17β-Estradiol modulates effects of insulin-induced changes in vascular contractility

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Summary

The protective role of estrogens against peripheral vascular and coronary disease in women is well documented; however, it is not present in diabetic women. Estrogens reduce tension development through non-genomic mechanisms that include changes in calcium concentrations in endothelial and smooth muscle cells, and regulation of nitric oxide synthase (NOS) in endothelial cells. Insulin increases endothelin-1 (ET-1) release from endothelial cells modulating smooth muscle calcium levels and elevating force generated by femoral and coronary arteries. This paper examines whether 17β-estradiol (E₂β) modulates changes in femoral and coronary artery contractility induced by insulin. Femoral and coronary arteries were obtained from male Wistar rats, placed in isolated tissue baths for in vitro studies, perfused with different solutions, and the contractile response to KCl 40 mmol/L was measured. Insulin increased arterial contraction induced by KCl. This increase was not present when the endothelium was removed. In the presence of E₂β, we observed a dose dependent reduction in the tension developed and this effect disappeared when the endothelium was removed. The insulin-induced contraction was significantly reduced in presence of E₂β. These data indicate that the effect of insulin on femoral and coronary arteries is modulated by 17β-estradiol.

Resumen

El 17β-ESTRADIOL MODULA LOS EFECTOS DE LOS CAMBIOS DE LA CONTRACTILIDAD VASCULAR PRODUCIDOS POR LA INSULINA

Los estrógenos protegen a la mujer contra enfermedades vasculares periféricas y centrales; sin embargo, su papel se pierde con la diabetes. Los estrógenos reducen la tensión en las arterias mediante cambios en el calcio intracelular en células endoteliales y musculares lisas y la regulación de la óxido nítrico sintasa en células endoteliales. La insulina incrementa la liberación de endotelina-1 (ET-1) en células endoteliales aumentando la fuerza generada por las arterias. En este estudio se examina si el 17β-estradiol (E₂β) modula los cambios en la contractilidad inducidos por insulina en las arterias femorales y coronarias. Las arterias se obtuvieron de ratas Wistar macho y se colocaron en cámaras para tejido aislado para perfundirse in vitro con distintas concentraciones de insulina y estrógenos estimulando la contracción con KCl 40 mmol/L. La insulina elevó la fuerza de la contracción inducida por KCl. Este incremento desapareció cuando se eliminó el endotelio. El E₂β disminuyó la tensión desarrollada por las arterias conforme se aumentó la dosis y el efecto desapareció al quitar el endotelio. El incremento en la tensión...
Introduction

The protective role of estrogens has been well documented and several reports have demonstrated that premenopausal women have lower incidence of peripheral and coronary vascular disease than postmenopausal women.\textsuperscript{1,2} However, in diabetic women the normal premenopausal gender related difference in the prevalence of cardiovascular disease is lost.\textsuperscript{3,4} Although the mechanisms of action of estrogens and insulin on vascular smooth muscle cells and on vascular endothelium have been extensively studied, they are still not completely understood and probably many interacting mechanisms may be involved in the effects of these hormones.

Steroids exert some of their effects on target cells by entering them, binding to high affinity cytoplasmic or nuclear receptors to constitute the steroid-receptor complex that interacts specifically with DNA. This complex regulates gene transcription and production of specific proteins that modify cell function.\textsuperscript{5} However, this mechanism does not account for all of the cellular effects of steroid hormones, signaling the existence of non-genomic mechanisms, particularly for the rapid, transient effects, which seem to be independent of hormone interaction with the cell nucleus.\textsuperscript{6} One of the alternative mechanisms of action proposed in endothelial cells is an increase in intracellular calcium \([Ca^{2+}]_i\) which is necessary for NO synthesis.\textsuperscript{7-10} In addition, cytosolic and plasma membrane estrogen receptors on vascular smooth muscles cells have been found and are known to block calcium entry through L-type calcium channels, reducing \([Ca^{2+}]_i\) and inducing relaxation.\textsuperscript{11}

In a previous paper we demonstrated that insulin increases force generated by femoral and coronary arteries and that these effects are mediated by ET-1.\textsuperscript{12} ET-1 induces elevation of \([Ca^{2+}]_i\) concentration in vascular smooth muscle cells\textsuperscript{11} and increases vascular tone. Therefore, the aim of the present report was to examine whether \(E_2\beta\) modulates changes of femoral and coronary artery contraction induced by insulin through rapid mechanisms, particularly affecting endothelial cell function rather than smooth muscle contraction.

Methods

Vascular tissue preparation and tension recording

Male Wistar rats weighing 250 to 350 g were used. The animals were killed by cervical dislocation, and the heart or femoral arteries were dissected immediately and kept in an oxygenated standard Tyrode solution of the following composition (mM): 136.9 NaCl, 5.4 KCl, 1 CaCl\(_2\), 1.05 MgCl\(_2\), 11.9 NaHCO\(_3\), 0.33 NaH\(_2\)PO\(_4\), and 5.5 glucose. Under a dissecting microscope, the femoral vessels were cleaned of surrounding tissue, avoiding damage to the endothelium. Arterial segments of 5 mm were cut, and two 250-µm-diameter S-shaped silver hooks (Medwire) were inserted into the lumen to measure the tension developed transversally. For experiments in coronary arteries, the first wire string was inserted into the left coronary artery in the intact heart. The wall of the vessel was lifted to insert the second wire. A coronary artery segment of 2 to 3 mm was then dissected from the ventricle wall and partially cleaned from cardiac muscle. One of the silver hooks was fixed to the bottom of an organ chamber, and the other was attached to an isometric force-displacement transducer (FT03, Grass Medical Instruments). The transducer was connected to a Grass polygraph model 79 D. The optimal basal passive tensions for rings of both types of vessels were previously found under our experimental conditions\textsuperscript{12} and applied: 500 mg for femoral arteries and 250 mg for coronary arteries. Basal passive tension for coronary arteries was smaller than the one applied to femoral arteries since these vessels are normally perfused during diastole in which arterial pressure is smaller than in arteries perfused during systole. The experiments were also done in arteries in which the endothelium was removed by gently rubbing the vessel with the silver strings against a wet piece of cloth.

Key words: Insulin. Estrogen. Endothelium. Coronary arteries. Femoral arteries.

integrity or absence of the endothelium was proved by testing the response to acetylcholine (10^6 M) at the end of the experiment.

**Experimental protocol**

The vessels were perfused with standard Tyrode solution bubbled with a 95% O₂, 5% CO₂ gas mixture. The chamber was continually perfused with warm (37°C) solution. The rings were allowed to equilibrate for 15 min. Contractions were induced by a Tyrode solution containing 40 mmol/L KCl; this concentration was chosen after concentration-response curves had been obtained since it produced submaximal responses (data not shown). After recording control-developed tension, the arteries were washed with normal Tyrode solution and were allowed to return to their basal tension level. The procedure was repeated, and the mean value of the force developed was taken as a 100% response.

After this, the arteries were subjected to one of the following situations in separate experiments repeating the stimulation with KCl to test the contractile response: 1. Arteries were perfused with different doses (150, 300, 600 pmol/L) of insulin (Eli Lilly & Company). These concentrations were chosen to include fasting (150 pmol/ L) and postprandial (300 pmol/L) normal concentrations. 2. Arteries were perfused with different doses (10 to 10,000 pmol/L) of E₂β. These concentrations were chosen to include estrus (350-450 pmol/L) and diestrum (80-120 pmol/L) normal concentrations in female rats from the same species. 3. Arteries were perfused with insulin (300 pmol/L) in the presence of E₂β (100 pmol/ L). 4. At the end of each experiment, arteries were bathed again with standard Tyrode solution and contracted with KCl to verify that there were no important effects secondary to the incubation time or to repeated exposures to KCl.

**Statistical analysis**

Values are expressed as percentages; basal contraction was considered as 100% and the percentage in each experiment calculated. Mean and standard errors of at least six different arteries were calculated. Statistical significance of the differences was analyzed using Students t test to compare femoral and coronary arteries under the same experimental situation and ANOVA followed by Newman-Keuls test for multiple comparisons. A value of P < 0.05 was considered statistically significant.

**Results**

To investigate the role of E₂β, as a modulator of insulin effect, we compared the effects of E₂β on the response to KCl in the presence and absence of insulin in femoral and coronary arteries, with and without endothelium. No effects on passive basal tension were observed as the vessels were perfused with solutions containing different insulin or E₂β concentrations. Nevertheless, when the arteries were contracted by the addition of KCl, tension development varied under the different experimental conditions. Basal contraction force in femoral and coronary arteries bathed with normal Tyrode (without insulin or E₂β) was 28 ± 15 and 134 ± 18 mg respectively and was considered as 100% in all experiments.

**Insulin-induced increases in vascular contractility. Endothelial-mediation in the effects**

We found a dose-dependent increase in force generated to KCl by femoral and coronary arteries with standard Tyrode solution and in the presence of different insulin concentrations. The maximal force increase for femoral arteries was found at 300 pmol/L, reaching 175.48 ± 12.20% (n = 10), whereas for coronary arteries the largest increase was found with a lower dose of 150 pmol/L and the force reached only 130.42 ± 7.8% (Fig. 1).

Mediation by the endothelium in the insulin effects on femoral and coronary arteries was tested after removing it. We observed that, when the endothelium was removed, KCl-induced contractions in the presence of insulin in femoral and coronary arteries were not significantly modified from the control contractions and therefore the insulin effect disappeared. (Fig. 2A).

**Effects of different E₂β concentrations on vascular contractility**

When femoral and coronary arteries were bathed with normal Tyrode solution, in the presence of E₂β, we observed a dose-dependent reduction in tension developed to KCl by the arteries. In both arteries, a significant decrease of approximately 35% of the control contraction (to 68.37 ± 4.14% in femoral and to 66.70 ± 4.87 in coronary arteries) was reached with the lower dose of 10 pmol/ L. The minimal response was reached in both arteries with the higher dose of 10,000 pmol/L representing a decrease of approximately 75%
of the control contraction (to 34.28 ± 3.17% in femoral and to 35.9 ± 4.31% in coronary arteries) (Fig. 3). Endothelial-mediation of the effect of E₂β was also tested. When the endothelium was removed in femoral and coronary arteries, the decrease in tension observed in vessels perfused with E₂β (100 pmol/L) disappeared and contractility returned to the basal level (Fig. 2B).

**E₂β modulator effect of insulin-induced changes in vascular contractility**

E₂β mediation of the insulin effects on femoral and coronary arteries was tested using a solution with insulin (300 pmol/L) and E₂β (100 pmol/L). When both hormones were applied simultaneously, only a slight non-significant increase on vascular tension was observed (to 119 ± 11.7% in femoral and 113 ± 7.22% in coronary arteries). Neither the increase in tension induced by insulin to 175.48 ± 12.20% and 130.42 ± 7.8% (n = 10) in femoral and coronary arteries respectively nor the decrease induced by estrogen of 35% (to 65 ± 11.86%) in femoral and of 24% (to 76.92 ± 2.93%) in coronary arteries were observed. These data indicate that the E₂β effects are strong enough to block the insulin-induced increase in vascular tension (Fig. 4).

**Discussion**

The incidence of cardiovascular disease with age is increasing, and elderly women constitute an important component of the aging population. Elderly women also have a relatively high incidence of diabetes. Although cardiovascular disease is less common in premenopausal women that in men, this difference begins to disappear after the onset of menopause, presumably related to decreased levels of female sex hormones. Diabetes mellitus removes the normal premenopausal gender-related differences in the prevalence of cardiovascular disease by mechanisms that are not clearly defined. Furthermore, the risk of death from cardiovascular disease in women with diabetes is more than 3 times that of nondiabetic women. Many factors contribute to the increase in cardiovascular disease in diabetic women as well as men. These include endothelial dysfunction, increased vascular oxidative stress, and abnormalities of platelet function, coagulation, fibrinolysis, and lipoproteins. The aim of the present study was to examine whether E₂β modulates the increases in femoral and coronary artery contractility induced by insulin.
that we have previously demonstrated,12 through rapid mechanisms affecting endothelial cells.

In our experiments, insulin increased the force generated by femoral and coronary arteries and these effects were larger as insulin concentrations rose. Although it is clear that diabetes mellitus is associated with an increase in vascular complications despite tight control of blood glucose, there is a controversy on the role of insulin on vascular contractility. Insulin has been reported to influence membrane components in different cells and situations that may increase or decrease intracellular calcium levels and contribute to the contradicting tension development changes found in vascular structures.8,15-18 Insulin has also been reported to modify endothelial cell secretion inducing both vasoconstriction and vasodilatation.19-22 It reduces α-adrenergic induced vasoconstriction by stimulating the production of endothelial nitric oxide through a pertussis toxinsensitive mechanism.23,24 However, in accordance with our data, other studies have shown a correlation between high insulin levels and hypertension.3,8,14,19 In bovine and porcine endothelial cells, insulin stimulates endothelin production and secretion and increases ET-1 gene expression.20 We have previously reported an increased femoral contraction with insulin that is mediated by endothelin.12

Several possible mechanisms of action of estrogens on human tissues have been proposed to explain the multiple observational clinical and experimental studies suggesting a marked reduction in risk of peripheral and coronary disease associated with postmenopausal estrogen use.4,25,26 In the intact organisms, changes in plasma lipoprotein levels, fibrinogen, antithrombin III, angiotensin type 1 receptor regulation, and antioxidant action have been proposed.27,28 Estrogens have also been found to have vascular local effects and their acute administration relaxes vascular smooth muscle, causing a reduction in vascular resistance and increased blood flow.29-31 These reports agree with our results, in which we found a decreased force of contraction as estrogen concentrations rose.

Several studies have proposed the classical signaling pathway for the genomic effect of estrogens. In this pathway, the estrogen receptor translocates to the nucleus and binds directly to response elements to regulate gene transcription.5 In addition, several non-classical pathways have been described to explain the rapid changes in-

Fig. 3. Effects of different E_2 concentrations on contractile response to KCl of femoral and coronary arteries. Values are means ± SEM, n = 6. *P < 0.05 compared to values without E_2. There were no significant differences between femoral and coronary arteries.

Fig. 4. Compensation of the insulin response (300 pmol/L) by the presence of E_2 (100 pmol/L) in femoral and coronary arteries. Values are means ± SEM, n = 6. *P < 0.05 when comparing the effects of the hormones to the control. ^P < 0.05 when comparing the response with insulin and E_2 against the contraction to each of the hormones independently.
duced by estrogens, which are clearly not compatible with the genomic mechanisms. It is not clear which of these estrogen mechanisms predominate in each of the different cell types. One of the genomic mechanisms described for Eβ that may explain in part our results in vessels is the stimulation of NOS-III gene expression in the rat uterus. Some of the non-genomic mechanisms that might help explain our results include the depolarization of the plasma membrane, in pancreatic β-cells eliciting electrical activity and intracellular calcium signals. Vascular smooth muscle responds to Eβ reducing [Ca2+]i by blocking L-type Ca2+ channels. Although our experiments with estrogens could be explained by this mechanism, the fact that relaxation disappeared in the absence of endothelium suggests that estrogens modify endothelial function rather than smooth muscle cell contractility. Our results suggest that an impairment of the endothelial function may be responsible for the effects of estrogens on isolated vessels and for protection from vascular disease. Endothelial cells have estrogen receptors whereas subendothelial cells do not. Estrogens activate endothelial NOS, which is responsible for the relaxing effect by mechanisms of action that remain to be elucidated. It could be possible that the mechanism involves the increase in [Ca2+]i level, which induces expression and/ or activity of endothelial NOS and NO release. Other vasodilator substances released by the endothelium such as prostacyclin, endothelium derived hyperpolarizing factor, epoxy-eicosatrienoic acids, and adenosine might also be involved in the relaxing effects of Eβ. Our results show that estrogens modulate the insulin effect only when endothelial cells are present. Therefore, Eβ counterbalances the vasoconstrictor effect of insulin by altering endothelial function. Estrogens have not been reported to modify or block the regulatory mechanisms of action induced by insulin and apparently they use independent mechanisms to induce relaxation. When both hormones are present at the same time, their effects are compensated by the antagonistic mechanism of the other hormone. The release of both vasoconstrictor and dilator substances is increased, having opposite effects and therefore neither the contractile effect of insulin nor the dilator effect of Eβ predominate. This might be one of the reasons why diabetic women are not protected against coronary and peripheral vascular disease during the reproductive age. Furthermore, it has been shown that male diabetic rats exhibit the deleterious effect of the disease and that chronic Eβ treatment reduces injury in male diabetic rats, providing vascular protection.

Our findings support the idea that endothelial dysfunction may represent the pathogenic mechanism in disease states associated with altered blood flow, such as diabetes, hypertension, and cardiovascular disease. Vascular endothelial cells have the ability to modulate local vascular tone by releasing relaxing factors or vasoconstrictor peptides. We conclude that Eβ, modulates the increases in femoral and coronary artery contractility induced by insulin, acting upon the endothelial cells. These results help explain the lost protective role of estrogens against peripheral and coronary vascular disease in diabetic women.

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