Mechanisms of 17β-estradiol on the production of ET-1 in ovariectomized rats

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Abstract

In order to clarify the mechanism underlying the possible preventive effect of estrogen on atherogenesis, we investigated the role of 17β-estradiol (E2) in the regulation of endothelin-1 (ET-1) production in ovariectomized rats, which may contribute to atherogenesis. Female Sprague–Dawly rats were randomly divided into three groups: sham-operated group (sham), ovariectomized group (OVX) and 17β-estradiol replacement group (OVX + E2, 20 μg kg⁻¹ d⁻¹ s.c.). 4 weeks after operation, the plasma concentration of ET-1, clearance of ET-1, functional ECE activity and preproET-1 mRNA expression in aorta were measured. Concentration of plasma ET-1 change from 107.8 ± 18.3 pg/ml (sham) and 135.5 ± 27.6 pg/ml (OVX + E2) to 190.7 ± 25.5 pg/ml (OVX) (n = 8, p < 0.05). There was no significant difference in the clearance of ¹²⁵IET-1 among three groups (p > 0.05). Functional ECE activity was increased in OVX group in comparison to that in sham group (p < 0.05). The OVX increased the preproET-1 mRNA expression in sham, whereas treatment with estrogen reversed these changes (p < 0.05). The present study have shown that estrogen down-regulates plasma ET-1 levels by inhibiting the preproET-1 mRNA expression and functional ECE activity. Clearance of ET-1 was not affected. Inhibition of ET-1 production mediated by modulating ECE activity may be one of the novel mechanisms of the protective of estrogens on the cardiovascular system.

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Introduction

The endothelins are a family of 21-amino-acid peptides consisting of three known members: endothelin-1, endothelin-2 and endothelin-3. Endothelin-1 (ET-1) is produced mainly by vascular endothelium cells and is a kind of potent vasoconstrictor and mitogen for vascular smooth muscle cells (Levin, 1995). Previous studies have shown that ET-1 may participated in the development of atherosclerosis: immunoreactive ET-1 and increased ET-1 mRNA levels have been found in atherosclerosis plaques (Zeiher et al., 1995) and a correlation has been reported between increased plasma levels of ET-1 and the severity of atherosclerosis (Dashwood and Tsui, 2002).

Clinical studies supporting a protective role for estrogen have demonstrated that restoration of estrogen via hormone replacement therapy (HRT) reduces cardiovascular risk by about half (Stampfer and Colditz, 1991; Mosca et al., 1997, 2001). Moreover, not all studies have demonstrated clear the benefits of HRT on cardiovascular disease (Hulley et al., 1998; Tolbert and Oparil, 2001). In fact, a recent advisory from the American Heart Association does not recommend initiation of HRT for the sole purpose of preventing cardiovascular disease. Despite of the ambiguity associated with clinical studies, it is very clear that estrogens exert specific effects on cardiovascular tissues. Experimental studies indicate that premenopausal monkeys develop only 50% as much coronary atherosclerosis as males or ovariectomized females, whereas HRT decreases plaque extent by the same percentage (Adams et al., 1990; Clarkson et al., 1993; Mikkola and Clarkson, 2002). Animal experiments have also shown the inhibitory effects of estrogen on the development of atherosclerosis (Bayard et al., 1999). In light of these studies, it seems clear that estrogen possesses anti-atherogenic action, but the molecular basis of this response is not completely understood.

Recently more studies have shown that estrogen has the effect on the production of ET-1: plasma ET-1 levels are higher in men than in age-matched women and fluctuated during the menstrual cycle (Polderman et al., 1993, 2000). ET-1 decreases during estrogen replacement therapy (Best et al., 1998) and in the absence of estrogen, ET-1 peptides and ET-1 mRNA increase (Ylikorkala et al., 1995).

However the mechanisms by which estrogen accomplish this are not completely known. In ET-1 processing, conversion of the precursor polypeptide, big ET-1, to mature ET-1 is the final key step. Endothelin synthesis depends on activity of endothelin-converting enzyme (ECE) which cleave the nondibasic bond of Trp$^{21}$-Val$^{22}$ from precursor big ET-1 resulting in mature ET-1 (Yanagisawa et al., 1988). In this study, we focus on preproET-1 mRNA expression, functional endothelin converting enzyme (ECE) activity and clearance of ET-1 to elucidate the effect of estrogen on the production of ET-1 in ovariectomized rats.

Materials and methods

Reagents

TianTAM one Tube RT-PCR kit was purchased from Boehringer Mannheim (German), TRIzol reagent from GibCO BRL (USA), 17β-estradiol, L-nitroarginine methylester(L-NAME), endothe-
lin-1 (ET-1), big-endothelin-1 (bigET-1) were obtained from Sigma Chemical Co (USA). ET-1 RIA kit was obtained from East Asia Immune Technology Inc (Beijing, China). Primer sequences were synthesized by SanGon Inc. (Shanghai, China).

Animal model

Female Sprague–Dawly rats (n = 32, 220 – 250 g) were randomly divided into three groups. Two groups of rats were ovariectomized and the third group was received sham operation. After a week recovery period, one group of the ovariectomized rats received a subcutaneous injection of 17β-estradiol (20 μg.kg⁻¹.d⁻¹) for four weeks (OVX + E₂), the other group of ovariectomized rats (OVX) and sham-operated rats (sham) received the same amount of solvent.

Measurement of ET-1

Blood sample (2 ml) was collected from each group rats in anesthesia state through an arterial catheter placed in the Carotid into a plastic tube containing ethylenediaminetetraacetic acid (EDTA) disodium (20 μl) and aprotinin (40 μl). Blood samples were centrifuged at 3000 g for 10 min at 4 °C and then was assay using commercially available ET-1 RIA kit.

Clearance studies

In the subset of animals (n = 5 per group), clearance studies were performed by injection of approximated 2000 cpm of radiolabeled ¹²⁵¹ET-1 peptide through the left femoral vein. 100 μl of blood sample from the carotid artery was collected over 2 minutes. Radioactivity was determined in a gamma counter.

Arterial preparations

Thoracic aorta were carefully isolated, removed and rinsed in 37 °C modified krebs’ solution (mM: NaCl 115.0, KCl 5.0, MgSO₄ 1.16, NaH₂PO₄ 1.16, NaHCO₃ 21.9, CaCl₂ 2.5 and Glucose 11.0, pH 7.2 ~ 7.4) aerated with 95% O₂ and CO₂. The aortic segments were carefully cleaned from perivascular tissue, and cut into rings (3 ~ 5 mm in length). In the end of the experiment, the rings were blotted dry and weighed. Contractile forces were calculated as gram⁻¹.gram⁻¹.wet tissue⁻¹.

Contractile experiment

Each rings was equilibrated for 2 hours with the rest tension of 1.5 g. During the equilibration, the rings were washed every 20 minutes. Before tacking data, the rings were stimulated with 100 mM KCl repeatedly to make sure that the later 100 mM KCl responses are reproducible and in similar extent, and were then equilibrated again for 30 minutes. Then, contractile to ET-1 and bigET-1 were obtained and normalized to KCl (100 mM). It’s need to indicated that all the experiments were performed in the L-NAME (1 μM) to eliminate the effect of nitric oxide (NO).
Function ECE activity was calculated using the ratio of contraction to bigET-1/ET-1 (1,3,10,30,100 nM) as arbitrary units.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total tissue RNA was isolated from the rest thoracic aortic after contractile experiment using TRIzol reagent. Concentrations of RNA were determined by measuring absorbance at 260 nm (A_{260}). The A_{260}/A_{280} ratio of the samples ranged from 1.8 to 2.0. Expression of preproET-1 mRNA was studied using Tian^{\text{TAM}} one Tube RT-PCR kit. PCR primers were as follows: ppET-1, the sense primer was 5'-CTG AGT CTA AGC GAT CTT TG-3' and the antisense primer was 5'-TTC TGG TCT CTG TAG AGA TC-3' which should amplify a 319 bp fragment; and GADPH sense primer 5'-CAT CAC CAT CTT CCA GGA GCC-3' and antisense 5'-TGA CCT TGC CCA CAG CCT TG-3' (443 bp). GADPH was used as an internal control for the coamplification. All the steps followed by application protocol of the kit. PCR products were electrophoresed by 1.5% agarose gel containing ethidium bromide. The band intensity was measured using a software (UVP-GDS8000, England), and the signals were expressed relatively to the intensity of GADPH amplicon in each coamplified sample.

Statistics

Values are expressed as means ± SEM. The data were analyzed using one-way ANOVA. P < 0.05 considered statistically significant.
Fig. 2. Ovariectomy and estrogen replacement had no effect on clearance of ET-1. Sham: sham-operated rats; OVX: ovariectomized rats; OVX + E2: ovariectomized and 17β-estradiol-replaced rats. n = 5, Values are expressed as mean ± SEM.

Fig. 3. Functional of endothelin converting enzyme activity (ratio of responses to bigET-1/ET-1, normalized to 100 mM) in aorta. Sham: sham-operated rats; OVX: ovariectomized rats; OVX + E2: ovariectomized and 17β-estradiol-replaced rats; n = 7, Values are expressed as mean ± SEM., *p < 0.05 vs. sham, **p > 0.05 vs. sham.
Results

Plasma ET-1 level

Plasma ET-1 level in OVX group rats was significantly increased in comparison to that in sham and OVX + E. Concentration of ET-1 from 107.8 ± 18.3 pg/ml (sham) and 135.5 ± 27.6 pg/ml (OVX + E2) to 190.7 ± 25.5 pg/ml (OVX) (n = 7, p < 0.05). The increase was 76.9% and 40.7% respectively. There was no significant difference in plasma ET-1 level between sham and OVX + E2. As shown in Fig. 1.

Fig. 4. Effects of ovariectomy and estrogen replacement on preproET-1 mRNA expression in aorta. A: RT-PCR products of preproET-1 mRNA and CADPH. B: the results of densitometric scanning for DNA bands of each group. Sham: sham-operated rats; OVX: ovariectomized rats; OVX + E2: ovariectomized and 17β-estradiol-replaced rats; n = 5, Values are expressed as mean ± SEM. *p < 0.05 vs. sham, **p < 0.05 vs. OVX.
Clearance of radiolabelled $^{125}$IET-1

Counts/minute versus time were fitted to a mono-exponential decay curve. The clearance of $^{125}$IET-1 was mostly took place within 10 seconds. At the time of 60 seconds, almost more than 90% $^{125}$IET-1 have been degraded. There was no significant difference in the clearance of $^{125}$IET-1 among three groups. This is shown that estrogen has no effect on the clearance of $^{125}$IET-1 (Fig. 2).

Functional ECE activity

Functional of endothelin converting enzyme activity (ratio of response to bigET-1/ET-1, normalized to 100 mM KCl) was not affected at the low concentration of bigET-1/ET-1 1,3, nM among three groups. But as the concentration of bigET-1/ET-1 increased to 30, 100 nM, Functional ECE activity was increased in OVX group in comparison to that in sham group (p < 0.05). The increase was as much as 21.3% at 10 nM, 42.3% at 30 nM and 34.1% at 100 nM respectively. Increased functional ECE activity was reduced by estrogen replacement in OVX + E2 group (vs. OVX, p < 0.05). As shown in Fig. 3.

Expression of preproET-1 mRNA

As shown in Fig. 4, the expression of preproET-1 mRNA in the aorta elevated in OVX group compared with that in sham group (ppET-1/GAPDH density: OVX,81.6 ± 10.3%; OVX + E2, 36.4 ± 7.5%; 23.5 ± 6.7% of sham, p < 0.05), and the elevated was reversed by estradiol replacement (vs. OVX, p < 0.05).

Discussion

Estrogen is believed to possess cardiovascular protective effects, and it seems to play a role in the development of cardiovascular diseases, but the mechanism of its anti-atherogenic actions are not fully understood. Because ET-1 plays a very important role in the pathogenesis of cardiovascular diseases, such as atherosclerosis, it’s possible that the effect of estrogen on the attenuated development of atherosclerosis may be related to modulation the ET-1 effects on the cardiovascular system.

In the present study, we demonstrated that plasma ET-1 level was increased in ovariectomy compared to sham, and estrogen replacement can reverse this effect. These findings clearly indicate that estrogen is involved in the regulation of plasma ET-1 level. The reduction of ET-1 may be responsible for some of the beneficial effect of estrogen on cardiovascular system. Previous studies (Polderman et al., 1993, 2000) have shown that that sex hormones can decreased plasma ET-1 levels. There was also other reports indicated that plasma ET-1 levels were higher in men than in women, and ET-1 levels decreased in male to female transsexuals (Miyauchi et al., 1992). Although those studies have shown that estrogen may influence plasma ET-1 level, it’s remain unclear whether the difference in plasma ET-1 level is a consequence of difference in production, activity of ECE or degradation of ET-1 in vivo. To investigate this issue, we focus on the effect of estrogen on the preproET-1 mRNA expression, functional endothelin converting enzyme (ECE) activity, and the clearance of ET-1.

Fig. 4 demonstrates results from RT-PCR showing mRNA expression of preproET-1 in aorta from sham, OVX, and E2-treated rats. The OVX increased the preproET-1 mRNA expression in sham,
whereas treatment with estrogen reversed these changes. Similar results also get by other investigators (David et al., 2001; Bilsel et al., 2000).

More recently studies have shown that ET-1 production can be regulated by transcriptional as well as post-transcriptional mechanism. Half-life studies have shown that estrogen can accelerated ET-1 mRNA degradation (Morey et al., 1998). Estrogen receptor binds to the estrogen responsive element of estrogen responsive genes and acts by regulating their transcription (Akishita et al., 1998). Since the known structure of estrogen responsive element is not found in the 5′ upstream region of preproET-1 gene (Inoue et al., 1989), it’s possible that some factors which is regulated by estrogen may affect the preproET-1 mRNA expression (Dubey et al., 2001). It was not possible in this study to determine whether the transcriptional regulation of the gene was due to the direct action of estrogen binding to the promoter regions or indirect modulation of another second messenger system. But results of this study indicate that the effect of estrogen on plasma ET-1 levels may be regulated at the transcriptional level. However these aspects still wait clarification. Other studies have shown that estrogen can through mediation NO production inhibition ET-1 gene expression (Bilsel et al., 2000).

The major observation in this study is that ovariectomy enhanced functional ECE activity to a greater extent than in sham and estrogen replacement. To our knowledge, this is the first demonstration of modulation of ECE by estrogen. ET-1 is cleaved from big endothelin-1 by endothelin-converting enzymes (ECEs) into the biologically active mature form that mediates vasoconstriction and cell proliferation (Xu et al., 1994; Chiou et al., 1994). Since ECE activity is difficult to assess in intact vascular rings, we applied an ECE bioassay incorporation response to bigET-1 and ET-1 which give an function estimate of ECE activity in intact rings (Barton et al., 1997a). Those findings first indicate that estrogen may not only affect the endothelin system by inhibition of the preproET-1 mRNA expression, but also by inhibiting its conversion. ECE is a member of zinc metalloprotease family. To date, more than one ECE has been cloned. It has also been observed that big ET-1 and big ET-3 are not converted by the same enzyme (Rizzi et al., 1998). ECE-1, the final key enzyme of endothelin processing, has been found three different isoforms (ECE-1a, ECE-1b and ECE-1c) (Schweizer et al., 1997). Therefore, which ECE activity was affected by estrogen and how much the increased functional ECE activity contributes to increased plasma ET-1 levels remain to be determined.

Synthesis and clearance are major determinants of ET-1 production. ET-1 is released into the circulation where it is rapidly taken up by vascular smooth muscle cells. Our results shown that the clearance of ET-1 is unaffected in sham, OVX and OVX + E₂. Similar result was also get by other investigators that estrogen has no effect on the clearance of ET-1 (Barton et al., 1997b). Those suggest that increase in plasma ET-1 content is likely not to be related to impair the clearance of circulatory ET-1.

In summary, the present study indicated that estrogen down-regulates of plasma ET-1 level by inhibiting the preproET-1 mRNA expression and functional ECE activity. Clearance of ET-1 was not affected. Inhibition of ET-1 production mediated by modulating ECE activity may be one of the novel mechanisms of the protective of estrogen on the cardiovascular system.

Acknowledgements

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