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17β-Estradiol modulates endothelin-1 expression and release in human endothelial cells

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Abstract

Objective: In this study the role of 17β-estradiol (E2) in the regulation of endothelin-1 (ET-1) mRNA expression and secretion was investigated in cultured human umbilical vein endothelial cells (HUVECs). **Methods:** Endothelial cells were either deprived of or treated with 17β-estradiol (10^{-9}, 10^{-7} M) for 48 h. After the incubation, the effect of E2 on ET-1 gene expression was evaluated by Northern blot analysis. ET-1 release into the media was measured by radioimmunoassay after 6 h of incubation under basal conditions and upon stimulation with thrombin (4 U/ml). In addition, the cyclic guanosine 5′-monophosphate (cGMP) content of cells was assayed by immunoassay. In order to exclude the role of nitric oxide (NO) in E2-induced effects on endothelin-1 gene expression and secretion, nitric oxide synthase (NOS) inhibitor, *N*-nitro L-arginine methyl ester (1 mM) (L-NAME) was added to the media of some cultures. **Results:** Incubation of HUVECs with 10^{-9} and 10^{-7} M E2 for 48 h resulted in a 30 and 47% inhibition of ET-1 mRNA expression, respectively. Incubation with E2 also decreased the basal and thrombin-stimulated ET-1 release while increasing the cGMP content of cells significantly. NOS inhibitor L-NAME increased the release of ET-1 from E2-incubated cells but did not alter the ET-1 release from hormone-deprived cells. However, ET-1 secretion of E2-treated cells were significantly less than the deprived ones. Northern blot analyses also demonstrated that inhibition of NOS only partly attenuated the effect of E2 on ET-1 gene expression. In the presence of L-NAME, treatment with 10^{-7} M E2 caused a 12% decrease in ET-1 gene expression. **Conclusion:** The results demonstrate that E2 may play both direct and indirect role in regulation of ET-1 gene expression and production in human endothelial cells. E2-induced increase in NO but decrease in ET-1 production may partly explain the mechanism of the protective effects of the hormone on the cardiovascular system.

Keywords: Endothelial function; Endothelins; Nitric oxide; Hormones; Gene expression

1. Introduction

The vascular endothelium plays an essential role in the regulation of vascular tone by producing vasoactive substances like nitric oxide (NO), prostacyclin and endothelin-1 (ET-1) in response to local and circulating factors [1]. There is increasing evidence that sex steroid 17β-estradiol (E2) acts directly on the endothelium and influences the vascular functions and reactivity. Studies on the influence of E2 on vascular function indicate that the effect of the hormone on endothelium may involve changes in the production of several endothelium-derived factors. These studies show that E2 potentiates endothelium-dependent vasodilation to acetylcholine in atherosclerotic arteries, acts on coronary vascular bed as a vasodilator, suppresses the contraction of various arteries, and has a significant antiatherosclerotic effect [2–8].

The exact mechanism underlying the effects of estrogen on vascular function has not yet been clearly defined. However, it has been established that NO plays an important role in the effects of estrogen on the vasculature [8,9]. In vivo and in vitro studies have shown that the release of NO is greater in females than in males, and E2 enhances basal NO formation in endothelial cells by

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stimulating the expression of nitric oxide synthase (cNOS) [10–13]. Due to the well known favorable effects of NO, the increased release of NO is proposed to play an important role in the beneficial effects of estrogen such as the low incidence of cardiovascular diseases in premenopausal women as compared to men.

Although much of the current research on the effect of estrogen on vascular function has focused on endothelium-derived NO, another possible mediator of estrogen action is ET-1. Endothelin-1 is a peptide which plays an important role in vascular physiology and disease [14–16]. It is a potent vasoconstrictor which also stimulates vascular smooth muscle cell proliferation, migration and monocyte adhesion [17–19]. The production of ET-1 is regulated by a variety of hormones, other vasoactive substances and vascular stress. Recent studies demonstrate that plasma ET-1 levels are also influenced by estrogen [20–22].

However, the mechanism by which estrogen modulate ET-1 production remains unclear and speculative. In this study, we aimed to investigate the effect of E2 on ET-1 gene expression, and basal and thrombin stimulated release from human umbilical vein endothelial cells (HUVECs). Moreover, we tried to investigate the role of NO in this process.

2. Methods

2.1. Endothelial cell culture

Endothelial cell cultures were prepared from human umbilical cord veins [23]. Cells were isolated by collagenase (0.1%, type IA, C-2674 Sigma, in HEPES buffer) treatment of veins. Endothelial cells obtained from two to four different cords were pooled and seeded in 24-welled culture dishes or in 25-cm^2 flasks for Northern analysis. The cultures were grown in medium 199 (M-2520 Sigma) supplemented with 10% fetal calf serum (FCS) (04-001-1B, Biol. Indus., Kibbutz Beth Haemak, Israel), and antibiotics (penicillin–streptomycin, P-3539, amphotericin B, A-9528 Sigma). The cultures were maintained at 37°C, with 5% CO_2 containing humidified air. The medium was changed every other day. The identification of endothelial cells in culture was based on typical cobblestone morphology and the presence of von Willebrand factor (Von Willebrand reagent, Dade Behring, Germany) in conditioned media which was absent in fresh media. When the cultures were nearly confluent, the cells were incubated with estrogen-free medium or medium containing 10^{-9} or 10^{-7} M E2 for 48 h.

During this incubation period, the NOS inhibitor, L-arginine analog *N*-nitro-L-arginine methyl ester (L-NAME) was also added to some culture media in order to investigate the effect of NO. The concentration of L-NAME (1 mM) used in the experiments has been shown to be effective at inhibiting NO release from HUVECs in our previous studies. At the end of this incubation period, cells were either frozen to be used for Northern analysis or washed and incubated for additional 6 h in the presence or absence of E2 with/without thrombin (4 U/ml) or L-NAME (1 mM) with medium which contained 0.1% bovine serum albumin (BSA) instead of FCS. Then the media and the cells were collected to be used for ET-1 and cGMP assessments. Culture media was stored at −20°C for up to 2 weeks before the ET-1 assay.

Hormone-free medium was phenol red-free (M-3769 Sigma) and contained FCS which was charcoal-stripped to remove estrogen-like substances [24]. Estrogen level of charcoal stripped FCS was measured by immunoassay and found negligible. Medium containing E2 was prepared by adding E2 to charcoal treated FCS. Stock solutions of E2 was prepared in 99.9% ethanol and stored at −20°C.

The investigation conforms with the principles outlined in the Declaration of Helsinki. The procedure was approved by Marmara University Medical Faculty Ethic Committee.

2.2. Northern blot analysis

Frozen HUVECs were lysed with 800 μl of a denaturing solution containing 4.0 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% sarcosyl and total cellular RNA was isolated by using the guanidium isothiocyanate method [25]. After suspension in diethypyrocarbonate (DEPC) treated water, the RNA was quantified by measurement of optical density at 260 nm. A 10-μg amount of total cellular RNA was fractionated on a 1.5% formaldehyde gel. The samples were tested for equal loading by ethidium bromide staining of the gel, and were then blotted onto nylon membranes (Gene-Screen Plus, Dupont, USA). The membranes were prehybridized for 4 h at 42°C in a solution containing 50% deionized formamide, 5×SSPE (20×SSPE: 3 M NaCl, 0.2 M NaH_2PO_4–H_2O, 20 mM EDTA-Na_2), 5× Denhardt's solution (1× Denhardt's solution: 0.02% BSA, 0.02 Ficoll, and 0.02 polyvinylpyrolidone), and 1% sodiumdodecyl sulfate (SDS). Hybridization was performed with the same solution at 42°C for 20 h using human ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes [ATCC, cat. no: 65699 (ET-1),and 57091 (GPDH)] labeled with [α-^{32}P] dCTP (Amersham, UK) according to the random priming method [26]. The membranes were initially washed at room temperature with 2×SSPE for 15 min, then washed at 65°C for 45 min in 2×SSPE with 2% SDS, and finally agitated at room temperature for 15 min with 0.1×SSPE. After hybridization with ET-1 probe, the membranes were stripped of the labeled probe following manufacturer's instructions and rehybridized with internal control GAPDH probe. Autoradiography was performed by exposing Kodak X-Omat film to the nylon membranes at −70°C. The amount of radioactivity in the individual bands on membranes was quantified by a densitometer

(Vilber Lourmat, France) within linear exposure range of the film and the relative amount of ET-1 messenger RNA (mRNA) in each sample was calculated as the ratio of ET-1 to GAPDH.

2.3. Measurement of endothelin-1

Immunoreactive ET-1 concentration in the culture media was assayed using commercially available RIA kit (RIK 6901, Peninsula Laboratories, USA). Media was extracted with Sep-Pak C_{18} cartridges and evaporated by blowing nitrogen gas. The reconstituted solution was applied to the RIA. According to the manufacturer intra-assay coefficient of variation is <5%, and inter-assay coefficient of variation is <15%. The cross-reactivity of the kit with human big endothelin-1 is 17%, with human endothelin-2 and -3 is 7%.

2.4. Measurement of cGMP

After 6 h of incubation under basal and stimulated conditions cGMP content of cells was measured with EIA kit (Biotrak, Amersham, UK). Cell extracts obtained from four wells were pooled for the assessments. Samples were prepared according to the acetylation assay procedure given in the kit. Results were expressed as fmol/well. Sensitivity of the assay was 2 fmol/well. According to the manufacturer, intra-assay coefficient of variation is 9.0% for a control of 871.8±65.1 fmol/well, and inter-assay coefficient of variation is 12.7% for a control of 240±22.9 fmol/well.

2.5. Statistical analysis

Student's unpaired *t*-test and one-way analysis of variance (ANOVA) were used, and if significance was found Tukey–Kramer multiple comparison test was used to test pairwise differences. Results are expressed as mean±SEM, *n* represents the number of separate wells used in the experiments.

3. Results

3.1. Northern blot analysis

Northern blot analysis, using a human ET-1 cDNA as a probe, demonstrated that incubation of HUVECs with 10^{-9} and 10^{-7} M E2 for 48 h resulted in a 30 and 47% inhibition of ET-1 mRNA expression, respectively (Fig. 1A). Inhibition of NOS with L-NAME partly attenuated the effect of E2 on ET-1 expression. Presence of both L-NAME and E2 (10^{-7} M), reduced ET-1 expression by 12% (Fig. 1B). The Northern blot analysis was repeated four times and similar results were obtained.

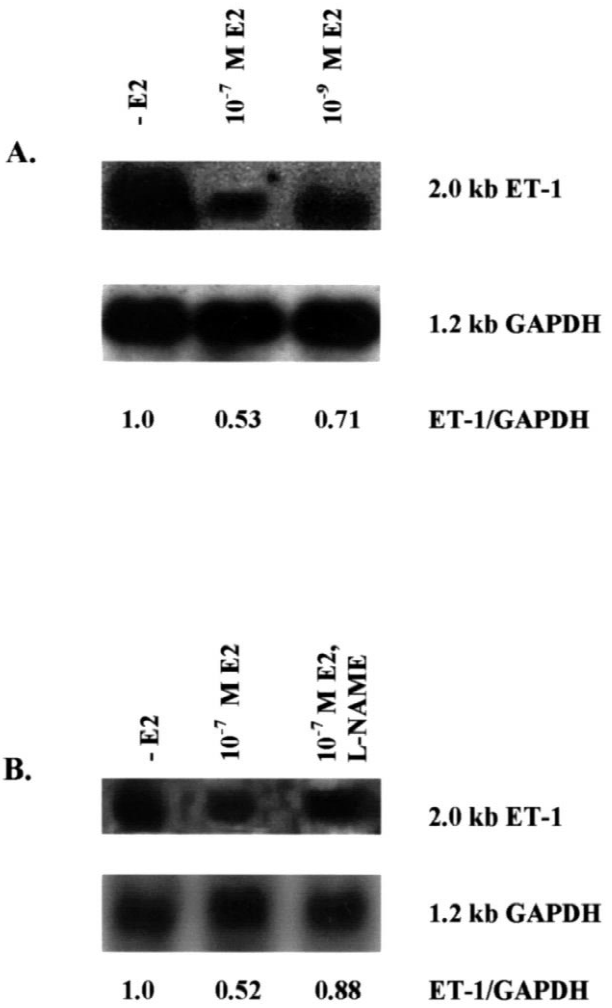

Fig. 1. Northern blot analyses of total RNA (10 μg) from E2-incubated (10^{-7} and 10^{-9} M) with or without L-NAME and E2-deprived endothelial cells hybridized to [α-^{32}P] dCTP-labeled human ET-1 and GAPDH probes. (A) Lanes: 1=E2-deprived control cells; 2 and 3=E2-incubated cells (10^{-7} and 10^{-9} M, respectively). (B) Lanes: 1=E2-deprived control cells; 2=10^{-7}M E2-incubated; 3=10^{-7}M E2-incubated cells in the presence of L-NAME for 48 h. Exposure time was 48 h.

3.2. Endothelin release

Basal production of immunoreactive ET-1 was assayed after 6 h of incubation with or without E2 and the amount was expressed as picograms ET-1 per milliliter of medium. Each well of cultures contained 0.550 ml medium, 190 mm^2 growth area, and ≈$1.9 \cdot 10^5$ cells. The basal ET-1 levels of E2-deprived culture media was 157.0±22 pg/ml (n=10), while that of 10^{-9} and 10^{-7} M E2-incubated culture media were 64.8±6.7 (n=10) and 30.4±3.2±2.06 pg/ml (n=5), respectively. The difference between basal ET-1 concentrations of E2-deprived and incubated cells was statistically significant ($P<0.001$ at 10^{-7} M and $P<0.05$ at 10^{-9} M E2) (Fig. 2). Vehicle-treated cultures

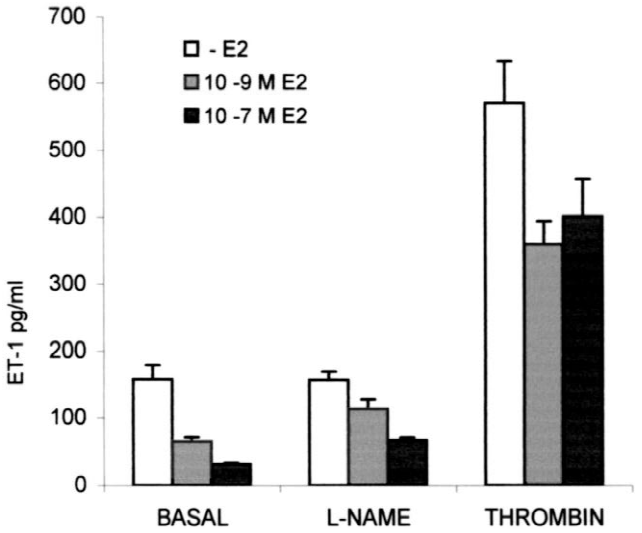

Fig. 2. Effect of 17β-estradiol on the production of endothelin-1 under basal conditions and after inhibition of NOS with L-NAME and upon stimulation with thrombin (4 U/ml) in HUVECs. The amount of immunoreactive ET-1 is expressed in pg/ml of medium after 6 h of incubation. The difference between basal ET-1 concentrations of E2-deprived and incubated cells was statistically significant ($P<0.001$ and $P<0.05$ at 10^{-9} and 10^{-7} M E2 concentrations). NOS inhibitor L-NAME (1 mM) did not effect the basal ET-1 production of E2-deprived cells, but significantly increased the ET-1 production of E2-treated cells ($P<0.05$, $P<0.0001$ at doses of 10^{-9} and 10^{-7} M, respectively, compared to basals). The difference between ET-1 levels of E2-incubated and E2-deprived cells was significant ($P<0.05$ at 10^{-7} M). After thrombin stimulation, the difference in ET-1 concentrations of E2-deprived and E2-treated cells was significant ($P<0.05$) whereas no significant difference was found between two concentrations of E2.

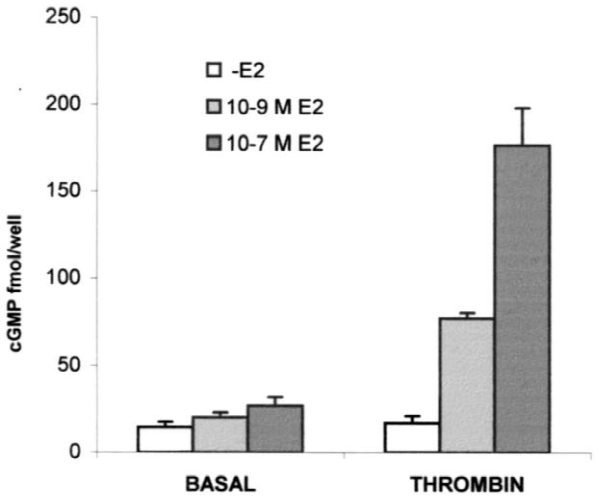

Fig. 3. Effect of 17β-estradiol on basal and thrombin (4 U/ml) stimulated cGMP content of HUVECs. The amount of cGMP is expressed as fmol/well. Thrombin stimulation did not cause a significant increase in cGMP in E2-deprived cells but increased the cGMP content of E2-treated cells in a dose dependent way at the 6 h time point studied ($n=5$, $P<0.01$, $P<0.001$ compared to E2-deprived control).

showed no significant difference in ET-1 release from untreated ones.

NOS inhibitor L-NAME (1 mM) did not effect the basal ET-1 production of E2-deprived cells, but caused a significant increase in the ET-1 release of E2-treated cells. After 6 h of incubation, the ET-1 levels of media were 113.2±14.4 ($n=8$), 67.0±4.2 pg/ml ($n=5$) in 10^{-9} and 10^{-7} M E2-incubated cells, respectively. Compared to basal conditions the increase was significant ($P<0.05$, $P<0.0001$ at doses of 10^{-9} and 10^{-7} M, respectively). However, ET-1 levels of the media of E2-incubated cells were still significantly less than those of E2-deficient cells ($P<0.05$ at 10^{-7} M E2) (Fig. 2).

Thrombin stimulated the production of ET-1 from HUVECs. After 6 h of thrombin stimulation (4 U/ml), the mean of ET-1 concentrations was 571.1±62.5 pg/ml ($n=12$) in E2-deprived and 359.0±35.4 ($n=11$), 401.0±56.6 ($n=12$) in 10^{-9} and 10^{-7} M E2-incubated HUVECs, respectively (Fig. 2). After thrombin stimulation, the difference in ET-1 concentrations of E2-deprived and E2-treated cells was significant ($P<0.05$) whereas no significant difference was found between two concentrations of E2.

3.3. cGMP content

The cGMP content of E2-deprived and -treated cultured HUVECs was assayed under both basal conditions and after thrombin stimulation. Under basal conditions E2-deprived cells contained 16.8±3.1 fmol/well cGMP while 10^{-9} and 10^{-7} M E2-incubated ones contained 20.1±2.9 and 26.8±5.1 fmol/well, respectively. Thrombin stimulation did not cause a significant increase in cGMP in E2-deprived cells but increased in the cGMP content of E2-treated cells in a dose dependent way at the 6 h time point studied. After thrombin stimulation cGMP contents of 10^{-9} and 10^{-7} M E2-incubated cells were 77.0±3.42 and 176.4±21.66 fmol/well ($n=5$, $P<0.01$, $P<0.001$ compared to E2-deprived ones) (Fig. 3).

4. Discussion

The present study shows that 17β-estradiol decreases the expression and release of ET-1 in cultured HUVECs. To our knowledge, this is the first demonstration of modulation of the expression of ET-1 by E2 in human endothelial cells. In the study, 48 h incubation of endothelial cells with E2 (10^{-9} and 10^{-7} M) significantly reduced the ET-1 mRNA expression compared to E2-deprived cells. E2-incubation also decreased basal and thrombin stimulated ET-1 release while increased cGMP content of cells. The rise in cGMP content indicates an increase in NO production.

ET-1 is a potent endothelium-derived vasoconstrictor, which is produced both under basal conditions and after

stimulation with agonist such as thrombin, arginine vasopressin, and angiotensin [17]. Although the physiological role of ET-1 has not been fully established, there is increasing evidence of its involvement in pathological conditions such as hypertension, atherosclerosis, myocardial infarction and congestive heart failure [18,19,27,28]. Therefore, E2-induced decrease in ET-1 production might have important pathophysiological implications, and the reduction of ET-1 may be responsible for some of the beneficial effects of estrogen on cardiovascular system.

In the literature, there is increasing evidence of the regulation of endothelin production by estrogen but the cellular mechanism remains unclear. Polderman et al. have reported that plasma endothelin levels were higher in men than in women, and ET-1 levels decrease in male to female transsexuals [20]. Miyauchi et al. have shown that sex hormones decrease plasma ET-1 level [21]. Akishita et al. have demonstrated that plasma ET-1 concentration and c-*fos* gene expression in aortic smooth muscle cells were higher in ovariectomized rats [29]. Recently, it was demonstrated that E2 inhibits endothelin release in HUVECs and the stimulated transcription of ET-1 in bovine aortic endothelial cell cultures [30,31]. But in contrast to these findings, Miller et al. have observed no difference in circulation concentration of endothelins or in ET-1 mRNA levels between female and male pigs [32]. Mikkola et al. reported that 17β-estradiol had no effect on ET-1 production in cultured HUVECs [33].

Although, it is established that vascular endothelial cells are the main source of circulating ET-1, the synthesis of endothelins may occur in many cell types and contribute to plasma levels. Therefore, this study provides direct evidence for the inhibitory effect of E2 on the release of ET-1 from HUVECs, and proposes a molecular mechanism for the previous findings such as lower ET-1 levels in females compared to males and lower ET-1 levels in postmenopausal women after estrogen replacement therapy [20,22].

There is substantial evidence that some of the cardioprotective effects of estrogen are mediated through NO. It is established that both acute and chronic estradiol treatment increase NO synthesis and play an important role in the tone and reactivity of vascular system [12,13]. The well known vasodilator, antiplatelet and antiproliferative effects of NO makes this molecule an ideal mediator of estrogen action and partly explains the vasculoprotective effects of estrogen. In addition, NO opposes the synthesis and the actions of ET-1. Various studies demonstrate that NO reduces ET-1 production in endothelial cells [34–36]. Antagonistic effect of NO on ET-1 production is mediated by cGMP-dependent pathway [34]. NO activates soluble guanylate cyclase and in turn increases the cGMP levels in endothelial cells. In the present study, slightly higher basal cGMP levels were observed in E2-treated cells. However, after thrombin stimulation, a dramatic increase in cGMP levels was observed in E2-treated cells (Fig. 3). Thrombin stimulates the gene expression and release of ET-1 as well as of NO in cultured cells [17,37,38]. Thus, higher cGMP levels in E2-treated groups confirm that E2-induces NO production. This finding may further indicate that the lower ET-1 levels in E2-incubated culture media may be due to the antagonistic effects of NO on ET-1 production. Based on these findings, there may be several possible mechanisms by which E2 can modulate the ET-1 production: it may be; (1) through the specific effect of the hormone on NO production and secretion (2) through a direct effect on ET-1 synthesis or (3) through a combination of both mechanisms with the possibility of involvement of unknown mechanism(s) in each case.

To investigate the role of increased NO synthesis in decreased basal ET-1 release from HUVECs, the NOS inhibitor L-NAME was used in the experiments. Cells were also treated with thrombin to find out the effect of E2 under stimulated conditions. The results show that inhibition of NOS, increases basal endothelin release in E2-treated but not in E2-deprived cells. The inability of L-NAME to inhibit the basal release of ET-1 may be due the insensitivity of the peptide production to NO at basal conditions. Similar results were also observed by Boulunger and Lüscher [34] with L-NMMA. After inhibition of NOS with L-NAME, increase in ET-1 release from E2 treated-cells suggests that NO is partly responsible for the effect of E2 on endothelin release. However, still significantly less ET-1 production in E2-treated group compared to the deficient one may suggest the presence of a direct effect of the hormone on ET-1 release. This finding is further supported by Northern blot analyses which show that L-NAME treatment causes partial but significant reduction of E2-induced inhibition of ET-1 expression.

After stimulation with thrombin, ET-1 production increases dramatically in both groups but similarly still the increase in E2 treated group is significantly less than in E2-deprived group. Our results also demonstrate that in thrombin-stimulated cells, although E2 causes a dose dependent increase in cGMP content, it does not cause a dose dependent reduction in ET-1 release (Figs. 2 and 3). This finding is in accordance with the results of Wingrove and Stevenson [30] who observed no further inhibition of thrombin-stimulated endothelin production at E2 doses >2 nM. This finding may be due to complex interactions of E2- and thrombin-induced effects on NO and ET-1 production or to their unknown effects on post-transcriptional modification of ET-1 mRNA. Although further studies are required to explain this result, this finding may further provide a hint for NO-independent regulation of ET-1 production.

In conclusion, the present study indicates that 17β-estradiol may play an important role in the regulation of ET-1 synthesis both directly by modulating the expression of endothelin-1 gene expression and indirectly by stimulating NO generation. Modulation of both NO and

endothelin-1 production in favorable ways might be one of the mechanisms of the protective effects of estrogen on the cardiovascular system.

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