

Cardiovascular protection by postmenopausal estrogen replacement therapy: possible mechanisms of the estrogen action

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Summary: Estrogen replacement therapy reduces the cardiovascular risk in postmenopausal women, but the mechanism has yet to be evaluated. There is growing evidence that estradiol administration results in direct vasodilatory effects on vessel walls. The biochemical mechanisms have been investigated in human arteries and veins with respect to different mediators and cell systems. Whereas estradiol had no direct effect on the prostaglandin system we found an endothelin-mediated stimulation of prostacyclin production in endothelial cell cultures. In experiments with the NO/cGMP-system estradiol activity could be demonstrated. In addition estradiol provoked an increase in the intracellular Ca^{2+} concentration. These results indicate that several mechanisms may be involved in the vasodilating effect of estradiol.

Key words: Coronary heart disease; Postmenopause; Estrogen replacement; 17β -estradiol; Prostacyclin; Endothelin; Nitric oxide/cGMP; Calcium antagonism.

INTRODUCTION

At least 25 epidemiological studies analysing the relationship between estrogen therapy and coronary heart disease agree that estrogen replacement therapy may have dramatic benefits in menopausal women by reducing morbidity or death from coronary heart disease by about 50% (^{1, 2}). However, much or all of the data comes from observational rather than from randomized controlled trials, and the mechanisms underlying the effects are still unclear. Only recently it has been shown

that the protection against coronary heart disease, that is mediated by estrogen-induced beneficial changes in lipids, can only account for less than half of the reduced risk (^{3, 4, 5}). Part or all of the protection may be due to direct vasodilating effects of estrogen on vessel walls. This is supported by recent clinical trials using Doppler ultrasound to measure blood flow in arteries of postmenopausal women who had been treated with transdermal estradiol (^{6, 7, 8}). These data are indicative of the direct vasodilating effects of estradiol and are supported by animal studies including measurements of increased cardiac output, increased heart rate and reduced vascular resistance (^{9, 10, 11}). However, our knowledge in this most important field of estrogen replacement therapy is incomplete, and the biochemical mecha-

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nisms by which the effects occur have still to be elucidated.

In the past, many questions with respect to effects on the cardiovascular system have been addressed by investigations of human blood vessels *in vitro* including mediators which could be responsible for the questionable mechanisms. Direct vasodilating effects of estradiol could be induced via muscle relaxation, whereby the following mediators may be operating:

- 1) Some prostanoids, especially prostacyclin.
- 2) NO which is synthesized from L-arginine and stimulates the production of cGMP by activation of a guanylatecyclase.
- 3) alterations in Ca^{2+} -homeostasis.
- 4) antagonism to vasoconstrictive agents, e.g., endothelin.

The aims of our *in vitro* studies in human arteries and veins were to investigate the different mechanisms given above by which estradiol may cause blood vessel relaxation.

MATERIALS AND METHODS

Human umbilical cords were obtained after normal vaginal deliveries and employed to prepare endothelial cell cultures or homogenates.

Cell cultures:

Cell dispersion was established by treatment with collagenase (0.1%) in phosphate buffer (pH 7.4) for 10 min at room temp., placed in culture dishes and allowed to replicate to confluence in Dulbecco's Modified Eagle Medium/Nutrient Mix F12 (1:5, v/v) that contained fetal calf serum (10% v/v), endothelial growth factor (20 $\mu\text{g}/\text{ml}$), heparin (50 $\mu\text{g}/\text{ml}$), streptomycin (100 $\mu\text{g}/\text{ml}$) and amphotericin B (2.5 $\mu\text{g}/\text{ml}$). Cell cultures were maintained in an atmosphere of CO_2 (5%) in air.

Tissue culture media and supplies, antibiotics and fetal calf serum were purchased from Gibco, Eggenstein, Germany. Endothelial cell growth factor was purchased from Boehringer, Mannheim, Germany.

Homogenates:

Fresh human umbilical cord arteries and veins or pieces of human leg veins were transported on ice. The vessels were homogenized in 5 ml sodiumphosphate buffer (50 mM, pH 7.4) with EDTA (2 mM) by means of a Ultraturrax homogenizer. The homogenate was centrifuged at 750 g for 10 min and the supernatant frozen at -70°C .

Determination of prostacyclin:

100 μl homogenate of umbilical cord vein or artery was mixed with the same volume of a solution of arachidonic acid (50 μM) and incubated for 30 min at 37°C . The reaction was terminated by addition of 100 μl of acetic acid (1 N). Estradiol (dissolved in ethanol) was included at a final concentration of 10^{-8} and 10^{-6} M. The basal value was determined by addition of the solvent mixture. Prostacyclin was determined by measurement of the stable, hydrolytic metabolite, 6-keto-prostaglandin $\text{F}_{1\alpha}$, by radioimmunoassay.

Determination of cGMP:

Homogenates of leg veins (250 μl) were mixed with 10 μl of each Cofactor solution (NADPH (0.1 mM), Calmodulin (0.1 μM)). Estradiol (dissolved in ethanol) was added in 10 μl at a final concentration of 10^{-6} , 10^{-7} , 10^{-8} M. The mixture was incubated at 37°C for 15 min and frozen until determination of cGMP by radioimmunoassay.

Endothelin-mediated effects on prostacyclin production:

The experiments were initiated in duplicates by the addition of 17β -estradiol (dissolved in ethanol) and/or endothelin 1 (dissolved in 0.9% NaCl-solution) to confluent cell cultures (passages 2-3) to give a final concentration of 10^{-8} and 10^{-9} M for endothelin 1 and 10^{-6} and 10^{-8} M for 17β -estradiol in the culture medium. The amount of ethanol was $< 0.2\%$ (v/v). Incubations were performed for 24h. All experimental values were corrected for blanks in samples which were treated with solvent mixture alone. At the end of the incubation period the culture medium was collected and 6-keto-PGF $_{1\alpha}$ and thromboxane B_2 were determined directly by radioimmunoassay.

Investigation of changes of intracellular calcium-concentration:

Confluent endothelial cell cultures from human umbilical cord veins (passage 2) were washed three times with physiological salt solution (PSS-buffer, 140 mM NaCl, 5 mM KCl,

1.5 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, 10 mM Hepes, pH 7.4) and incubated with PSS-buffer for 15 min. After preincubation the cells were incubated with a solution of $^{45}\text{Ca}^{2+}$ (ca 0.2 $\mu\text{Ci}/\text{ml}$) in PSS-buffer and estradiol (10^{-6} , 10^{-8} , 10^{-10} M) in ethanol for 5 min. The basal value was determined by addition of $^{45}\text{Ca}^{2+}$ in PSS and ethanol. The final amount of ethanol was 1.25% (v/v).

Culture medium was aspirated and the cells washed twice with H_2O and treated with 0.6 N trichloroacetic acid. The cells were scraped off, ultrasonicated for 10 s and the activity of intracellular ^{45}Ca measured by a Beta-counter.

Statistical analyses were conducted by the Student t-test.

RESULTS

The influence of estradiol on prostacyclin production in 5 different homogenates of human umbilical arteries or veins after addition of exogenous arachidonic acid is shown in figure 1. The addition of arachidonic acid stimulated the prostacyclin production in the artery by 36-47% and in the vein by 16-21%. However in neither of the estradiol concentrations could significant change in the prostacyclin production be observed, compared to samples with exogenous arachidonic acid alone.

The effects of estradiol on cGMP production in homogenates from human leg veins is shown in figure 2. A concentration of estradiol (10^{-8}M) induced an increase of cGMP production ($p = 0.05$) compared to the basal value ($15.1 \pm 5.6\%$, $\bar{X} \pm \text{SD}$, $n = 8$).

Figure 3 shows the effect of estradiol and/or endothelin 1 on the prostacyclin production in endothelial cell cultures from human umbilical veins. Estradiol alone had no influence on the prostacyclin production in all concentrations tested compared to the basal value. Endothelin 1 significantly increased the prostacyclin production in endothelial cell cultures from human umbilical cord veins in small but definite amounts i.e. $9.5 \pm 1.1\%$ and $7.2 \pm 0.09\%$ at concentrations of 10^{-8}M and 10^{-9}M ($p < 0.05$), respectively. The addition of estradiol in the concentration of 10^{-6}M to both endothelin concentrations did not change the prostacyclin production much, either. In contrast, addition of the lower dosage estradiol (10^{-8}M) showed a statistically significant increase ($p < 0.05$) of the prostacyclin production of both endothelin concentrations. With en-

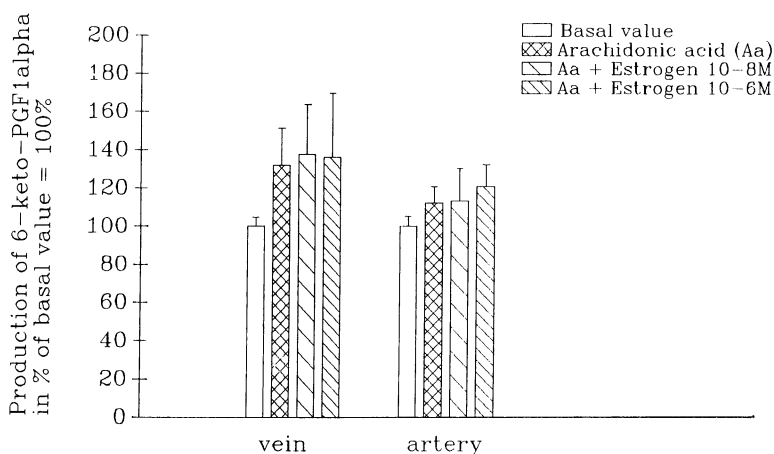


Fig. 1. — Relative prostacyclin production in percent of basal value (=100%) in homogenates from human umbilical cord vessels after addition of estradiol (means \pm SD, $n = 5$).

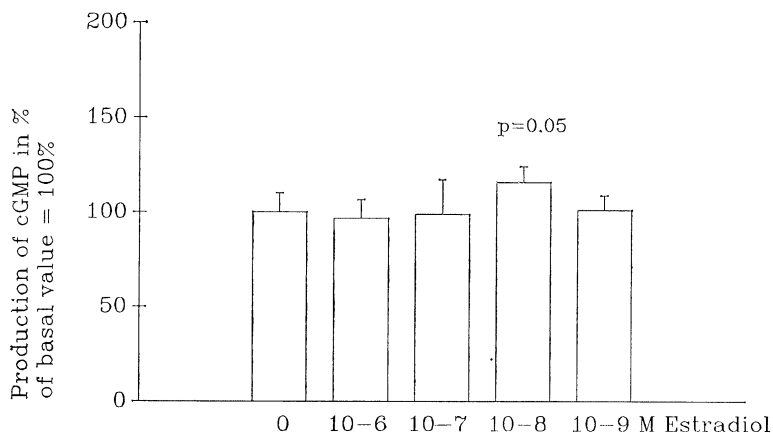


Fig. 2. — Relative cGMP-production in percent of basal value (= 100%) in homogenates from human leg veins after addition of estradiol (means \pm SD, n = 8).

dotelin at a concentration of 10⁻⁸M an increase of 60.0 \pm 22.5%, and at 10⁻⁹M an increase of 39.5 \pm 22.1% was observed.

Thromboxane B₂ production was not affected by the addition of endothelin 1 or estradiol, alone or in combination.

The effect of estradiol on the calcium

influx in endothelial cell cultures from human umbilical cord veins is shown in figure 4. Estradiol concentrations of 10⁻⁶ and 10⁻⁸M induced a statistically significant reduction of calcium influx of 11.2 \pm 4.6% and 12.3 \pm 5.0% (p < 0.05), respectively.

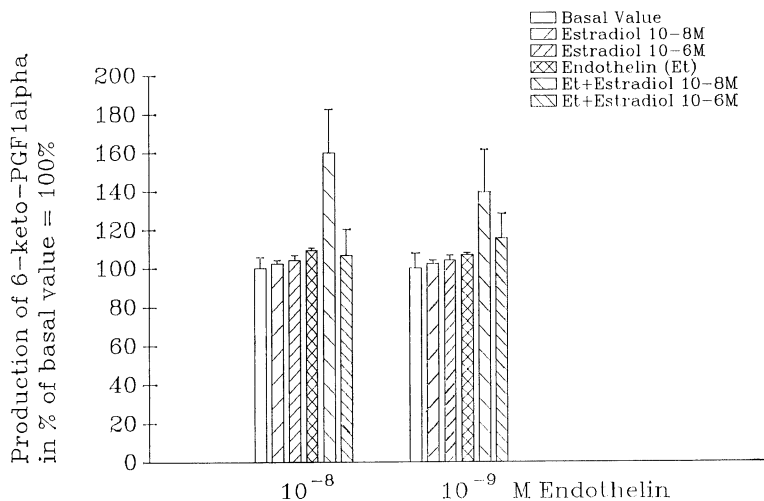


Fig. 3. — Relative prostacyclin production in percent of basal value (= 100%) in endothelial cell cultures from human umbilical cord veins after addition of endothelin 1 and estradiol, alone or in combination (means \pm SD, n = 5).

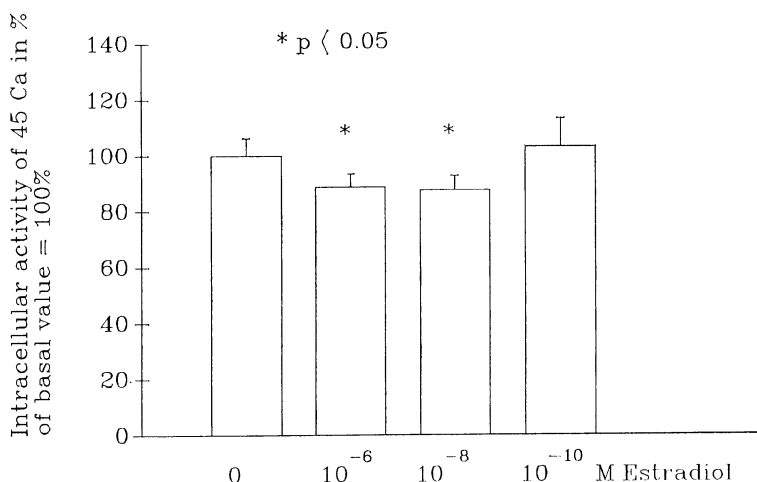


Fig. 4. — Relative intracellular ⁴⁵Ca-activity in percent of basal value (= 100%) in endothelial cell cultures from human umbilical cord veins after addition of estradiol (means ± SD, n = 5).

DISCUSSION

In 1988, a protein related to the estradiol receptor was identified in the intima of major vessels in humans, suggesting that estradiol may exert direct vasodilating action on the arterial wall through a conventional steroid receptor mechanism (¹²). In animals estradiol increases cardiac output and arterial flow velocity, decreases vascular resistance and systolic and diastolic blood pressure (^{10, 11}). Recent studies with Doppler ultrasound showed a beneficial effect of transdermal estradiol application in postmenopausal women increasing blood flow in the uterine and carotid artery (^{6, 7, 8}). Those haemodynamic effects of estrogens *in vivo* may only be explained by determining biochemical actions *in vitro*, and human tissues are known to represent the best *in vivo* conditions.

We have investigated the vasodilatory properties of estradiol in different *in vitro* systems and with some mediators. In *in vitro* experiments, estradiol had no significant effect on prostacyclin production. This was true both for studies in cell homoge-

nates and in endothelial cell cultures (¹³). This is in contrast to the results of others (¹⁴) who found an increase of prostacyclin metabolites in urine after percutaneous or oral application of estradiol. However, our results are in agreement with Corvazier *et al.* (¹⁵) and Busse R. (¹⁶).

The release of endothelium-derived relaxing factor (EDRF), now identified as nitric oxide (NO), can be induced by acetylcholine (¹⁷), suggesting that estradiol may alter vascular tone by an NO-dependent mechanism involving acetylcholine responses. Experiments with animals are suggestive that the NO/cGMP-system may play a role in vasodilatation by estradiol (^{9, 11}). Our results with homogenates showing that estradiol enhanced the cGMP production support this hypothesis. We do not know how important these *in vitro* results may be in explaining cardiovascular protection by estrogen replacement therapy in postmenopausal women.

Another mechanism by which estradiol induces vasodilatation may be through an alteration of the sensitivity of endothelial cells to endothelin 1. The importance of

endothelin 1 release from the endothelium in patients with myocardial infarction, hypertension and atherosclerosis is unknown; an inhibitory effect of estradiol might be possible and beneficial. It is known that endothelin 1, a vasoconstrictory substance, stimulates prostacyclin synthesis in different cell system⁽¹⁸⁻²⁰⁾. This can be interpreted as a counterbalance to vasoconstriction. In human umbilical cord endothelial cells, we found that estradiol potentiated this effect of endothelin on prostacyclin production⁽²¹⁾. This could point to an antagonist triggered mechanism.

In uterine smooth muscle cells estradiol provokes vasodilatation by mechanisms that include Ca^{2+} -antagonism⁽²²⁾. Our experiments showed that estradiol effects Ca^{2+} movements, i.e. reduced Ca^{2+} -influx, at two different concentrations in endothelial cell cultures. The results support the hypothesis that estradiol may have calcium antagonistic effects, which may contribute to the cardiovascular protection in postmenopausal women.

In summary, the biochemical mechanisms by which estradiol induces vasodilatation are complex. We found, that there is no direct effect on prostacyclin production but rather a potentiation of other stimulative agents (e.g. endothelin). The role of the NO/cGMP-system has yet to be defined. Effects of estradiol on Ca^{2+} -homeostasis give strong support that estradiol may have calcium antagonistic properties. If the vasoactive biochemical effects could be established in further evaluations this could have important clinical consequences with respect to the multidisciplinary concepts in preventing and treating cardiovascular diseases of menopausal women.

Some of these results were presented at the 7th International Congress on the Menopause, Stockholm, Sweden, June 20-24, 1993.

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