In vitro effect of oestradiol on thymidine uptake in pulmonary vascular smooth muscle cell: role of the endothelium

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1 The effect of different concentrations of oestradiol-17 β (3–300 nM) on [³H]-thymidine uptake was studied in segments from canine pulmonary artery, and cultures of rat pulmonary vascular smooth muscle cells (VSMC).

2 Incubation with oestradiol-17 β for 24 h, potentiated in a concentration-dependent manner [³H]-thymidine uptake in VSMC cultures.

3 Oestradiol-17 β potentiated thymidine uptake by pulmonary arterial segments but only when the endothelium had been removed. Autoradiography showed dense incorporation of radioactive thymidine in the vascular smooth muscle cells of the media.

4 The non-steroidal oestrogen, stilboestrol (300 nM), also significantly potentiated [³H]-thymidine uptake, in both VSMC cultures and pulmonary artery segments. Testosterone was ineffective at a similar concentration.

5 Pre-incubation of the pulmonary VSMC with the anti-oestrogen tamoxifen (1 μ M) antagonized the potentiating effect of oestradiol-17 β on [³H]-thymidine incorporation. The effect of tamoxifen was less pronounced in pulmonary arterial segments.

6 These data suggest that oestrogen may promote proliferation of pulmonary VSMC. Endothelial injury or dysfunction may be an important factor in the expression of the oestrogenic effect.

7 We speculate that plasma oestrogen may be a contributing factor to the proliferative lesion observed in certain forms of pulmonary vascular injury in women.

Keywords: Oestrogen; endothelium, pulmonary; vascular smooth muscle; [³H]-thymidine uptake, tamoxifen

Introduction

Primary pulmonary hypertension is reported to be more common in young sexually mature women (Wagenvoort & Wagenvoort, 1970; Rich *et al.*, 1987) than in their male counterparts, and may be exacerbated in conditions associated with high oestrogen levels (Savajiyani *et al.*, 1981). An early morphological study showed that lungs from female rats show a higher incidence of muscularized arteries than those from males, in response to hypoxic challenge (Smith *et al.*, 1974). This is of interest since the pathological lesion in pulmonary hypertension is characterized by medial hypertrophy and myointimal thickening as a result of migration and proliferation of pulmonary vascular smooth muscle cells (Heath *et al.*, 1987). Oestrogen is known to promote growth of many cell lines, including breast cancer cells (Lippman *et al.*, 1976; Chabos *et al.*, 1982), mouse Leydig cells (Nishizawa *et al.*, 1988) and rat hepatocytes (Francavilla *et al.*, 1989). However, there is no direct evidence to indicate that oestrogen stimulates vascular smooth muscle cell growth. Clinically, oestrogen replacement therapy is associated with decreased incidence of coronary heart disease (Stampfer *et al.*, 1991). Oestradiol-17 β also protects against the development of experimental atherosclerosis (Renaud, 1970; Foegh *et al.*, 1987; Clarkson *et al.*, 1990). The effect of oestrogen on pulmonary vascular smooth muscle cell (VSMC) growth and proliferation has not been investigated.

In this study we describe the *in vitro* effect of oestradiol-17 β on pulmonary vascular smooth muscle cells using intrapulmonary arterial segments and VSMC cultures and [³H]-thymidine uptake as an index of cell proliferation (Fisher-Dzoga *et al.*, 1983). The specificity of the oestrogenic response was assessed by use of the synthetic oestrogen, stilboestrol, as well as testosterone, and the oestrogen-receptor antagonist, tamoxifen.

Methods

Pulmonary vascular smooth muscle cell cultures

Rat pulmonary VSMC were obtained from Dr C. Diglio (Detroit, MI, U.S.A.). About 10⁴ cells, between the 10th and 17th passages, were cultured in 6-well Falcon tissue culture plates (Becton Dickinson, NJ, U.S.A.), and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), gentamycin (25 μ g ml⁻¹) and 10% foetal bovine serum (FBS). Cells were incubated in a humidified atmosphere at 37°C in the presence of 5% CO₂. At confluence, cell growth was arrested by withdrawing the serum from the culture medium. All experiments were performed in synchronized cells.

The effect of oestrogen on [³H]-thymidine uptake was studied in growth-arrested cells, incubated overnight in DMEM containing 10% FBS, and different concentrations (0, 3, 30 and 300 nM) of oestradiol-17 β , and [³H]-thymidine (2 μ Ci ml⁻¹). The same experiment was repeated in the presence of 300 nM concentrations of testosterone and the synthetic oestrogen stilboestrol. All hormones were dissolved in ethanol and were added to the medium at a final alcohol concentration of 1%.

We also determined the effect of the oestrogen receptor antagonist, tamoxifen, on oestradiol-induced cell growth. VSMC were incubated with tamoxifen (1 μ M) for 3 h before treatment with oestradiol. After 3 h, the incubation medium was changed overnight to one containing oestradiol-17 β (300 nM) and [³H]-thymidine.

[³H]-thymidine uptake determination

The uptake of radiolabelled thymidine was used as an index of proliferation. Following overnight incubation with [³H]-thymidine, VSMC were incubated for 5 min in phosphate

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buffer saline (4°C), containing unlabelled thymidine and then digested overnight in NaOH (0.5 N). Aliquots were then used for determination of both radioactive count (Beckman Scintillation System, model LS 3150T) and protein content by the method of Lowry *et al.* (1951). Results are expressed as counts min⁻¹ mg⁻¹ protein.

Pulmonary artery segments

Lungs were obtained from anaesthetized male mongrel dogs (17–25 kg) and preserved in ice cold oxygenated Krebs-Heinsleit buffer solution. Intrapulmonary arteries were isolated, cleaned of connective tissue and cut into 4–5 mm segments. In some segments, the endothelium was removed by gentle rubbing of the intima (Furchgott & Zawadzki, 1980). Segments were incubated overnight in DMEM, and 24 h later, transferred to fresh medium with 10% FBS, to which oestradiol-17 β (0, 3, 30, 300 nM) and [³H]-thymidine (2 μ Ci ml⁻¹) were added. As in cell culture studies, the specificity of the oestrogenic response was also tested with testosterone (300 nM), stilboestrol (300 nM), and tamoxifen (1 μ M).

Autoradiographic studies

Autoradiographic studies were performed in order to localize the site of uptake of radioactive thymidine by pulmonary arterial segments. Following incubation with [³H]-thymidine, some pulmonary artery segments were washed with PBS and fixed in 10% formalin in phosphate buffer for 3 days. Tissues were then dehydrated, embedded, mounted and stained with Mayer's haematoxylin. Stained sections were then rehydrated overnight and coated with photographic emulsion NTB₂. After 2 weeks of exposure, the sections were developed with Dektol for 45 s and fixed with GBX emulsion for 5 min. The site of [³H]-thymidine incorporation was determined by light microscopy.

Statistical analysis

Statistical analysis was performed by unpaired Student's *t* test, and statistical significance was achieved *P* < 0.05. All data are expressed as means $\pm$ s.e.

Materials

Oestradiol-17 β , testosterone, stilboestrol and tamoxifen were purchased from Sigma; Dektol, GBX and NTB₂ emulsions from Eastman Kodak; Dulbecco's modified essential medium (DMEM), foetal bovine serum (FBS), phosphate buffer saline (PBS) and trypsin from Biofluids.

Results

Effect of oestradiol 17 β on [³H]-thymidine uptake

Pulmonary VSMC Incubation with 10% foetal bovine serum and oestradiol-17 β (3–100 nM) increased in a concentration-dependent manner [³H]-thymidine uptake in cultured pulmonary vascular smooth muscle cells. The potentiating effect was significant (*P* < 0.01) at all concentrations, and reached 100 $\pm$ 10% at 300 nM oestradiol (Figure 1b).

Pulmonary arterial segments

Oestradiol 17 β also significantly (*P* < 0.01) increased the uptake of [³H]-thymidine in segments of canine intrapulmonary artery, in which the endothelium was removed. Oestradiol was ineffective when the endothelium remained intact (Figure 2). The increase in [³H]-thymidine uptake in arterial segments without endothelium was concentration-dependent

Figure 1 Effect of oestradiol 17- β on [³H]-thymidine uptake in: (a) segments of canine intrapulmonary artery (rings) and (b) cultures of rat pulmonary vascular smooth muscle cells (Cells). Both cells and rings were incubated overnight in DMEM supplemented with 10% FBS, in presence of [³H]-thymidine (2 μ Ci) and 3 nM (solid columns), 30 nM (hatched columns) or 300 nM (stippled columns) oestradiol-17 β . Results are expressed as percentage potentiation of [³H]-thymidine uptake as compared to control uptake in presence of vehicle (ethanol). Experiments were triplicated with *n* = 4–6.

Figure 2 Effect of the endothelium on oestradiol-17 β -induced uptake of [³H]-thymidine in canine intrapulmonary artery. Intact (hatched columns) and arterial segments without endothelium (solid column) were incubated overnight in DMEM + 10% FBS, in presence of [³H]-thymidine (2 μ Ci) and 300 nM oestradiol-17 β or vehicle (ethanol). Results are expressed as percentage potentiation of [³H]-thymidine uptake as compared to control uptake in presence of ethanol. Experiments were triplicated with *n* = 4–6.

and ranged from 11 $\pm$ 2 to 47 $\pm$ 11% between 3 and 300 nM (Figure 1a).

The site of radioactive thymidine incorporation in the vessel segments was determined by autoradiography. The results show dense labelling of the nuclei in the media suggesting heavy uptake of [³H]-thymidine by vascular smooth muscle cells (Figure 3).

Effect of stilboestrol and testosterone

Incubation of pulmonary VSMC with 300 nM of the non-steroid estrogen, stilboestrol, increased [³H]-thymidine incorporation by 70 $\pm$ 5% (Figure 4b). Similar results were observed in pulmonary arterial segments, where the same concentration of stilboestrol increased [³H]-thymidine uptake by 30 $\pm$ 2% (Figure 4a). Testosterone, at the same concentration, pro-

Figure 3 Autoradiograph of segment of canine intrapulmonary artery showing uptake of [^3H]-thymidine by nuclei of vascular smooth muscle cells. Arterial segments without endothelium were incubated overnight in DMEM + 10% FBS in presence of [^3H]-thymidine and 300 nM oestradiol-17 β . The segments were then washed with PBS, fixed in 10% formalin, and processed for autoradiography. The autoradiograph shows dense granulation of nuclei in the media of the vascular wall, suggesting heavy uptake of [^3H]-thymidine by vascular smooth muscle cells.

duced only a weak and not significant effect in both preparations (Figure 4).

Effect of tamoxifen

Incubation of cell cultures with the anti-oestrogen, tamoxifen (1 μM), a partial agonist, caused a moderate (15%) but significant increase in [^3H]-thymidine uptake. However, tamoxifen significantly ($P < 0.01$) antagonized the potentiating effect of oestradiol in this preparation (Figure 5b). A similar qualitative effect of oestradiol was observed in the pulmonary arterial segments, but with values not reaching the level of significance (Figure 5a).

Discussion

Our data show that oestradiol-17 β potentiates [^3H]-thymidine uptake in cultured pulmonary vascular smooth muscle cells, and in canine isolated pulmonary arterial segments, in which the endothelium has been compromised or removed. In both preparations, potentiation of [^3H]-thymidine uptake is also observed with the non-steroidal oestrogen stilboestrol, but not with testosterone. Furthermore the effect of oestradiol is antagonized by the anti-oestrogen, tamoxifen. This drug is a partial agonist (Jordan, 1984), which induces a moderate increase in [^3H]-thymidine uptake. Thus this effect of oestradiol appears to be associated with oestrogenic activity and

Figure 4 Comparison of the effect of testosterone (stippled columns), stilboestrol (hatched columns) and oestradiol-17 β (solid columns) on [^3H]-thymidine uptake in (a) segments of canine intrapulmonary artery (rings) and (b) pulmonary vascular smooth muscle cell culture (cells). Cells and rings were incubated overnight in DMEM + 10% FBS in presence of [^3H]-thymidine (2 μCi) and ethanol or 300 nM concentrations of testosterone, stilboestrol or oestradiol. Results are expressed as percentage potentiation of [^3H]-thymidine uptake as compared to control uptake in presence of vehicle. Experiments were triplicated with $n = 4-6$.

Figure 5 Effect of tamoxifen on basal (stippled columns) and oestradiol-induced (hatched columns) uptake of [^3H]-thymidine, compared to the effect of oestradiol-17 β alone (solid columns) (a) segments of canine intrapulmonary artery (rings) and (b) cultures of rat pulmonary vascular smooth muscle cells (Cells). Rings and Cells were incubated with tamoxifen (1 μM) for 3 h before addition of ethanol or 300 nM oestradiol-17 β to the medium. Results are expressed as percentage potentiation of [^3H]-thymidine uptake as compared to control uptake in presence of ethanol. Experiments were triplicated with $n = 4-6$.

may be mediated by specific oestrogen binding sites in pulmonary vascular smooth muscle cells. Ligand-binding studies have demonstrated the presence of oestrogen binding sites in vascular smooth muscle cells of different animal species (Harder & Coulson, 1979; Lin *et al.*, 1982).

Fisher-Dzoga *et al.* (1983) have studied the effect of oestradiol on [^3H]-thymidine incorporation in rabbit aortic medial tissue cultures. They found that oestradiol (20 ng ml $^{-1}$) does not affect growth of cells incubated in normal rabbit serum, but inhibits hyperlipaemic serum-induced cell proliferation. Our present data show that in cultured pulmonary vascular smooth muscle cells incubated in presence of normal foetal bovine serum, similar concentrations of oestradiol-17 β elicited a significant increase in [^3H]-thymidine uptake. Our results are in agreement with the observed effects of oestrogen in other cell lines. Francavilla *et al.* (1989) have shown

that oestrogen, in the presence of 5% normal rat serum, stimulated rat hepatocyte DNA synthesis as determined by [³H]-thymidine incorporation and the labelling index. Similarly, oestrogen-responsive mouse Leydig cell tumour shows an oestrogen-dependent enhancement of cell proliferation in medium supplemented with foetal bovine serum (Nishizawa *et al.*, 1988).

Oestrogen may promote cell proliferation by directly altering gene expression (Davidson & Lippman, 1989). Injection of ovariectomized rats with oestradiol-17 β induces a rapid and transient increase in c-fos gene transcription in the luminal and glandular cells of the endometrium (Papa *et al.*, 1991). The c-fos oncogene plays a key role in the regulation of normal cell growth, and its transcriptional activation occurs in a number of cell types during the early phases of the proliferative response to hormones (Cohen & Curran, 1989). Oestrogen may also have an autocrine role in promoting the synthesis and/or release of peptide growth factors by growing cells, and their subsequent expression. Oestradiol regulates the synthesis and mRNA coding of mitogenic proteins in MCF-7 and T47D human breast tumour cell line (Adams *et al.*, 1980; Chalbos *et al.*, 1982; Vignon *et al.*, 1986), and endometrial carcinoma biopsies (McGuire *et al.*, 1986). Non-stimulated transformed mouse B1 Leydig cells, grown in serum-free medium also secrete a growth-promoting substance, the levels of which can be enhanced by oestrogen (Nishizawa *et al.*, 1988). Moreover, growth stimulation of many breast carcinomas by oestradiol-17 β has been linked to induction of EGF-related polypeptide growth factors (Dickson *et al.*, 1986). Oestrogen may also indirectly promote cell proliferation by inactivating the action of growth-inhibiting factors present in the serum (Soto & Sonnenschein, 1985). Whether any of these mechanisms is involved in the mitogenic effect of oestradiol in pulmonary vascular smooth muscle, remains to be explored.

Epidemiological and clinical studies have supported a protective role of oestrogen against coronary heart disease (Stampfer *et al.*, 1991). Oestradiol also inhibits experimentally induced atherosclerosis in rat and rabbit (Moskowitz *et al.*, 1956; Renaud *et al.*, 1970), as well as myointimal proliferation in aorta and cardiac allograft associated with abdominal aorta transection and surgical re-anastomosis in the rabbit (Foegh *et al.*, 1987; Cheng *et al.*, 1991). Studies in the pulmonary circulation however, suggest a facilitating role of oestrogen on the development of pulmonary vascular wall

hypertrophy associated with pulmonary hypertension. Smith *et al.* (1974) has shown that lungs from female rats exposed to chronic hypoxia develop a higher number of muscularized arteries than those from males. Moreover, clinical observations have reported a higher incidence of primary pulmonary hypertension in young sexually mature females (Wagenvoort & Wagenvoort, 1970), which may be exacerbated by pregnancy and the use of oral contraceptives (Savajiyani *et al.*, 1981). The difference in the oestrogenic response between pulmonary and systemic vessels may suggest the presence of two pathways, one for stimulation and one for inhibition, induced by oestrogen-mediated events (Sato *et al.*, 1986). These observations may also relate to differences in the endocrine, paracrine and metabolic properties of the various vascular beds.

The present data also show that oestrogen potentiates [³H]-thymidine uptake by segments of dog pulmonary artery in which the endothelium has been removed. Autoradiographic studies indicate that the incorporation of radio-labelled thymidine occurs in the medial layer of the vascular segments which consists mainly of smooth muscle cells. The effect of oestradiol on VSMC proliferation in arterial segments however, is absent when the endothelium is intact. The vascular endothelium releases a number of anti-proliferative mediators, such as endothelium-derived relaxing factor (Garg & Hassid, 1989) and prostacyclin (Zidek *et al.*, 1987) which may compete with the mitogenic effect of oestrogen on VSMC. In addition, disruption of the endothelial barrier may facilitate exposure of the basal membrane and underlying smooth muscle layer to platelets, leukocytes and circulating mitogens (Roth & Reindel, 1980). Thus an intact endothelium may protect against oestradiol-induced VSMC proliferation in the pulmonary vascular wall.

In conclusion, myointimal hyperplasia is a component of the pathological lesion observed in chronic pulmonary hypertension. Our data suggests that oestradiol-17 β enhances the proliferation of pulmonary vascular smooth muscle cells. However, in pulmonary artery segments, oestrogen-induced thymidine uptake was only observed when the endothelium was damaged. We speculate that oestrogen may promote VSMC proliferation in pulmonary vessels in the presence of endothelial injury or dysfunction.

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