SHORT COMMUNICATION

17β-Oestradiol inhibits stimulated endothelin release in human vascular endothelial cells

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Abstract
Oestrogen is believed to possess cardiovascular protective properties. Endothelin-1 (ET-1) is a potent vasoconstrictor and mitogen for vascular smooth muscle cells. We have investigated release of ET-1 from human endothelial cells in response to 17β-oestradiol. Serum was found to stimulate significantly ET-1 release during the first 4 h of culture. 17β-Oestradiol significantly reduced ET-1 immunoreactivity in the media of treated cells by up to 49% of control values after 4 h. This effect was found to be inversely related to the dose of 17β-oestradiol. Significant dose-dependent increases in nitric oxide synthase expression were observed in response to oestrogen after 24 h but not after 4 h. Serum-free experiments demonstrated that low doses of oestrogen were able to inhibit thrombin-induced ET-1 release whilst supraphysiological levels did not. These results provide a further perspective on the ability of oestrogens to maintain vascular health.

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Introduction
Cessation of ovarian function in women, whether natural (1) or following oophorectomy (2), is associated with increased risk of coronary heart disease (CHD). A decrease in production of the female sex hormone, oestrogen, may be the cause of this increased CHD risk. Indeed, hormone replacement therapy (HRT) has been associated with reductions in risk of CHD of up to 50% in postmenopausal women (3). Direct effects on the blood vessel wall could account for a significant proportion of the beneficial properties of oestrogen on CHD risk. It has been demonstrated that the naturally occurring oestrogen, 17β-oestradiol, is capable of upregulating production of the vasodilatator prostacyclin (PGI₂) (4), as well as increasing levels of constitutive nitric oxide synthase (5). In vivo studies suggest that an important endothelial product, endothelin-1 (ET-1), is significantly reduced by exogenous oestrogen administration (6). This 21-amino acid peptide is the most potent naturally occurring vasoconstrictor thus far discovered (7) and is also a powerful mitogen for vascular smooth muscle cells (8). In this study we investigated ET-1 release from human vascular endothelial cells in culture.

Materials and methods
Unless otherwise stated all chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, UK). Cell culture plastics were supplied by Marathon Laboratory Supplies (London, UK). Human umbilical cords were donated from a local hospital after approval by the local Ethics Committee. Endothelial cells (human umbilical vein endothelial cells, HUVECs) were extracted from the veins according to a published protocol (9). Culture media and components were phenol red-free. Cultures were plated out on 25 cm² flasks precoated with 2% (w/v) gelatin in M199 (Life Technologies, Paisley, Strathclyde, UK) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS), 2 KU/l penicillin, 100 mg/l streptomycin, 70 mg/l heparin, 75 mg/l endothelial cell growth supplement. Cultures were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. Cultures in passages 1–3 were used. A standard 17β-oestradiol preparation requiring initial dissolution in ethanol and a water-soluble cyclodextrin encapsulated form were used (range: 2 × 10⁻¹⁰ mol/l to 2 × 10⁻⁷ mol/l). Cells were plated onto gelatin-coated 24-well plates and grown to confluence. Cultures were washed in M199 prior to addition of media. For experiments conducted in the presence of serum the medium was M199, 10% (v/v) FCS. For serum-free experiments the medium was M199, 0.5% (v/v) BSA. Cultures were incubated in the absence or presence of test reagents for 4 h. Supernatants were harvested and stored at −20°C until assayed. Cell lysates were prepared...
Results

Basal production of ET-1 at 4 h from 10^5 cells was 69 ± 12 pmol/l and by 24 h this had only increased to 253 ± 31 pmol/l reflecting a 39.8 ± 2.5% (P < 0.01) decline in the rate of ET-1 accumulation in the media (n = 12) between 4 and 24 h. This suggested that ET-1 release was most rapid during the initial 4 h most probably due to stimulation by fresh serum. In the absence of serum, the release of ET-1 from HUVECs was found to be reduced by 77.7 ± 4.3% (P < 0.001) over 4 h (n = 6). We investigated the effect of 17β-oestradiol on serum-stimulated ET-1 release from HUVECs (Table 1). All doses of 17β-oestradiol yielded significant reductions in ET-1 immunoreactivity in the media over 4 h of culture. There was no significant difference between the physiological doses (0.2, 2 nmol/l) of 17β-oestradiol but both these doses were significantly different (P < 0.05) from the supraphysiological dose (200 nmol/l). No significant difference was found between cultures treated with ethanol-dissolved and cyclodextrin-encapsulated 17β-oestradiol. Vehicle-treated cultures showed no significant difference in ET-1 release from untreated controls.

Immunoblotting of control and 17β-oestradiol-treated cultures revealed a dose-dependent increase in eNOS expression after 24 h (Fig. 1). In separate experiments (n = 3) a dose of 0.2 nmol/l 17β-oestradiol elicited a doubling of basal eNOS levels yielding 216 ± 6% (P < 0.001) increase in expression over the same time period. No alterations in eNOS levels were observed in samples harvested at 4 h at any concentration of 17β-oestradiol.

Under serum-free conditions addition of thrombin (2 U/ml) increased ET-1 release from these cells by 42.8 ± 6% (P < 0.01) (n = 4). Addition of thrombin in the presence of 17β-oestradiol (0.02 nmol/l) showed a 6.5 ± 2.3% (not significant) increase in ET-1 immunoreactivity that was significantly different from thrombin alone (P < 0.001). A highly supraphysiological dose of 17β-oestradiol was tested to confirm the observation that increasing the dose of oestrogen decreased the inhibitory effect. 17β-Oestradiol (200 µmol/l) showed a 36.8 ± 5.1% (P < 0.01) increase in ET-1 immunoreactivity in the presence of thrombin that was not significantly different from the effect of thrombin alone.

Discussion

The reduction in risk of CHD observed in postmenopausal women receiving oestrogen is believed to occur largely through direct actions on the vessel wall. This study demonstrates for the first time a marked inhibitory effect of the naturally occurring oestrogen, 17β-oestradiol, on the release of the potential

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**Table 1** Effect of 17β-oestradiol on ET-1 accumulation. HUVECs were incubated for 4 h in M199/10% (v/v) FCS in the presence or absence of the indicated concentrations of 17β-oestradiol. The results are expressed as a percentage of control values ± S.E.M. where n is the number of replicate determinations.

<table>
<thead>
<tr>
<th>17β-Oestradiol (nmol/l)</th>
<th>n</th>
<th>ET-1 (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>100 ± 3</td>
<td>—</td>
</tr>
<tr>
<td>0.2</td>
<td>12</td>
<td>59 ± 9</td>
<td>0.001</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>59 ± 9</td>
<td>0.001</td>
</tr>
<tr>
<td>200</td>
<td>12</td>
<td>77 ± 11</td>
<td>0.05</td>
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by solubilisation in boiling SDS-PAGE sample buffer. ET-1 in supernatants was measured using EIA (Amersham Life Sciences, Amersham, Bucks, UK). Intra- and interassay coefficients of variation were 3.6% (3.2 × 10^-10 mol/l; n = 6) and 16.5% (1.6 × 10^-10 mol/l; n = 6) respectively. Endothelial nitric oxide synthase (eNOS) was measured following separation of cell lysates by SDS-PAGE and enhanced chemiluminescent immunoblotting (ECL, Hyperfilm, Amersham, Bucks, UK) using a mouse monoclonal antibody raised against the human protein (Affiniti Research Products, Cambridge, UK). Blots were stripped and reprobed using an antibody (DAKO, High Wycombe, Bucks, UK) to an endothelial marker protein, von Willebrand factor (vWF). Comparisons of significance were estimated using Student’s unpaired t-test. The results are expressed as the mean ± standard error from the mean.
vasoconstrictor, ET-1, from human vascular endothelial cells. This corroborates recent in vivo results that showed a significant decrease in circulating ET-1 levels in women receiving HRT (6). Using two physiological doses and a supraphysiological dose we showed that 17β-oestradiol significantly reduces stimulated ET-1 release from these cells. The maximum effect was observed at the lower menstrual cycle doses of 17β-oestradiol, whilst at supraphysiological doses the effect was diminished. The interpretation of the data as resulting from a biphasic inhibition of stimulated ET-1 release was strengthened by studies conducted under serum-free conditions. Thrombin caused a significant increase in ET-1 accumulation. A low physiological dose of 17β-oestradiol completely reversed this effect. Furthermore, a supraphysiological dose which failed to affect thrombin-stimulated ET-1 release confirmed the inverse relationship between the dose of oestrogen and the inhibition observed.

Nitric oxide (NO) is a feedback inhibitor of ET-1 release (10) and increases in the release of NO would be expected to decrease ET-1 accumulation in the media of treated cells. Our results confirmed that oestrogen is associated with increased expression of NO synthase; however this effect was not concomitant with the observed inhibition of ET-1 release. It is our assumption that the pathway by which NO inhibits ET-1 release is maximally stimulated under basal conditions, and it is unlikely that increases in NO output from these cells are the causative means by which oestrogen inhibits ET-1 release.

Thrombin and serum stimulate ET-1 release through increasing the activity of phospholipase C (PLC). This, in turn, modulates intracellular calcium and protein kinase (PK) C which enhances expression of prepro-ET-1 mRNA (11). In other studies, cyclic nucleotides have been postulated to affect calcium transients and inhibit PLC-stimulated, but not basal, ET-1 production (12). This may be of relevance to the observations in this study. Intracellular calcium demonstrates a bell-shaped curve with respect to ET-1 release with very low or very high concentrations being inhibitory (13). Oestrogen has been reported to act as a calcium channel blocker in vascular smooth muscle cells (14) and recent studies have shown that oestrogen is able to increase transiently intracellular calcium in endothelial cells (15). Additionally, 17β-oestradiol has been found to act directly upon calcium-calmodulin (16). The mechanisms for these effects are unknown; however they raise the possibility that either a long-term blockade of calcium channels or a short-term increase in intracellular calcium may inhibit stimulated ET-1 secretion. This hypothesis is supported by studies that show that 17β-oestradiol can affect PLC, either potentiating or attenuating stimulated PLC activity in a tissue-dependent manner (17, 18). Further studies will be required to elucidate whether the effect of 17β-oestradiol on ET-1 release is effected through modulation of intracellular calcium and the activity of PLC.

Physiological concentrations of 17β-oestradiol are associated with a decreased rate of serum-stimulated ET-1 release in the short term. This may explain, in part, the beneficial effects that oestrogens can exert on the vascular system such as the immediate improvements in myocardial ischaemia seen in postmenopausal women with established CHD (19).

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