BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **221,** 8–14 (1996)
ARTICLE NO. 0535

17β-Estradiol and Smooth Muscle Cell Proliferation in Aortic Cells of Male and Female Rats

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Received February 16, 1996

The low incidence of cardiovascular disease in women before menopause or during hormone replacement therapy suggests a protective effect of estrogens. The mechanism(s) are uncertain but may involve effects on lipids, coagulation and the endothelium. Vascular smooth muscle cell (VSMC) proliferation also contributes to atherosclerosis. Hence, we investigated whether 17β-estradiol (E_2) inhibits VSMC proliferation. VSMC of 6 female and 6 male Wistar Kyoto rats (WKY; age 10–12 weeks) were incubated for 24 h with E_2 and/or fetal calf serum (FCS). E_2 (10^{-9}–10^{-5} M) alone reduced [^{3}H]thymidine uptake at 10^{-5} M ($n = 8$, $p<0.05$ vs. control) in female cells only. In female and male VSMC, FCS (1%) increased [^{3}H]thymidine uptake (4.5-fold, $p<0.05$ vs. control). When given simultaneously, E_2 did not prevent this effect of FCS (1%). However, when cells were preincubated for 24 h with E_2 and then stimulated with FCS, [^{3}H]thymidine uptake was reduced by E_2 at 10^{-5} M in female VSMC ($n = 7$, $p<0.05$ vs FCS alone), while in male VSMC this effect was minimal (n.s.): Both female and male VSMC expressed estrogen receptors as demonstrated by RT–PCR. Pretreatment of female VSMC cells with the E_2 receptor antagonist tamoxifen prevented the antiproliferative effects exerted by E_2. In aortic VSMC of female rats, E_2 moderately inhibited proliferation on its own and during stimulation with FCS, while this effect was small in VSM of male rats. The expression of the E_2 receptor in female and male VSMC and the effects of tamoxifen suggest that this effect is mediated by E_2 receptors.

Sex hormones have been recognized to be involved in a wide range of diseases. In the cardiovascular system, the role of estrogen is protective as demonstrated by epidemiological, anatomical and clinical studies (1). Before menopause or during hormone replacement therapy women have a lower incidence of coronary artery disease as well as other cardiovascular events compared to age-matched males (2–5). The beneficial effects of estrogen might be due to changes in classical cardiovascular risk factors such as lipids, blood pressure, blood glucose and/or insulin (6,7). The presence of estrogen receptors within the arterial wall and in particular in smooth muscle cells of animals and humans (8–12) supports the hypothesis that female sex hormones might directly interfere with the blood vessel wall. Indeed, estrogen enhances acetylcholine-induced vasodilation in animal and human coronary arteries via an endothelium-dependent and endothelium-independent mechanism (13,14). In addition, estrogen attenuates abnormal coronary vasomotion in patients with angina pectoris as well as in atherosclerotic animals (13,15–17). Furthermore, in animal models of transplantation vasculopathy, estrogen treatment reduces structural changes (18,19). Finally, estrogen reduces neointima formation after balloon injury (20).

Hence, it is likely that estrogens directly interfere with the function of vascular smooth muscle cells and in turn exert an anti-atherosclerotic effect. However, the effect of estrogens on proliferation of vascular smooth muscle cells (SMC) is still controversial. Some reports have suggested an inhibitory role (20,21), while others found no effect or actually proliferative effects of the hormone (22,23). As VSMC proliferation is a key event in the pathogenesis of atherosclerosis, restenosis and bypass graft disease (24–26) such an effect of estrogens may be clinically important.

The aim of the present study is to evaluate the anti-proliferative effects of 17β-estradiol on cultured aortic SMC of male and female Wistar Kyoto rats (WKY).

0006-291X/96 $18.00

MATERIAL and METHODS

Supplies

Wistar Kyoto rats were from Charles River, Wiga, Germany; RPMI medium, HEPES solution, L-Glutamine, penicillin, streptomycin, trypsin-EDTA, fetal calf serum as well as TRIzol reagent were purchased from GIBCO Life Technologies (Basel, Switzerland); bovine serum albumin, 17β-estradiol (E_2), tamoxifen, monoclonal smooth muscle α-actin antibodies and all the chemicals used were from Sigma (Buchs Switzerland). [^{3}H]thymidine was obtained from Amersham (Zürich Switzerland).

Experimental Animals

Six female and 6 male Wistar Kyoto rats (WKY; 10–12 weeks of age) were used. Weight averaged 210 ± 8 grm. for females and 248 ± 14 grm. for males (n.s.). 17β-estradiol plasma levels were 0.13 ± 0.04 nM in females and 0.06 ± 0.01 nM in males ($p < 0.001$).

Cell Culture

Rat aortic smooth muscle cells (SMC) were isolated using explant technique and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 mM HEPES solution, 100 U/L penicillin, 100 mg/L streptomycin and 20 mM L-glutamine (27). Subconfluent cells were harvested and passaged by trypsinization in T-150 flasks or 24-well plates. The purity of SMC was estimated to be >90% by cell morphology and by positive immunostaining to monoclonal smooth muscle α-actin antibodies (1:1000) at the third passage. The growth medium was changed every third day until confluence.

DNA Synthesis

DNA Synthesis in SMC was assessed in each experiment by measurement of [^{3}H]thymidine incorporation into the cells. During the last 4 hours of each experiment the cells were pulsed with 0.5 μCi/well of [^{3}H]thymidine. Then DNA synthesis was measured by the radioactivity incorporated into the trichloroacetic acid-insoluble fraction of the cells counted by a β-counter.

RNA Preparation

Total cellular RNA was extracted from the same SMC used for the experiments above. The cells were seeded at a density of 10^5/ml in petri dishes (7 ml per plate). Subconfluent cells were treated in the same way as in the above experiments and finally lysed with TRIzol reagent according to manufacturer's instructions.

Reverse Transcription–Polymerase Chain Reaction (RT–PCR) Analysis

Total cellular RNA was isolated from SMC and MCF-7 cells (as positive control) as described above, and first-strand cDNA was then synthesized by adding 200 U Superscript-reverse transcriptase in a 20 μl reaction buffer, and incubated for 1 h 42°C. Control reactions (to exclude genomic DNA contamination) were performed under the same conditions using an aliquot of total cellular RNA omitting Superscript reverse transcriptase.

To study estrogen receptor expression, PCR was performed using first strand cDNA as template, and the following synthetic oligonucleotide primer sets: forward primer: 5′-AATTGGATCCGCAAGTCTTGGGAGCGTGAT-3′, and reverse primer 5′-CATGGAATTCATGCAAAGGGGTCTGTGTCT-3′, which correspond to nucleotide 5672-5691, and 6062-6081 of the published human estrogen receptor (28), respectively. RT-PCR using forward primer 5′-CAGGAATTCGGTGAAGGTCGGGAGTCAACGG-3′, and reverse primer 5′-AGTGGATCCGGTCATGAGTCCTTC-CACGAT-3′ of the housekeeping gene GAPDH were used as a control. PCR reactions were performed in a Biometra-Trioblock thermo-cycler for 40 cycles at an annealing temperature of 60°C for 30 seconds, denaturation at 94°C for 30 seconds, and primer extention at 72°C for 1 min. PCR products were then separated by 1% agarose gel electrophoresis, and visualized and photographed using the Visionary gel documentation system (Fotodyne).

Protocols

For the following experiments, culturedaortic smooth muscle cells of each gender were used. The cells were seeded at a density of 5×10^4/well in 24-well plates. Sub-confluent (70–80% of confluence) SMC were growth arrested for 48 hours in serumfree medium. Because of the partial estrogen-like agonist action of phenol red (29), phenol red free RPMI medium was used. Cells of passage 3 to 6 were used. Preliminary experiments were performed to evaluate if there was a difference in responsiveness to proliferative stimuli between male and female SMC. FCS was chosen as an non-specific but potent growth promoter and the SMC were tested with increasing concentrations of FCS from 0.5% to 10%.

Protocol 1: Direct effect of 17β-estradiol (E_2) on FCS-induced proliferation of SMC. To establish if E_2 could have a direct effect on SMC proliferation, the cells were treated either with increasing concentrations of E_2 ($10^{-9} - 10^{-5}$ M) alone,

or with 1% of FCS alone, or with the combination of both E_2 and 1% FCS for 24 h at 37°C, pulsed with [^{3}H]thymidine for 4 hours, washed, and incorporated [^{3}H]thymidine was measured.

Protocol 2: Prolonged 17β-estradiol (E_2) pretreatment and FCS-induced proliferation of SMC. To test if prolonged preincubation of SMC with E_2 would be required to observe an antiproliferative effect, cells were first treated with 17β-estradiol (10^{-9}–10^{-5} M) for 24 hours, then stimulated with 1% FCS for another 24 hours in the presence of the same concentration of E_2. Thymidine incorporation was measured to assess SMC proliferation.

Protocol 3: Effect of tamoxifen on E_2-pretreated SMCs. To examine whether E_2 could mediate its action through its receptor, SMCs were preincubated with increasing concentrations of tamoxifen (10^{-11}–10^{-6} M, suspended in 0.1% ethanol) for 4 hours before the pretreatment with E_2 for 24 hours as described in protocol 2, cells were then stimulated with 1% FCS for another 24 hours in the presence of E_2, and finally thymidine incorporation was measured.

Data Analysis and Statistics

All experiments were performed in triplicate and all values are expressed as mean±SEM; n refers to the number of the experiments done. ANOVA followed by Bonferroni's test was used to evaluate all the concentration-response curves and Student's paired t test was applied when a single effect was tested. A two-tailed value of $P < 0.05$ was considered statistically significant.

RESULTS

Fetal Calf Serum-Induced Proliferation in Male and Female SMCs

The effects of increasing concentrations of fetal calf serum (FCS) on [^{3}H]thymidine incorporation was determined in male and female SMCs. FCS concentration-dependently increased [^{3}H]thymidine incorporation in SMCs of both genders ($p < 0.001$ and $p = 0.018$ vs. control for females and males respectively). The absolute increase in [^{3}H]thymidine incorporation, however, was much more pronounced in female SMCs than in male SMCs, although the increase in percent of control did not differ between the two genders. In female SMC [^{3}H]thymidine incorporation increased from 5358 ± 1771 cpm/well to 48175 ± 19021 cpm/well ($p < 0.001$), while in males control values averaged 6240 ± 1581 cpm/well, and increased to 23047 ± 4035 cpm/well after maximal stimulation with FCS (10%; $p < 0.005$; n.s. vs. females).

Direct Effects of 17β-Estradiol on FCS-Induced SMC Proliferation

E_2 (10^{-9}–10^{-5} M) alone did not exert any anti-proliferative effects in unstimulated male SMC. In contrast, in female SMC, [^{3}H]thymidine uptake was significantly reduced from the control value of 3465±537 cpm/well to 2217±352 cpm/well in the presence of 10^{-5} M of E_2 ($p<0.05$).

FCS (1%) alone significantly increased SMC proliferation as compared to control values both in male and female SMC ($p<0.05$). In the presence of 1% FCS [^{3}H]thymidine uptake averaged 13611±2728 cpm/well in male and 16512±2385 cpm/well in female SMC ($p<0.05$).

In parallel experiments, male and female SMC were treated with 1% FCS and increasing concentrations of E_2 (10^{-9}–10^{-5} M) were given at the same time. Using this protocol (protocol 1), FCS-induced proliferation could not be inhibited by E_2 either in male and female SMC (data not shown).

Effect of 17β-Estradiol Preincubation on FCS-Induced SMC Proliferation

As in the previous experiments, 1% FCS induced an increase in [^{3}H]thymidine uptake (**Fig. 1;** $p<0.05$). In female SMC pretreatment with E_2 for 24 hours reduced [^{3}H]thymidine incorporation in a concentration-dependent manner which reached significance at 10^{-5} M E_2 **(Fig. 1 left panel).** This effect was not as pronounced in male SMC and did not reach statistical significance (n.s.). In female cells, the reduction in FCS-induced proliferation was also significant compared to FCS alone, while this did not reach statistical significance in male SMC **(Fig. 1 right panel).**

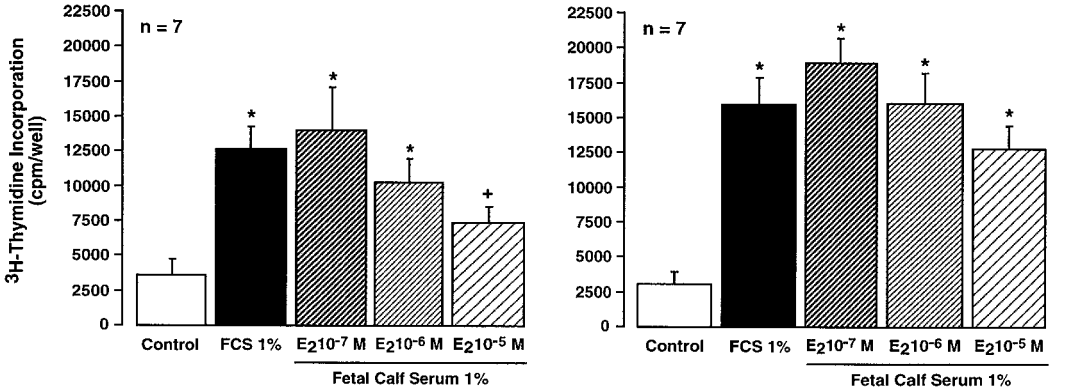

FIG. 1. Effects of 17β-estradiol (E_2) on proliferation of rat aortic smooth muscle cells obtained from female rats (left panel) or male rats (right panel). The cells were preincubated for 48 hours with different concentrations of E_2 and then exposed to fetal calf serum (FCS; 1%). Values are means ± SEM. * = $p<0.05$ vs. control; $^+$ = $p<0.05$ vs. FCS 1%.

Effect of Tamoxifen on 17β-Estradiol-Pretreated SMC

To examine if E_2 mediated its effect through estrogen receptor, we tested the effect of tamoxifen, an estrogen receptor antagonist, on female SMC. Tamoxifen (10^{-11}–10^{-6} M) alone did not exert any effect on SMC as compared to control (n = 6; data not shown). When tamoxifen was added 4 hours before E_2 preincubation, tamoxifen antagonized the effects of E_2 in a concentration-dependent fashion; 10^{-10} M tamoxifen prevented the inhibition of FCS induced proliferation in the presence of 10^{-5} M E_2 (1% FCS: 11552 ± 2920 cpm/well and tamoxifen plus E_2 and FCS: 8957 ± 2321 cpm/well; $p < 0.05$ for both vs. E_2 + FCS: 4151 ± 1372 cpm/well).

Estrogen Receptor Expression in Aortic SMC

Using RT-PCR techniques the expression of the classical E_2 receptor mRNA could be demonstrated in both female and male aortic SMC, although at much lower levels than in MCF-7 cells, a human breast carcinoma cell line, which served as positive control. A PCR-product of the expected size of 429 bp was detected in SMCs obtained from both male and female rat aorta **(Fig. 2).**

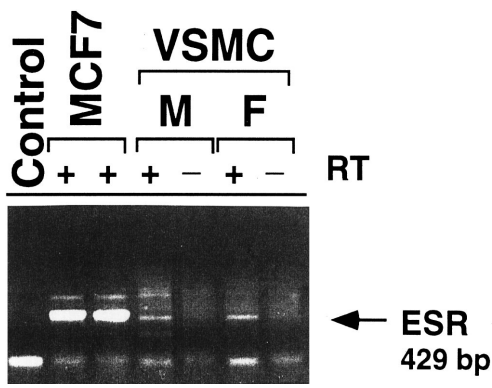

FIG. 2. Reverse transcriptase polymerase chain reaction (RT–PCR) analysis of cultured rat aortic vascular smooth muscle cells (VSMC) obtained from female (F) and male (M) rats. A PCR-product of expected size (429 bp) for estrogen receptor (ESR) is detected in both female and male VSMC. MCF7 cells (a human breast carcinoma cell line) served as positive control, and experiments without reverse transcriptase (RT) served negative controls.

DISCUSSION

This study aimed to characterize the antiproliferative effect of 17β-estradiol (E_2) in rat aortic smooth muscle cells in culture obtained from age-matched female and male animals. The results demonstrate that (1) in unstimulated SMC E_2 alone reduced proliferation in female SMC, while this effect was less pronounced in male cells, (2) that pretreatment with E_2 was capable of reducing proliferation induced by fetal calf serum in female SMC, but not in male SMC, (3) pretreatment with the estrogen receptor antagonist tamoxifen prevented the antiproliferative effects of E_2 and (4) RT-PCR demonstrated the presence of estrogen receptor in vascular smooth muscle cells of both female and male rats. Taken together, these results suggest that E_2 exerts a moderate antiproliferative effect in rat vascular smooth muscle cells.

Proliferation of vascular smooth muscle cells is considered to contribute to hypertensive vascular disease, atherosclerosis and restenosis (25,26). Although normally vascular smooth muscle cells of the media are in a contractile phenotype they may change their property in disease states and start to proliferate; the latter property is shared by vascular smooth muscle cells in culture. To avoid any interference with estrogen receptors in our cell culture experiments, we used a phenol red-free medium, as phenol red is a weak estrogen receptor agonist (29). Fetal calf serum, which was used to stimulate proliferation in our cells, also may contain some estrogen. However, when fetal calf serum was added to the culture dishes in our experiments, the final concentration of E_2 in the medium was less than 0.1 pM. Hence, under our experimental conditions we can be quite certain that estrogen-like compounds able to activate estrogen receptors other than the 17β-estradiol (E_2) are very unlikely to confound our results. Hence, any antiproliferative effects of E_2 on vascular smooth muscle cells must be related to the compound added in the concentrations given. As under in vivo conditions a large number of growth factors are likely to be involved in the pathogenesis of vascular disease in hypertension, atherosclerosis and restenosis, we choose fetal calf serum rather than a distinct growth factor to induce proliferation. In a preliminary series of experiments, we have determined the proliferative effects of fetal calf serum in male and female smooth muscle cells. In both cell lines, fetal calf serum induced concentration-dependent increases in [^{3}H]thymidine incorporation. Surprisingly, male rats showed a similar sensitivity to fetal calf serum, but the maximal response was markedly decreased. Obviously this might have altered the effects of estrogens under our experimental conditions. Hence, we choose two concentrations of fetal calf serum to stimulate the cells, i.e. 1% fetal calf serum which corresponds to about a half-maximal concentration in female cells and 0.5% fetal calf serum which corresponds to about a third of the maximal effect in the male cells. Under both conditions of VSMC stimulation in the male, however, the effects of E_2 much less pronounced in male than in female cells.

When E_2 was applied together with fetal calf serum at the same time, the estrogen was unable to affect proliferative responses in either female or male cells. However, with a 24h preincubation, E_2 concentration-dependently and significantly reduced [^{3}H]thymidine uptake. The degree of antiproliferation was modest and reached maximum at relatively high concentrations of the estrogen (i.e. 10^{-5} M). The fact that prolonged preincubation of E_2 was required to achieve the antiproliferative effects strongly suggests that E_2 might bind to the classical estrogen receptor and induce a secondary factor(s) required to inhibit cell replication.

The mechanism of the antiproliferative effects of E_2 in rat SMC involve the classical estrogen receptor. Indeed, female and male SMC do express estrogen receptors as demonstrated by RT-PCR technique. Furthermore, the estrogen receptor antagonist tamoxifen was able to prevent the antiproliferative effects of E_2. The level of estrogen receptor expression, however, was much lower than in MCF-7 breast carcinoma cell lines, and was not detectable in Northern blot analysis (unpublished observation). The latter interpretation is also compatible with the fact that relatively high concentrations of E_2 were required to induce antiproliferative effects in these cells.

In summary, this study demonstrates antiproliferative effects of E_2 in rat aortic smooth muscle

cells. These antiproliferative effect of E_2 in rat smooth muscle cells need to be confirmed in human vascular tissue and may indicate that E_2 indeed might exert protective effects in hypertensive vascular disease, atherosclerosis and possibly restenosis.

ACKNOWLEDGMENTS

The authors thank Dr. Niklaus A. Bersinger, Universitäts-Frauenklinik, Bern, Switzerland for measuring plasma estrogen levels. This work was supported by the Swiss National Research Foundation (Grant 32-32541.91/2). Emma Espinosa was supported by a stipend of the University of Ancona, Italy (1992), and the Ciba-Geigy Foundation (1993).

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