Effects of 17β-Estradiol on the Plasminogen Activator System in Vascular Smooth Muscle Cells Treated with Lysophosphatidylcholine

Byung-Koo Yoon,1,2 Young-Hee Kang,2 Won-Jong Oh,2 Dong-Yun Lee,1 Duk-Kyung Kim,3 Bruce Kessel,4 Chi-Dug Kang5

1Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea, 2Samsung Biomedical Research Institute, Samsung Medical Center, Seoul, Korea, 3Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea, 4Department of Obstetrics and Gynecology, University of Hawaii, Honolulu, HI, USA, 5Department of Biochemistry, Pusan National University School of Medicine, Yangsan, Korea

Objectives: When administered soon after menopause, hormone therapy can prevent coronary heart diseases in women. To explore the mechanism underlying the cardioprotective actions of estrogen, we investigated the effects of 17β-estradiol (17β-E2) on the plasminogen activator system using cultured vascular smooth muscle cells (VSMCs).

Methods: VSMCs were isolated from rat aortas. Protein expression of plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (t-PA) were evaluated using Western blotting and enzyme-linked immunosorbent assay, respectively. The enzyme activity of PAI-1 in a conditioned medium was assessed via reverse fibrin overlay zymography and that of t-PA was assessed via fibrin overlay zymography. Gene expression was quantified using real-time reverse transcription-polymerase chain reaction.

Results: Following pre-treatment for 24 hours, 17β-E2 suppressed both protein expression and enzyme activity of PAI-1 stimulated by lysophosphatidylcholine (lysoPC) in a significant and dose-dependent manner at a near physiological concentration. Moreover, 17β-E2 (10^{-7} M) inhibited PAI-1 gene expression, and ICI 182,780—a specific estrogen receptor antagonist—blocked the effects of 17β-E2 on the PAI-1 protein. 17β-E2 did not affect t-PA secretion but significantly enhanced free t-PA activity through reduced binding to PAI-1. Furthermore, 17β-E2 suppressed intracellular reactive oxygen species production and nuclear factor-kB-mediated transcription.

Conclusions: In VSMCs stimulated with lysoPC, 17β-E2 reduced PAI-1 expression through a non-receptor-mediated mechanism via antioxidant activity as well as a receptor-mediated mechanism; however, it did not alter t-PA secretion. Of note, 17β-E2 suppressed PAI-1 activity and concurrently enhanced t-PA activity, suggesting a beneficial influence on fibrinolysis.

Key Words: Estrogens, Fibrinolysis, Myocytes, smooth muscle, Plasminogen inactivators, Tissue plasminogen activator

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of mortality in women. Although the pathogenesis of CHD has not been fully understood, thrombosis plays a crucial role in the development and progression of atherosclerosis [1]. Thrombosis is normally balanced by fibrinolysis, which is under refined regulation by tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) [2]. The t-PA from vascular cells converts plasminogen into plasmin, which degrades the fibrin polymer into small
fragments, and thus helps to dissolve clots. In contrast, PAI-1 binds to t-PA and inhibits the activation of plasminogen. Indeed, an increased blood level of PAI-1 is associated with increased risk of CHD [3].

If started early after menopause, menopausal hormone therapy (MHT) prevents CHD, one of the late health problems associated with estrogen deficiency after menopause [4,5]. In addition to beneficial systemic impacts on lipid profiles, blood pressure, and glucose metabolism, direct actions of MHT on arteries may be a major mechanism for cardio-protection [6].

Oxidative stress, which induces the oxidative modification of low-density lipoprotein (LDL), is another key component in the development of atherosclerosis. Oxidized LDL exerts arterial impacts in various adverse ways [7]. We reported that lysophosphatidylcholine (lysoPC), an active component of oxidized LDL, induced PAI-1 expression in vascular smooth muscle cells (VSMCs) [8]. Further lysoPC stimulated the enzyme activity of PAI-1 and inhibited that of t-PA, probably leading to decreased fibrinolysis. The present study was undertaken to investigate the direct effect of 17β-estradiol (E2) on the plasminogen activator (PA) system in cultured VSMCs.

MATERIALS AND METHODS

This study was performed according to materials and methods described previously by our group [8]. Here, the materials and methods will be described briefly.

Materials

Sprague–Dawley rats were purchased from Charles River Japan (Hino, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and DMEM/F-12 without phenol red, fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA), and penicillin-streptomycin were purchased from GIBCO BRL (Grand Island, NY, USA). Human plasminogen and bovine fibrinogen were obtained from Enzyme Research Laboratories, Inc. (Swansea, UK). Human urokinase and bovine thrombin were purchased from Calbiochem (Darmstadt, Germany). Monoclonal antibody for α-smooth muscle actin was purchased from DAKO (Glostrup, Denmark). 2′,7′-dichlorofluorescin diacetate (DCFDA) was obtained from Molecular Probes (Eugene, OR, USA) and was dissolved in dimethyl sulfoxide (DMSO). 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were purchased from Tocris Bioscience (Bristol, UK). LysoPC and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). E2 was dissolved in ethanol (EtOH), whereas ICI 182,780, MPP, and PHTPP were dissolved in DMSO. All of the other chemicals were dissolved in water.

Cell culture

VSMCs were isolated from thoracic aortas of 3-month-old Sprague-Dawley rats (160–180 g) using a specific enzyme digestion method and were grown in DMEM/F-12 (50:50) without phenol red, containing antibiotics and 10% FBS. The cells were stained positively for α-smooth muscle actin. In order to obtain quiescent cells, the cells were incubated for 48 hours in a defined serum-free (DSF) medium containing insulin (0.5 µM), transferrin (5 mg/mL), and ascorbate (0.2 mM).

This study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (IACUC No. C-A3-220-2), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and abides by the guide of the Institute of Laboratory Animal Resources.

Western blot analysis

Cells were lysed for 30 minutes on ice in RIPA buffer (50 mM Tris-HCl [pH, 7.5], 200 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride. Thirty micrograms of cellular proteins were separated by SDS-PAGE, electrotransferred to a polyvinylidifluoride transfer membrane (Schleicher & Schuell, Keene, NH, USA), and then blocked and incubated with rabbit polyclonal anti-PAI-1 antibody (American Diagnostica Inc., Stamford, CT, USA). After washing, blots were incubated with anti-rabbit/anti-mouse horseradish peroxidase conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK), and the bands were detected with ECL reagents (Amersham Biosciences). β-actin was used as a control.

RNA preparation and real-time reverse transcriptase–polymerase chain reaction assay

Total RNA was extracted from cells using the easy-spin™ Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Korea). Conversion to cDNA was
achieved through the PrimeScript™ RT Master Mix (Takara Bio USA, Inc., Mountain View, CA, USA). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using SYBR Premix Ex Taq™ (Takara Bio USA, Inc.). The PCR reactions were run in an Applied Biosystems QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and the relative expression of PAI-1 was calculated after normalization to glyceraldehyde 3-phosphate dehydrogenase using the difference in the cycle threshold (ΔCt) method. Primers for rat PAI-1 were 5’-CAATGGAAGACCCCCTTCTTAGAG-3’ (forward) and 5’-CATGGGCAGGGAGATGGT-3’ (reverse). Primers for rat glyceraldehyde 3-phosphate dehydrogenase were 5’-GTATCGGACGCCTGGTTACC-3’ (forward) and 5’-TTGATGGCAACAATGGTCCACTTTG-3’ (reverse).

Enzyme-linked immunosorbent assay

The t-PA levels were quantified using enzyme-linked immunosorbent assay (ELISA; Molecular Innovations Inc., Novi, MI, USA). The color reaction was performed with a 3,3’,5,5´-tetramethylbenzidine substrate, and measured at 450 μm by a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA).

Fibrin overlay zymography/reverse fibrin overlay zymography

Conditioned media (CM) was subjected to electrophoresis using a 9% polyacrylamide gel containing 0.1% SDS. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 1 hour to remove the SDS. In order to detect PA activity with the fibrin overlay assay, the gel was placed on an opaque fibrin indicator gel containing 1% low-gelling-temperature agarose (FMC Corporation, Philadelphia, PA, USA), human plasminogen (12.5 μg/mL), bovine thrombin (0.5 U/mL), and bovine fibrinogen (2 mg/mL). Afterwards, this combined gel (polyacrylamide/fibrin indicator) was incubated at 37°C, and PA activity was detected by the appearance of clear bands in the opaque indicator gel. In addition, activity of PA inhibitor was detected by a reverse overlay assay, which is similar to the fibrin overlay assay described above, except that it also contained human urokinase (1.5 U/mL). Since the fibrin in the reverse indicator gel was lysed in the absence of PAI, opaque bands on the indicator gel indicated the presence of PAI.

Analysis of intracellular formation of reactive oxygen species

Intracellular free radical production was determined using DCF-DA. To analyse the reactive oxygen species (ROS) formation quantitatively, flow cytometry analysis was used. After incubation of the quiescent cells with 10 μM DCF-DA and 5 μM lysoPC, the cells were trypsinized and resuspended in PBS containing 1 mM EDTA, and then analyzed immediately by flow cytometry (10,000 cells/sample) using a beam of 488 nm excitation. The median intensity of fluorescence was determined by CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA).

Determination of nuclear factor-κB activation

Activation of nuclear factor-κB (NF-κB) was determined in the VSMCs after transfection with a reporter plasmid containing the luciferase reporter gene linked to five repeats of the NF-κB binding sites. The VSMCs (1 × 10^5 cells/well) were plated in 24-well plates and grown to about 70% confluence. Cells were then transiently cotransfected with 1 μg of NF-κB–luciferase reporter plasmid and 1 μg of β-galactosidase plasmid using Lipofectamine plus (Invitrogen, Carlsbad, CA, USA). At 6 hours post-transfection, cells were starved for 48 hours before stimulation with lysoPC. Transfected cells were exposed to 5 μM lysoPC for the indicated time periods. Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI, USA) with signal detection for 5 seconds in a luminometer (Panomics Inc., Fremont, CA, USA). A β-galactosidase enzyme assay (Promega) was used to determine the β-galactosidase activity with a SmartSpec 3000 spectrophotometer at 420 nm. The results are expressed relative to the NF-κB activity compared with controls after normalizing for β-galactosidase activity and protein concentration.

Data analysis and statistics

Data are presented as mean ± standard error of the mean, and N is the number of performed experiments. The Kruskal-Wallis test and Wilcoxon rank sum test were carried out for statistical analysis using Statistics Package for Social Sciences Version 20.0 (IBM Corp., Armonk, NY, USA). A two-tailed value of P < 0.05 was considered statistically significant.
Fig. 1. Effects of 17β-estradiol (E₂) on the plasminogen activator system in lysophosphatidylcholine (lysoPC, 5 µM)-treated vascular smooth muscle cells after 24-hour pre-treatment. (A) Time-course effects and (B) dose-response effects of 17β-E₂ on plasminogen activator inhibitor type 1 (PAI-1) in cell lysates as determined by Western blot analysis. Representative blots are shown in the upper section of each panel. The intensity of the bands was densitometrically determined and normalized to that of corresponding β-actin bands. Graphic data are depicted in the lower part of the panel. (C) Effects of 10⁻⁷ M 17β-E₂ on gene expression of PAI-1 as evaluated by real-time reverse transcriptase-polymerase chain reaction. (D) Time-course effects of 10⁻⁷ M 17β-E₂ on secreted tissue-type plasminogen activator (t-PA) in the conditioned media as measured by enzyme-linked immunosorbent assay. Data are expressed as mean ± standard error of the mean. DSF: defined serum-free, EtOH: ethanol. a, b, c, d, e P < 0.05.
**Fig. 2.** Effects of 17β-estradiol (E₂) on enzymatic activities of the plasminogen activator system in the conditioned media harvested from cultured vascular smooth muscle cells stimulated with lysophosphatidylcholine (lysPC, 5 µM) after 24-hour pre-treatment. Representative zymographs are shown in the upper section of each panel. The intensity of the bands was densitometrically determined, and graphic data are depicted in the lower part of the panel. (A) Time-course effects and (B) dose-response effects of 17β-E₂ on plasminogen activator inhibitor type 1 (PAI-1) as determined by reverse fibrin overlay zymography. (C) Time-course effects of 10⁻⁷ M 17β-E₂ on tissue-type plasminogen activator (t-PA) as examined by fibrin overlay zymography. Data are expressed as mean ± standard error of the mean. DSF: defined serum-free, EtOH: ethanol. a, b, c, d P < 0.05.
RESULTS

17β-estradiol inhibited lysophosphatidylcholine-induced expression of plasminogen activator inhibitor type 1 protein and mRNA with no change in tissue-type plasminogen activator secretion in the vascular smooth muscle cell.

Our group had previously shown that lysoPC significantly increased PAI-1 expression in the VSMC at 5 µM or higher concentration of lysoPC in a dose-dependent manner [8]. In Western blot analysis, co-treatment of 17β-E2 did not alter PAI-1 expression stimulated with lysoPC (data not shown). We then tested the effects of 17β-E2 after 24 hours of pre-treatment. In a time-course study, 10−7 M of 17β-E2 decreased PAI-1 expression induced with 5 µM lysoPC, reaching statistical significance after 8 hours (Fig. 1A). EtOH (0.1%), a vehicle, did not change PAI-1 expression. Compared to...
vehicle-treated controls, PAI-1 expression was reduced in a dose-dependent manner when checked at 8 hours of treatment with lysoPC, and significant reductions were observed at 7.5 × 10⁻⁸ M or a higher concentration of 17β-E₂ (Fig. 1B). The effect of 17β-E₂ was also investigated at the level of gene expression after 24 hours of pre-treatment. We had previously reported maximal expression of the PAI-1 gene at 4-hour treatment of lysoPC [8]. EtOH (0.1%) had no influence on PAI-1 gene expression. Compared to vehicle-treated controls, lysoPC-induced expression of PAI-1 mRNA at 4 hours was significantly down-regulated with 10⁻⁷ M 17β-E₂, as assessed by RT-PCT (Fig. 1C).

In addition, t-PA change in CM was analysed by ELISA. We have reported that neither production nor secretion of t-PA was altered by lysoPC [8]. Constitutive secretion of t-PA into the CM was undetectable and increased with time following lysoPC treatment. t-PA secretion was not affected by 10⁻⁷ M 17β-E₂ after pre-treatment for 24 hours (Fig. 1D). These results suggest that 17β-E₂ inhibits lysoPC-induced expression of PAI-1 protein and mRNA without influencing t-PA secretion in the VSMC.

17β-estradiol suppressed enzyme activity of plasminogen activator inhibitor type 1 and enhanced that of free tissue-type plasminogen activator in the conditioned media

Time-course experiments using reverse fibrin overlay zymography demonstrated that PAI-1 activity in response to 5 μM lysoPC was significantly reduced after 12 hours by pre-treatment with 17β-E₂ (10⁻⁷ M) for 24 hours (Fig. 2A). In dose-response experiments performed at 12 hours after lysoPC treatment, EtOH (0.1%) did not affect PAI-1 activity. In comparison to vehicle-treated controls, 17β-E₂ treatment suppressed PAI-1 activity in a dose-dependent fashion. Significant reductions were observed at 7.5 × 10⁻⁸ M and higher concentrations of 17β-E₂ (Fig. 2B).

Enzyme activity of t-PA was also examined using fibrin overlay zymography (Fig. 2C). As reported previously [8], lysoPC increased higher molecular weight complexes after 8 hours, which are presumed to be inactive t-PA bound to PAI-1. Parallel to the change in bound form, free t-PA activity was barely detected with lysoPC. After 24-hour pre-treatment, 17β-E₂ (10⁻⁷ M) significantly reversed t-PA responses: bound-form activity was significantly lowered after 8 hours, while free-form activity was elevated after 12 hours. These results suggest that 17β-E₂ suppresses enzymatic activity of PAI-1 possibly through downregulation of PAI-1 levels and consequently leads to an increased free form of t-PA in the CM.

Mechanisms of 17β-estradiol action

LysoPC increases PAI-1 expression via oxidative stress and downstream signaling involving NF-κB-mediated transcriptional activity [8]. Therefore, the influence on intracellular ROS production was explored first. EtOH (0.1%) did not change ROS production in response to 2-hour 5 μM lysoPC treatment. Compared to vehicle-treated controls, the increased ROS production by lysoPC was significantly reduced by pre-treatment with 17β-E₂ (10⁻⁷ M) for 24 hours (Fig. 3A). While NF-κB activity after 2-hour treatment with 5 μM lysoPC was not altered with EtOH (0.1%), 17β-E₂ suppressed the lysoPC-stimulated NF-κB activity, as compared to vehicle-treated control (Fig. 3B).

Estrogen receptors (ERs) α and β are expressed in rat VSMCs [9]. Whether 17β-E₂ action is mediated by ERs was also examined (Fig. 3C). DMSO (0.1%) did not affect PAI-1 protein expression in response to 17β-E₂. ICI 182,780 (10⁻⁶ M), an antagonist against both ERs α and β, significantly abolished 17β-E₂ responses as compared to vehicle-treated controls. In particular, MPP (10⁻⁶ M), a specific antagonist against ERα, significantly restored PAI-1 expression suppressed by treatment with 17β-E₂ (10⁻⁷ M). Meanwhile, PHTPP (10⁻⁶ M), a specific antagonist against ERβ, showed a similar blocking effect without statistical significance. These results suggest that 17β-E₂ reduces PAI-1 expression via receptor-mediated and non-receptor-mediated mechanisms including inhibition of NF-κB, which can be activated by lysoPC.

DISCUSSION

This study was carried out to examine the direct effects of 17β-E₂ on the PA system in cultured VSMCs. We report that 17β-E₂ diminished lysoPC-induced PAI-1 protein and activity levels and concomitantly promoted t-PA activity in VSMCs.

Higher PAI values are found in myocardial infarction and re-infarction patients [3]. Further, elevated blood PAI-1 levels have a causal effect on CHD risk [10]. Estrogen therapy decreases blood PAI antigen and activity and increases release of active t-PA in postmenopausal women [11]. Estrogen is reported to decrease PAI-
1 production in hepatocytes [12] and adipose tissue [13]. In the present study, we focused on the PA system within the artery.

In CHD, local fibrinolysis systems are critically important for arterial thrombosis in response to endovascular injury. As well as in mouse [14], the level of PAI-1 mRNA in human arteries is correlated with the degree of atherosclerosis [15]. Although PAI-1 expression is detected primarily in vascular endothelial cells (VECs) of healthy arteries, PAI-1 is also found in intimal VSMCs in early atherosclerotic lesions and the fibrous cap in advanced atheromatous plaques [16]. Accordingly, direct modulation of the PA system in VSMCs might be more clinically relevant than in VECs.

We have reported that lysoPC might negatively affect fibrinolysis in VSMCs by increasing protein expression and activity of PAI-1 and decreasing enzyme activity of t-PA [8]. The present study demonstrated that after 24-hour pre-treatment, 17β-E₂ suppressed PAI-1 protein expression and activity in VSMCs stimulated with lysoPC in a dose-dependent fashion. These effects of estrogen occurred at near a physiologic concentration of 7.5 × 10⁻⁸ M. PAI-1 mRNA levels were also down-regulated by 17β-E₂, which was restored by ICI 182,780. These observations support a receptor-mediated effect of estrogen. In the present study, specific antagonists against ERα (MPP) or β (PHTPP) showed a similar trend of inhibition, even though ERα antagonist effects alone reached statistical significance. In contrast, opposing regulation of PAI-1 promoter activity by two ER isoforms was reported in VECs: ERα activated, whereas ERβ suppressed the promoter [17]. Further study is needed to investigate the underlying molecular mechanisms for differential control of PAI-1 gene expression by estrogen in vascular cells. This study also showed that PAI-1 activity in the CM was inhibited in parallel with the reduction of PAI-1 protein levels. On the whole, 17β-E₂ suppressed lysoPC-induced PAI-1 in VSMCs at all levels of mRNA, protein and enzyme activity.

LysoPC induces PAI-1 expression in VSMCs via oxidative stress [8]. As for further mechanisms of action, 17β-E₂ rapidly reduced intracellular ROS production in the present study, which reproduced previous data published by our group [9]. Moreover, 17β-E₂ suppressed NF-κB activity, one of major redox-sensitive signal pathways [18]. These findings support the antioxidant effects of estrogen.

With respect to t-PA, lysoPC does not affect t-PA production and secretion in VSMCs [8]. We did not observe a change in t-PA secretion with 17β-E₂ but demonstrated an increase in free t-PA activity concomitant with a decrease in bound-form activity in the CM. This increase is likely due to inhibited PAI-1 synthesis by 17β-E₂ in VSMCs.

To our knowledge, this is the first study to report favorable impacts of 17β-E₂ on PA system in VSMCs. Besides, positive influences of 17β-E₂ on PA system in VECs are also published. 17β-E₂ upregulated mRNA expression and activity of t-PA [19] and decreased secretion of PAI-1 [20,21]. Taken together, estrogen might enhance vascular fibrinolytic function, which serves as an important mechanism for cardio-protection with postmenopausal estrogen therapy.

On the other hand, PAI-1 plays a crucial role in vascular remodeling. PAI-1 is involved in pathological intimal hyperplasia [22]. PAI-1 also reduces VSMC migration, likely resulting in a thinned fibrous atheroma cap [2]. Further, PA system is implicated in vascular inflammation. Plasmin can amplify the inflammatory response of monocyte [23]. Beneficial effects of estrogen on PAI-1 in VSMCs demonstrated in this study might contribute to attenuating atherosclerosis progression including neointima formation and plaque instability. Additional studies are required to better understand how estrogen modulates the PA system in CHD.

Although using VSMCs derived from rat aortas is an established in vitro model, cells from human coronary arteries might be more appropriate for further experiments to study the direct vascular effects of estrogen.

In conclusion, 17β-E₂ inhibits PAI-1 expression via non-receptor mediated mechanism by antioxidant activity and receptor-mediated mechanism, but does not alter t-PA secretion in VSMCs treated with lysoPC. Importantly, 17β-E₂ suppresses PAI-1 activity and concurrently enhances t-PA activity, suggesting a beneficial influence on fibrinolysis.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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