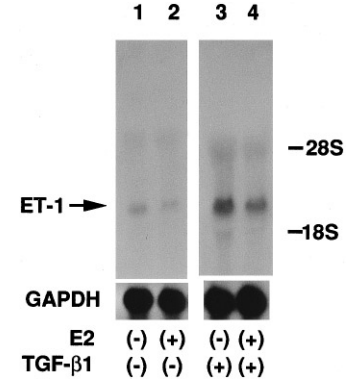

FIG. 2. Effect of 17β-estradiol (E2) on the expression of endothelin-1 (ET-1) mRNA by cultured endothelial cells (ECs). ECs were pretreated with (E2 (+), lane 2 and 4) or without (E2 (−), lanes 1 and 3) 10 nM E2 for 24 h. Then, 2.0 ng/ml transforming growth factor-β1 (TGF-β1 (+), lane 3 and 4) or vehicle (TGF-β1 (−), lanes 1 and 2) was added and the cells were incubated for 4 h. RNA was extracted and 20 μg total RNA was used for Northern blot analysis of ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

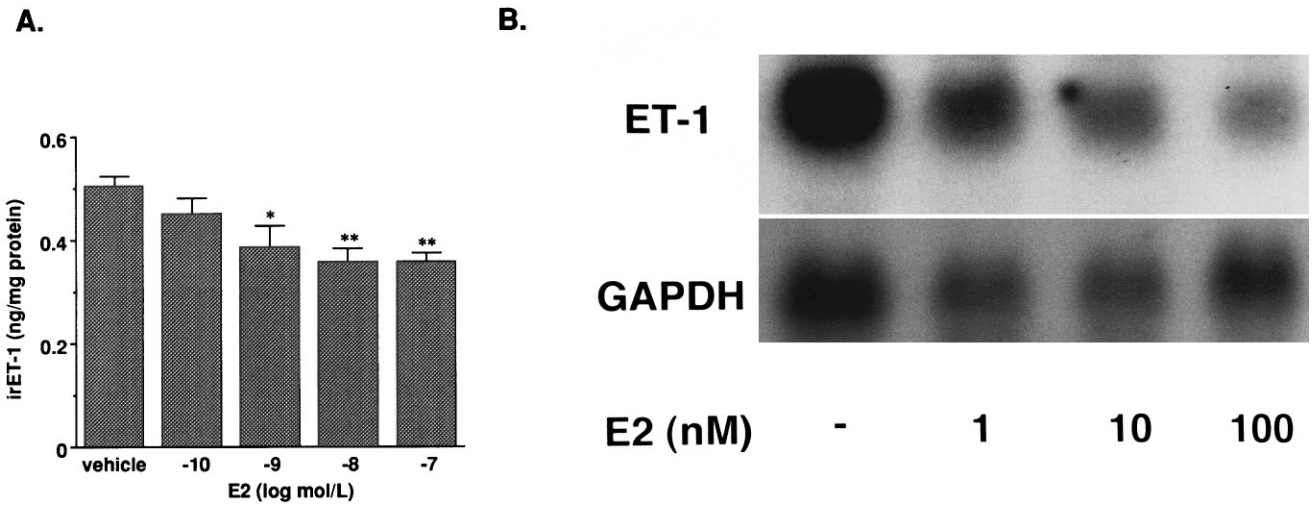

FIG. 3. Dose-dependent effect of 17β-estradiol (E2) on the secretion of endothelin-1 (ET-1) immunoreactivity (A) and the expression of ET-1 mRNA (B) by cultured endothelial cells (ECs). ECs were pretreated with the indicated concentrations of E2 for 24 h. Then, 2.0 ng/ml transforming growth factor-β1 was added to stimulate ET-1 production. (A) Immunoreactive ET-1 (irET-1) in the culture supernatant was measured and normalized by cellular protein. Values are expressed as mean ± SEM. *, **$p < 0.05$, 0.01 vs vehicle. (B) Twenty micrograms of total RNA was used for Northern blot analysis of ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

plasma ET-1 concentration but also the ET-1 expression in VSMCs *in vivo* (18). However, since ET-1 is expressed in ECs abundantly compared with other tissues (19), the effect of estrogen on plasma ET-1 concentrations may be due to the attenuated production of ET-1 by ECs. The present study strongly suggests this possibility. Moreover, locally regulated expression of ET-1 by ECs may play a more important role as a paracrine factor for VSMCs than circulating ET-1. Indeed, Lerman *et al.* (27) have reported that acetylcholine-induced production of ET-1 by ECs contributes to the coronary vasoconstriction in humans. Filep (28) reported that endothelin receptor antagonists decreased blood pressure in rats when nitric oxide production was blocked by N^G-nitro-L-arginine methyl ester. Thus, estrogen may regulate vascular tone and VSMC proliferation by inhibiting circulating and locally produced ET-1.

Estrogen is reported to modulate the production of other vasoactive substances as well as the endothelial function. Hayashi *et al.* (24) have reported that estrogen upregulates endothelial nitric oxide synthase and stimulates nitric oxide production by human umbilical vein ECs and bovine aortic ECs. Seillan *et al.* (10) have reported that estrogen induces the secretion of prostaglandins including prostacyclin in piglet ECs. Endothelium-dependent vasodilatory actions of estrogen (7) may be due to these vasodilatory substances as well as ET-1. On the other hand, it has been shown that estrogen stimulates the proliferation of ECs and protects ECs from cell death. Morales *et al.* (29) have reported that estrogen promotes angiogenic activity *in vitro* and *in vivo*. Krasin-

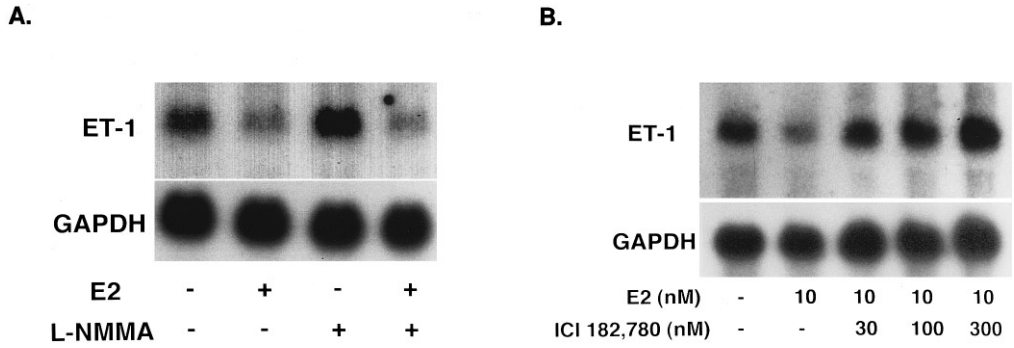

FIG. 4. Effect of 17β-estradiol (E2) on endothelin-1 (ET-1) mRNA expression by cultured endothelial cells (ECs); influence of a nitric oxide synthase inhibitor (A) and an estrogen receptor antagonist (B). (A) ECs were pretreated with or without 10 nM E2 and with or without 10 μM N^G-monomethyl-L-arginine (L-NMMA) for 24 h. (B) ECs were pretreated with or without 10 nM E2 and with the indicated concentrations of ICI 182,780 for 24 h. Then, in both experiments, 2.0 ng/ml transforming growth factor-β1 was added and incubated for 4 h. Twenty micrograms of total RNA was used for Northern blot analysis of ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ski *et al.* (30) have reported that estrogen accelerates reendothelialization after arterial injury. Recently, Spyridopoulos *et al.* (31) reported that estrogen protected human umbilical vein ECs from apoptosis induced by tumor necrosis factor-α. Interactions of vasoactive substances described above and possible involvement of these substances in estrogen-mediated endothelial protection remain to be defined.

In the present study, although we have shown that estrogen attenuates the expression of ET-1 mRNA, it is not determined whether estrogen regulates the transcription or the stability of ET-1 mRNA. Estrogen receptor binds to the estrogen responsive element of estrogen responsive genes and acts by regulating their transcription (32). Since the known structure of estrogen responsive element is not found in the 5′ upstream region of ET-1 gene (33), it is possible that some factor which is regulated by estrogen may affect ET-1 mRNA expression. Also, the result that estrogen treatment for up to 8 h did not inhibit ET-1 mRNA expression suggests this possibility. Thus, we examined the possible role of nitric oxide as a candidate for the mediator. However, the inhibitory action of estrogen was not affected by N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase, suggesting that nitric oxide is not involved in this estrogen action. Recently, a new subtype of estrogen receptor, estrogen receptor β, was cloned (34), and is reported to be expressed in ECs (35). In this study, we demonstrated that the inhibitory effect of estrogen on ET-1 expression was blocked by an estrogen receptor antagonist, ICI 182,780. However, since estrogen receptor antagonists can work via both estrogen receptor β and the classical estrogen receptor, estrogen receptor α (36), it is unknown whether this blockade was mediated by estrogen receptor α or β. A further study is needed to clarify the molecular mechanism by which estrogen inhibits ET-1 mRNA expression.

In conclusion, estrogen attenuates ET-1 production by ECs via an estrogen receptor-dependent pathway. This finding adds a new insight into the mechanism underlying the anti-atherogenic action of estrogen.

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