Sex hormones induce death or cell proliferation in various cell lines and in primary cultures. However, the signal transduction pathways involved in the regulation of proliferation and apoptosis in endothelial cells have not been fully elucidated. Here, we report that progesterone and testosterone induce apoptosis in HUVECs in a p38- and JNK-dependent manner, and that estradiol promotes proliferation via the activation of ERK2. We showed that, at physiological doses, progesterone and testosterone promoted p38, but not JNK, phosphorylation. Hormone inhibitors, on the other hand, prevented p38 phosphorylation. When supraphysiological doses were applied, both p38 and JNK were phosphorylated, causing apoptotic cell death. The addition of hormone inhibitors at an appropriate concentration did not prevent cell death or the phosphorylation of p38 and JNK. Estradiol, at physiological doses, promoted an increase in ERK2 phosphorylation that was blocked by fulvestrant. At physiological and supraphysiological doses, it promoted a proliferative effect. In conclusion, these findings suggest that JNK has an important pro-apoptotic function following progesterone and testosterone treatment in human endothelial cells, and that ERK2 has a proliferative effect following estradiol treatment.

Introduction

Apoptosis seems to be the main mechanism of death of the endothelium under physiological conditions. Moreover, the balance between proliferation and apoptosis in endothelial cells plays a critical role in the formation and regression of blood vessels, especially the arterioles and capillaries. Therefore, excessive apoptosis in endothelial cells may be involved in both endothelial dysfunctions and in the inhibition of angiogenesis, as described in various vascular diseases (1-3).

Hormone concentrations are subject to strict regulation to ensure that an unbalance in hormone secretion, resulting in the loss of cellular homeostasis, does not occur. Under certain circumstances, certain hormones act to stimulate cell proliferation (4,5).

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation and cell survival/apoptosis. The extracellular regulated kinase (ERK)1/2 MAPK pathway consists of a protein kinase cascade linking growth and differentiation signals (6). p38 and Jun N-terminal (JNK) are members of the stress-activated MAP kinases, and their activation has been implicated in various forms of apoptosis (7,8). Estradiol, progesterone, and testosterone can act non-genomically via membrane-associated receptors to activate MAPKs, as can various other ligands acting through heterotrimeric G protein receptors (9-11). The purpose of this study was to determine whether the hormones present in normal human plasma have an effect on the MAPK signaling pathway in human umbilical vein endothelial cells (HUVECs), and to establish their role in the balance between proliferation and apoptosis. We therefore investigated the effects of estradiol, progesterone and testosterone on the activation of MAPKs in HUVECs to fully understand the intracellular signaling response to these hormones.

Materials and methods

Sample collection. Blood was collected using siliconized vacutainer tubes containing 1/10 volume 3.8% trisodium citrate 2H2O. The tubes were then centrifuged twice at 1500 x g for 25 min at room temperature. The plasma was separated, placed in pyrogen-free microcentrifuge tubes, immediately frozen and stored at -80°C until use.

Cell culture and treatments. HUVECs were isolated from normal pregnancies with 0.1% collagenase type 1 (12). Cells were cultured in RPMI-1640 medium (Gibco, Scotland)
supplemented with endothelial cell growth factor (Sigma, St.
Louis, MO, USA), L-glutamine, 2-mercaptoethanol, heparin,
penicillin and streptomycin, and 10% fetal bovine serum
(FBS; Gibco), then incubated at 37°C, 5% CO2 in a humidified
incubator. Cells from different umbilical cords were mixed
and used to test hormones. HUVECs were used between
passages three and six.

Cells were cultured in 6-well dishes until 80% confluence
was attained. Before exposure to the HUVECs, the plasma
was heparinized (100 μg/ml) to prevent clotting of the dilute
sodium citrate plasma. It was then added to the cultured
HUVECs at a final concentration of 10% plasma. The results are reported as the means ± SD of 4 separate experiments, with p≤0.05 as determined by ANOVA. Cells were stimulated with (A) E2, E2+fulvestrant (Fu), (B) E2, E2+starvation (SFB), (C) P, P+Fu,
(D) T, T+flutamide (F). (E) The apoptosis of HUVECs stimulated by normal male plasma, normal female plasma, normal female plasma-pregnant, E2, T or P. After stimulation, cells were harvested and the percentage of apoptotic cells was measured by flow cytometric analysis. The results are the means ± SD of 3 separate experiments. *p≤0.05 as determined by ANOVA.

Figure 1. 17ß-estradiol (E2) promotes cell survival. Testosterone (T) and progesterone (P) promote cell death and apoptosis in HUVECs at non-physiological doses. To evaluate the growth inhibitory effect of T and P on HUVECs, the MTS assay was used. Data are reported as the means ± SD. The OD of control samples was regarded as 100. Each condition was performed with 4 wells. The results are reported as the means ± SD of 4 separate experiments, with p≤0.05 as determined by ANOVA. Cells were stimulated with (A) E2, E2+fulvestrant (Fu), (B) E2, E2+starvation (SFB), (C) P, P+Fu,
(D) T, T+flutamide (F). (E) The apoptosis of HUVECs stimulated by normal male plasma, normal female plasma, normal female plasma-pregnant, E2, T or P. After stimulation, cells were harvested and the percentage of apoptotic cells was measured by flow cytometric analysis. The results are the means ± SD of 3 separate experiments. *p≤0.05 as determined by ANOVA.

Cell proliferation assay. HUVECs were seeded at a density of
5000 cells/well in a gelatin-coated tissue culture plate. Twelve
hours after seeding, the HUVECs were treated. Cell prolif-
eration was measured using the MTS proliferation assay
(Promega, WI) at 48 h post treatment. The test protocol for
cytotoxicity evaluation was adopted from previously published
studies (13,14). After completion of the exposure period, the
absorbance of the formazan product was read at 492 nm. Two internal controls were set up for each experiment consisting of cells only and medium only. Background absorbance due to non-specific reactions between test compounds and the MTS reagent was deducted from exposed cell values.

**Apoptosis assay by flow cytometry.** Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine in the plasma membrane (15) and is used, as in this study, for the analysis of apoptotic damage in HUVECs. Subconfluent HUVECs were treated for 48 h in a humidified incubator. Treated cells were harvested, washed and labeled with Annexin V-FITC and propidium iodide apoptosis kits (Calbiochem, Darmstadt). The cells were analyzed using the Beckton Dickinson FACScan flow cytometer with a commercial available software program (CellQuest).

**Western blot analysis.** HUVECs were starved of FBS for 6 h and then treated for 1 h at 37°C. Cells were washed once with ice-cold PBS and immediately lysed by the addition of boiling Laemmli sample buffer (16). Proteins were subjected to electrophoresis in 12% SDS-PAGE and transferred to a nitrocellulose membrane. The mouse monoclonal antibodies Phospho Detect anti-ERK1/2 kinase and anti-ß-actin were from Sigma. The rabbit monoclonal antibodies used were the Phospho Detect anti-p38 MAPK (Calbiochem, Darmstadt) and the anti-JNK activated (diphosphorylated JNK) clone JNK-PT48 (Sigma). The secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Sigma). Blots were visualized using ECL Western blotting detection reagents (Sigma) and propidium iodide apoptosis kits (Calbiochem, Darmstadt). The assays were probed with a phospho-specific ERK1/2 antibody and then reprobed for ß-actin. (A) Anti-phospho-MAPK Western blot densitometric analysis. Relative amounts of phospho-ERK1/2 were obtained by normalizing against ß-actin. Data are represented as the fold increase in the phosphorylated form relative to ß-actin levels. The results are the means ± SD of 3 separate experiments. *p ≤ 0.05 as determined by ANOVA. (B) Representative Western blot analysis of cytosolic lysates.

**Statistical analysis.** Differences between the results of experimental treatments were evaluated by ANOVA and post-hoc testing using the Student-Newman-Keul test. p ≤ 0.05 was considered significant.

**Results**

**17ß estradiol promotes cell survival induced by the deprivation of serum and activates the ERK2 pathway.** The results for E2 are shown in Fig. 1A and B, which represents the cell response to increasing concentrations of E2 (0.5-4 nM). Fig. 1A shows that different concentrations of E2 did not promote cell survival, and that neither did the addition of Fu. When cells were starved, E2 treatment resulted in significant differences without FBS (Fig. 1B).

Using different systems, it has been demonstrated that the mitogenic effect of E2 requires the activation of the ERK cascade. To examine this, we tested whether incubating HUVECs with E2 increased ERK1/2 phosphorylation. Fig. 2 shows that there was a significant increase (p ≤ 0.05) in ERK2 phosphorylation after 1 h of stimulation with E2. However, a significant increase in phosphorylated ERK1 was not found (Fig. 2B). Fig. 2B shows that incubation with Fu reversed the E2-induced phosphorylation of ERK2. There was no significant difference between HUVECs treated with NFP, NFP-p, NMP or the control (Fig. 2A, Table I).

**Progesterone and testosterone induce apoptosis in HUVECs and promote p38 and JNK phosphorylation.** The MTS assay showed an inhibition of cell growth after the addition of...
increasing concentrations of P (0.3-8 μM) (Fig. 1C). The use of physiological concentrations of P did not produce a change in cell viability, although exposure to supraphysiological concentrations induced a significant decrease (p<0.05) in cell survival (80±4 to 52±11%). Fig. 1D shows the change in cell viability with increasing T concentrations (0.3-9.6 μM). Incubation with T (75±12 to 28±19% cell survival) produced the same inhibition of cell growth as treatment with P.

Since the MTS test does not discriminate between necrosis or apoptosis, the Annexin V/IP test by flow cytometry was performed. Treatment for 24 and 48 h with P and T at supraphysiological doses and TNF-α as a control resulted in a significant increase (p<0.05) in apoptosis (Fig. 2, Table I). There was a significant increase (p<0.05) in the percentage of apoptosis in cells treated with 2 μM (8±1%) and 4 μM (20±2%) P. Cells treated with 1 μM P did not differ significantly from the controls. When HUVECs were exposed to 0.06 μM T for 24 h, a low number of Annexin V-positive cells were detected. When supraphysiological concentrations were used, the Annexin V-positive cells increased significantly (p<0.05), from 21±2% to 4.8 μM and 34±2% to 9.8 μM (Fig. 1, Table II).
To study the possible action of p38 as an intermediary in apoptotic signaling in HUVECs, we carried out two runs using P and T in physiological and supraphysiological concentrations. We found that regardless of the dose used, P and T produced a significant increase (p<0.05) in p38 phosphorylation (Fig. 3A, B, D and G). Inhibitors for P and T reversed the effect of the increased phosphorylation of p38 when physiological concentrations were used, but neither Fu nor F was able to reverse the effect at supraphysiological concentrations (Fig. 3A, B, D and G). It was noted that, at supraphysiological concentrations, P and T significantly increased (p<0.05) JNK phosphorylation (Fig. 4B and C). Furthermore, it was observed that hormones at physiological concentrations were not able to activate JNK (Fig. 4A and C). Inhibitors of P and T were able to reverse the effects of JNK phosphorylation when hormones were used at physiological, but not supraphysiological, concentrations. All phosphorylation differences were verified by an average of the intensities of pixels (Tables III and IV). There was no significant difference in the phosphorylation of ERK1/2 in HUVECs treated with NMP, T, P or the control. (Fig. 2A, Table II).

Discussion

In recent years, there have been numerous symptoms and side effects associated with an imbalance in estrogen-androgen levels. On the one hand, declining levels of androgens in women leading to a predominance of estrogens has been linked to the emergence of numerous side effects, including an increased incidence of gynaecological tumours. At the other extreme, we identified several situations in which the imbalance favored androgens, which reached supranormal levels (hormone replacement therapy, trans male-to-female sexuality). These high levels of androgens have also been correlated with the occurrence of adverse effects on the female...
Table III. Statistical analysis of the phosphorylation of p38 in HUVECs incubated with NMP, NFP, NFP-p, E2, P and T.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>p-value</th>
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<tr>
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<td>0.90</td>
<td>0.615</td>
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<tr>
<td>NMP</td>
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<td>NFP</td>
<td>5.8</td>
<td>1.86</td>
<td>0.502</td>
</tr>
<tr>
<td>NFP-p</td>
<td>1.0</td>
<td>0.27</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>E2</td>
<td>0.6</td>
<td>0.15</td>
<td>1.000</td>
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<tr>
<td>T</td>
<td>4.7</td>
<td>0.89</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>T+F</td>
<td>2.1</td>
<td>0.55</td>
<td>0.190</td>
</tr>
<tr>
<td>P</td>
<td>5.3</td>
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<td>&lt;0.050</td>
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<td>P+Fu</td>
<td>0.6</td>
<td>0.17</td>
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<tr>
<td>F</td>
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<td>0.89</td>
<td>0.702</td>
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<tr>
<td>Fu</td>
<td>0.6</td>
<td>0.26</td>
<td>1.000</td>
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B. Phosphorylation of p38 at supraphysiological doses.

<table>
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<th></th>
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<td>P</td>
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<td>0.55</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>P+Fu</td>
<td>2.3</td>
<td>0.26</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>F</td>
<td>0.5</td>
<td>0.10</td>
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<tr>
<td>Fu</td>
<td>0.6</td>
<td>0.26</td>
<td>0.351</td>
</tr>
</tbody>
</table>

ANOVA relative to respective control; post-hoc test, Student-Newman-Keul. p≤0.05 was considered significant. C, control; NMP, normal male plasma; NFP, normal female plasma; NFP-p, normal female plasma-pregnant; E2, 17ß-estradiol; T, testosterone; P, progesterone; Fu, fulvestrant; F, flutamide.

Our results indicate that, at physiological concentrations, E2 has a proliferative effect on endothelial cell growth, and that activation of the ERK2 pathway may play a role in estrogen-mediated endothelial cell protection.

The data from this study indicate that progesterone in supraphysiological concentrations promotes apoptosis in HUVECs and activates p38 and JNK. A study in pregnant Sprague-Dawley rats showed an increase in the activity of caspases 2, 8 and 9 that corresponded to the peak concentration of progesterone in the pregnant females, resulting in an increase in the number of apoptotic cells in the ovary (23). Our assumption is in accordance with these results, in terms of the increase in apoptotic cells in the presence of progesterone. We reported for the first time that supraphysiological concentrations of progesterone are capable of increasing the phosphorylation of p38 and JNK. Upon the activation of JNK, c-jun, which is a component of transcription factor AP-1, is phosphorylated and activated, thus regulating a set of genes involved in apoptosis (24). The effects of the phosphorylation of p38 are similar to those of JNK (25), involving transcription factors and proinflammatory cytokines. Therefore, progesterone should induce apoptosis in HUVECs by activating p38 and JNK. We found no literature documenting the activation of p38 and JNK in HUVECs due to the action of progesterone. However, previous work in a breast cancer cell line (T47D-YB) showed that the incubation of these cells with R5020 (an
analog of progestin) and EGF produced an increase in the phosphorylation of ERK1/2, p38 and JNK (26). Although the cells and the conditions used by Lange et al to visualize the activation of JNK and p38 by progesterone are different from those used in this study, their investigation provides additional evidence that the hormone has a direct action on the MAPK pathway.

It is known that testosterone promotes apoptosis in endothelial cell lines, with a reduction in the expression of Bcl-2 and an increase in the number of apoptotic cells (27,28). The same phenomenon was observed in the present study, albeit at higher-than-physiological concentrations of the hormone. Perhaps the difference in the effect of concentrations resides in the choice of primary cultures or cell lines, and in the number of hormone receptors that each has. Shimada et al (29) observed that it was necessary to activate p38 and JNK for prostate cancer cells (LNCaP) incubated with dihydrotestosterone to enter into programmed cell death. This led us to hypothesize that, with an increase in the number of apoptotic cells after incubation with testosterone (or progesterone), JNK could be active in addition to p38. The results of this study confirm that supraphysiological concentrations of testosterone produce an increase in the phosphorylation of p38 and JNK.

In conclusion, the proliferative effect of estradiol on HUVECs occurs through an increase in the phosphorylation of ERK2, which is induced by the deprivation of serum. Furthermore, treatment with progesterone and testosterone at supraphysiological concentrations promotes apoptosis via the activation of p38 and JNK.

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References