Vasorelaxant Effect of 17α-Ethynylestradiol on Human Saphenous Vein

Ahmad Reza Jodati¹, Hossein Babaei²³⁸, Yadollah Azarmi³, Sahar Fallah³, Afsaneh Gharebageri³, Danial Fadaei Fouladi³, Naser Safaei¹

¹Cardiovascular Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
²Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
³School of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Abstract

Purpose: A protective effect for estrogens against cardiovascular problems has long been known. The aim of this study was to investigate the vasorelaxant effect of 17α-Ethynylestradiol (17α-EE) on human saphenous vein.

Methods: The veins were suspended horizontally between two triangular stainless steel hooks for the measurement of isometric tension in individual organ baths containing 10ml Krebs solution, at 37°C and gassed with carbogen under 3gr optimum tension. The effect of different concentrations of 17α-EE (2-40 μM) on vascular tone was investigated in veins precontracted with PGF2α. Relaxation was measured after 40min and expressed as the percent decrease of initial contraction. To determine the involvement of potassium channels, endothelium, nitric oxide synthase, guanylylcyclase and prostanolins in the vasorelaxant effect of estrogen, the veins were incubated with tetaethyl ammonium, N-nitro-L-arginine methyl ester, methylene blue or indomethacin, respectively for 20min prior to experimentation. Responses to 17α-EE were directly compared to those obtained in the same tissues in the absence of the inhibitors.

Results: The mean relaxations induced by 17α-EE with concentrations of 2, 5, 10, 20 and 40µM in tissues precontracted with PGF2α were 19.8 ±5.5%, 26.1±10.8%, 32.2±7.4%, 48.6±10.8% and 56±7.6%, respectively. The results of the inhibition of potassium channels, nitric oxide synthase, guanylylcyclase, cyclooxygense and removing endothelium in relaxation induced by 17α-EE on precontracted veins with PGF2α proved no significant differences.

Conclusion: This study showed that 17α-EE has significant vasorelaxant effect on human saphenous vein in a concentration-dependent manner. This effect is probably independent of potassium channels, nitric oxide synthase, guanylylcyclase, prostanolins synthesis and endothelium functions.

Introduction

Estrogens are known for their cardiovascular protective properties through direct vasorelaxing mechanisms. Despite extensive research, however, the exact mechanisms underlying estrogen-induced vasorelaxation are unclear. In addition, the underlying mechanism(s) of vasorelaxation by estrogens varies from type to type. Actions of estrogenic compounds on the vascular wall include alteration/ modulation of ion influxes, receptors on smooth muscle cells, and endothelium-derived factors production and activity.

Guanylatecylase enzyme has been proposed as a mediator in estrogen-induced vasodilation. However, their exact role and mechanism is highly controversial. A number of reports indicate that nitric oxide (NO) may play a pivotal role in mediating the effects of estrogens on the vasculature. This potent vasodilator is produced in the vascular endothelial cells. A direct action on the vascular smooth muscles has been also reported as another underlying mechanism of vasorelaxation incited by estrogens. In a previous study, the authors demonstrated that the relaxant effect of 17β-estradiol on human saphenous vein was elicited by calcium-dependent and -independent pathways. The role of K⁺ channels was also underlined in another study.

It has been reported that estrogens could also modulate peripheral vascular synthesis of vasodilatory hormones. Prostacyclin is one of these hormones, which its production has been proposed as a pathway in estrogen-mediated vasorelaxation. Altogether, estrogens appear to reduce the risk of cardiovascular disease through a combination of mechanisms including changes in lipid profile, endothelial NO synthesis, cell proliferation and angiogenesis, and the regulation of vascular muscle cell (VSMC) Ca²⁺ and K⁺ channels. These effects may be mediated through genomic and/or nongenomic pathways. Naderali et al. showed that 17α-estradiol lacks any effect on classic estrogen receptors. Accordingly, they

*Corresponding author: Hossein Babaei, Tel: +98 (411) 33363311, Fax: +98 (411) 33363231, Email: babaeih@tbzmed.ac.ir
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concluded that the known complications of estrogen-contained oral contraceptive pills, such as endometrial carcinoma and thrombosis, might not be seen with 17α-estradiol.

The mechanisms involved in the rapid vasorelaxant effects of 17α-Ethynylestradiol (17α-EE) are not well understood and are drastically controversial. In addition, there is lack of data in terms of the effects of 17α-EE on veins.

Since it is usually used in coronary artery bypass graft (CABG) surgery, the saphenous vein is of great important to physicians. Furthermore, the incidence of acute closure and accelerated atherosclerosis has been reported high for this vein.

The present study aims to examine, for the first time, the possible relaxant effects of 17α-EE on human saphenous vein in vitro and its possible mechanism(s).

Materials and Methods

Tissue preparation and isometric change recording

After being approved by the Ethics Committee of Tabriz University of Medical Sciences and in conformity with the principles outlined in the Declaration of Helsinki, vein segments were taken from excessive portions of saphenous veins from patients undergoing CABG surgery (n=71), including 58 males (mean age: 57.50±10.32, range: 36-74) and 13 females (mean age: 58.80±2.47, range: 44-71) at Madani Educational Heart Centre, Tabriz University of Medical Sciences, Tabriz, Iran.

Our preliminary experiments showed that there is no significant difference between the contractile or relaxant responses of the saphenous vein rings obtained from male patients and those obtained from female patients. However, to avoid any interference of confounding variables (cardiovascular health, smoking, medications, etc.) in each set of experiments control rings employed from the same male or female patients.

The acquired segments were immediately transferred from operating room to laboratory in ice-cold Krebs solution, trimmed of adjacent tissues and cut into 3-5 mm rings.

The presence and functionality of the endothelium was proven routinely at the beginning of the experiment when there was significant relaxation (more than 50%) to acetylcholine (6 µM) in veins precontracted by phenylephrine (0.1µM).

To investigate the role of endothelium in 17α-EE-induced relaxation, the luminal surface of some vein rings was thoroughly scraped off by using cotton thread. The lack of endothelium was confirmed when there was no relaxation in response to acetylcholine (6µM) in the rings precontracted with phenylephrine (0.1µM).

For isometric change recording, two stainless steel triangle hooks introduced through the lumen of the vein rings, one fixed to the bottom of organ bath, while the other connected to a force-displacement transducer (LETICA, Spain). A computer-assisted data acquisition system (ADInstruments, Power Lab/4SP) recorded the changes in isometric tension during the experiments.

The employed organ bath (10 ml) consisted of modified Krebs-Ringer bicarbonate solution (NaCl: 118mM, KCl: 4.7mM, KH₂PO₄: 1.2mM, NaHCO₃: 25mM, MgSO₄.7H₂O: 2.1mM, CaCl₂: 2.5mM, glucose: 11.1mM). The solution was aerated with a mixture of 95% O₂ and 5% CO₂, maintaining a pH of 7.3-7.4. The temperature was held constant at 37°C. The optimal tension was adjusted at 3g in all primary tests and throughout the experiments as previously established.

Each preparation was allowed to equilibrate for at least 60 min prior to initiation of experimental procedures, during which the bath solution was refreshed every 15 min and the tension was adjusted.

Effect of 17α-EE on isolated human saphenous vein rings

Since KCl and PGF₂α were shown to produce a stable and long-lasting contraction in human saphenous vein, they were chosen as contractile agents in the present study. In order to obtain the submaximal concentration of these contractile agents, a concentration-response curve to each of them was constructed.

After achieving stable contraction by using either KCl or PGF₂α, different concentrations of 17α-EE (2, 5, 10, 20 and 40 µM) were added for 40 min as a standard cutoff time in a non-cumulative manner. Each preparation was exposed to only one concentration of 17α-EE. From 17α-EE concentration response curve, 20 µM was chosen as an optimal submaximal concentration and used in all mechanistic experiments. The vehicle (ethanol, at final bath concentration no greater than 0.1%, v/v) alone had no significant relaxant effect on the contractile responses of the vein rings.

Effect of endothelium-removal on 17α-EE-induced vasodilation

To assess the effect of endothelium, 17α-EE (20µM) was added to both intact and endothelium-denuded vein rings at the plateau of contraction produced by PGF₂α (0.8µM). Forty minutes later, the amount of relaxation was assessed and compared with control group.

Effect of guanylatecyclase (GC) inhibition on 17α-EE-induced vasodilation

Methylene blue inhibits GC by oxidizing its hem iron, and reduces the relaxation elicited by sodium nitroprusside and NO. The effect of methylene blue on 17α-EE-induced relaxation was examined to determine a possible role of soluble GC. For this purpose, vein rings with intact endothelium were incubated with methylene blue (10 µM) for 20 min before induction of contraction with PGF₂α (0.8µM). Afterward, the relaxant responses to 17α-EE were documented.

Effect of NO synthesis inhibition on 17α-EE-induced vasodilation

To assess the role of NO synthesis in 17α-EE-induced relaxation, vein rings with intact endothelium were challenged with N-nitro-L-arginine methyl ester.
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(L-NAME, 200µM) for 20 min prior to inducing contraction with PGF$_{2\alpha}$ (0.8µM). The relaxant responses to 17α-EE were evaluated thereafter.

**Effect of cyclooxygenase inhibition on 17α-EE-induced vasodilatation**

Indomethacin, an inhibitor of cyclooxygenase, was used for 20 min at a dose of 10µM in some vein rings with intact endothelium. After using contractile agents, 17α-EE (20µM) was added for 40 min in these tissues. As indomethacin was dissolved in pure ethanol, a group of vein rings using only pure ethanol (10µL) served as the control.

**Effect of potassium channel inhibition on 17α-EE-induced vasodilatation**

Tetraethyl ammonium (TEA) is a potassium channel blocker, with nonselective effect at high concentration. In order to examine the effect of potassium channel blockade on 17α-EE-induced relaxation, a group of vein rings with intact endothelium were incubated with TEA (5mM) for 20 min before contraction with PGF$_{2\alpha}$ (0.8µM). Subsequently, the relaxant responses to 17α-EE were investigated.

**Chemical Reagents and Drugs**

Acetylcholine, 17α-EE, indomethacin, N-nitro-L-arginine methyl ester, methylene blue and tetraethyl ammonium all were purchased from Sigma. Phenylephrine was obtained from SinaDaru Co (Iran) and PGF$_{2\alpha}$ from Abureyhan Co. (Iran). Indomethacin and 17α-EE were dissolved in pure ethanol and the rest in distilled water. Ingredients of Krebs solution obtained from Merck.

**Statistical analysis**

The SPSS software version 12 (SPSS, USA) was used for statistical comparisons. Data are presented as mean ± standard error of the mean (SEM). The relaxant responses induced by 17α-EE were expressed as percentage of the initial contraction produced by either KCl or PGF$_{2\alpha}$. Linear regressions were performed by least square method for calculation of EC$_{50}$. The Student’s t-test (Independent samples) was used to compare the data and p value less than 0.05 was considered as statistically significant.

**Results**

**Effect of KCl and PGF$_{2\alpha}$ on isolated human saphenous vein rings**

Both KCl (2-120 mM) and PGF$_{2\alpha}$ (0.05-2 µM) induced contraction in human saphenous vein tissues in a concentration-dependent manner. These responses reached a plateau after about 15-20 min, remaining stable for 60-70 min. Concentration-response curves to KCl and PGF$_{2\alpha}$ are depicted in Figure 1A and Figure 1B, respectively. Submaximal concentrations of PGF$_{2\alpha}$ and KCl (~ EC$_{50}$) were chosen for further experiments (0.8 µM and 60 mM, respectively).
**Effect of endothelium on 17α-EE-induced vasodilatation**

The mean percent decrease in contractile response after adding 17α-EE was comparable between the two groups containing endothelium-free and endothelium-intact saphenous veins (60.4±9.6% vs. 48.6±10.8%, respectively (Figure 4). Therefore, denuding endothelium from the human saphenous vein rings did not change the vasorelaxant effect of 17α-EE significantly (p=0.21).

**Effect of NO synthesis inhibition on 17α-EE-induced vasodilatation**

The mean 17α-EE-induced relaxation was 38.0±9.4% in the tissues incubated with L-NAME (NO synthase inhibitor), and 45.0±8.7% in the cases without L-NAME. There was not a significant difference between the two groups in this regard (p=0.23) (Figure 6).

**Effect of GC inhibition on 17α-EE-induced vasodilatation**

There was no significant difference between the veins incubated with indomethacin (cyclooxygenase inhibitor) and the control veins in terms of 17α-EE-induced relaxation (38.2±9.3% vs. 32.9±11.0%; p=0.33) (Figure 7).
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**Effect of potassium channel inhibition on 17α-EE-induced vasodilatation**

The mean 17α-EE-induced relaxation did not differ significantly between the veins incubated with TEA (potassium channel-inhibitor) and the control veins (38.6±6.1% vs. 45.2±10.7%, respectively; p=0.22) (Figure 8).

**Discussion**

Estrogen-induced vasorelaxation is believed to be rendered through interacting with vascular smooth muscle, endothelial cells, and vessel wall.\(^{30-32}\) Despite extensive research, however, the exact mechanisms underlying estrogen-induced vasorelaxation are unclear.\(^{1}\) In addition, the underlying mechanism of vasorelaxation by estrogens varies from type to type.\(^{2,4}\)

In the present study, the acute vasorelaxant effect of 17α-EE on isolated human saphenous vein rings precontracted with KCl or PGF\(_{2α}\) was investigated.

A direct effect of estrogens on the endothelium has been proposed as a possible mechanism of vasorelaxation.\(^{31}\) In the present work, however, there was no significant difference between endothelium-denuded and endothelium-intact vein rings in terms of the mean percent decrease in contractile response after adding 17α-EE. It should be taken into consideration that estrogen-induced vasodilatation can be either endothelium-dependent or –independent.\(^{34,35}\) Likewise, other factors such as gender and hormonal status may play a role in this regard. For example, Martínez et al.\(^{36}\) showed that while gender and hormonal environment had no effect on the estrogen-induced, endothelium-independent component of the relaxation, both of them significantly modulated the estrogen-induced, endothelium-dependent component of the relaxation in rat aorta strips. The role of these possible confounding factors needs to be examined in further studies.

The other factor, which is proposed to have an effect on the vasorelaxation mediated by estrogens, is guanylatecyclase enzyme. In an attempt to clarify the role of this enzyme, methylene blue, an inhibitor of guanylatecyclyase, was used.\(^{37,38}\) In line with some previous reports, adding methylene blue did not modify estrogen-induced relaxation.\(^{3}\)

Nitric oxide production is another mechanism that has been proposed in estrogen-induced vasorelaxation.\(^{39,40}\) Thus, in another part of the present work the role of nitric oxide in 17α-EE-induced vasorelaxation was examined. Accordingly, there was no significant difference in the vein rings relaxation produced by 17α-EE in the presence or absence of L-NAME, a potent inhibitor of nitric oxide synthase.\(^{25,26}\) In conformity with this finding, in a study on human omental artery, Vedernikov et al.\(^{41}\) concluded that the vasorelaxant effect of estrogen (17beta-estradiol) does not involve the products of endothelial nitric oxide. Shaw et al.\(^{42}\) examined the vasorelaxing effects of 17-beta-estradiol on pre-contracted pressurized (50 mmHg) isolated rat mesenteric and coronary arteries. The vasodilatory responses in both types of artery were unaffected by L-NAME. Similar finding was also reported in another study in rabbit carotid artery.\(^{43}\)

It is believed that estrogen stimulates nitric oxide production through both endothelium-dependent and independent pathways.\(^{44}\) Since L-NAME exhibits some selectivity for the inhibition of particular isoforms of nitric oxide synthase,\(^{45}\) it is possible that other ways of...
nitric oxide release remain unblocked even in the presence of L-NAME. To reach a definite conclusion in this regard, however, further clarifying studies are mandatory.

Some studies have suggested that the cardiovascular protection by estrogens may be exerted through the stimulation of prostaglandin synthesis in vessel wall. It has been demonstrated that chronic treatment by estrogen increases in vivo production of prostacyclin and attenuates the level of blood thromboxane in rabbits fed with highly atherogenic diet in comparison with placebo-treated animals. To determine the possible role of prostaglandins in 17α-EE-induced vasorelaxation, we compared indomethacin-incubated tissues with control group. Again, and in conformity with a previous report, no significant difference was detected between the two groups.

A widely discussed probable mechanism by which estrogen may exert its vasorelaxant effect is the alteration in potassium (K') channels. To assess this function in the present work, we assessed the 17α-EE-induced relaxation in the presence of TEA, an inhibitor of potassium channels. There was not a significant difference between the two groups in this regard.

As discussed earlier, the results of various studies are widely heterogeneous, and sometimes inconclusive. The type of estrogen and contractant, the presence or absence of endothelium, and the employed methodology to examine the acute effects may contribute to the variability in the response. In addition, the vasodilatory effects of different estrogens could widely vary between specific species and/or vascular beds in systemic circulation.

**Conclusion**

This is the first study, which examines the vasorelaxant effect of 17α-EE in human saphenous vein. This concentration-dependent relaxant effect occurs acutely (non-genomic), independent of the mechanisms associated with guanylatecyclase, cyclooxygenase, endothelium, NO and potassium channels.

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**Ethical Issues**

Not applicable.

**Conflict of Interest**

The authors report no conflicts of interest.

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