Effects of 17β-Estradiol on Preadipocyte Proliferation in Human Adipose Tissue: Involvement of IGF1-R Signaling

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Abstract

Estrogens are known to stimulate the proliferation of human preadipocytes. However, the molecular mechanisms underlying the increased cell growth by these steroids are poorly understood. In the present study, we have demonstrated that the proliferative effect of 17β-estradiol involves the induction of both cell cycle gene expressions, c-myc and cyclin D1. Moreover, the mitogenic effects of 17β-estradiol are suppressed by the pure antagonist ICI 182,780 suggesting that estradiol action is mediated by estrogen receptor α isoform. We also showed that 17β-estradiol is able to inhibit human preadipocyte apoptosis capacity as reflected by DNA fragmentation experiments and the mRNA expression of the pro- and anti-apoptotic genes. Finally, 17β-estradiol *via* ERα significantly induces both mRNA and protein expression of IGF1 receptor in human preadipose cells and thus reinforces the signalling pathway of the proliferative factor, IGF1.

Taken together, these data reinforce the concept of cross-talk between IGF1- and estrogen receptor-signalling pathways in preadipocytes and indicate that IGFI may be a critical regulator of estrogen-mediated preadipose growth.

Introduction

It is well established that adipose tissue development and distribution are tightly regulated by sex steroid hormones [1, 2]. Different studies reveal that these hormones and more particularly estrogens affect fat mass by altering both adipocyte number and size [3]. Indeed, recent experiments demonstrated that estrogens modulate lipid storage and mobilization capacities in human adipocytes principally i) through decreasing expression and activity of lipoprotein lipase, an enzyme that regulates lipid uptake by adipocytes [4], ii) through inducing the lipolytic enzyme hormone-sensitive lipase [5] and iii) attenuating α_2-adrenergic antilipolytic receptor expression [6]. Two other studies revealed that in human adipose tissue estrogens may increase the rate of proliferation *in vitro* [7, 8] without altering the differentiation process [1]. The two specific nuclear receptors (ER) α and β which mediate biological effects of estrogens are present in both human precursors and mature fat cells indicating a auto/paracrine action of these hormones [9]. It has been demonstrated that ERα is the main receptor in adipose cells [10, 11]. However, the molecular mechanisms by which estrogens modulate adipose biology are not clearly understood.

In various cell types, regulation of growth by estrogens seems to be mediated, at least in part, through changes in the expression of some growth factors and/or theirs receptors. Among these growth factors, Insulin-like Growth Factor 1 (IGF1) is considered as an important mitogenic factor in human adipose tissue [12]. Indeed, a growing body of evidence suggest that IGF1- and estrogen-mediated signalling pathway are cross-linked [13]. First, estrogens have been reported to alter expression of nearly all of the IGF family members including IGF1, IGF-binding proteins, IGF1 receptors (IGF1-R), and insulin receptor substrate 1 [14, 15]. Futhermore, ligand-bound ER has been described to activate IGF1 pathway by direct binding and activation of IGF1-R [16] or by directly binding to the p85 subunit

of PI3 kinase and the tyrosine kinase Src leading to activation of the PI3 kinase [17, 18]. Reciprocally, recent studies have reported that IGF1 signalling pathway may enhance ER expression and function by inducing phosphorylation of ER [19, 20]. Moreover, several studies have described synergistic effects of the combination of estrogens and IGF1 on cell cycle regulation in breast tumor cell lines [13].

In the present study, we have explored the *in vitro* effects of estrogens on human adipose growth in order to identify target genes that could be important for the estrogen action on adiposity with particular focus on the role of IGF1-R signalling.

Materials and Methods

Materials

DMEM-Ham's F12 (50:50 mix), penicillin, streptomycin, leupeptin, aprotinin, AEBSF, 17β-estradiol and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, Mo, USA). Collagenase was from Roche Molecular Biochemicals (Mannheim, Germany). Superscript II Rnase H-RT was provided by Gibco BRL (Grand Island, NY, USA), Taq polymerase and RNA guard were by Pharmacia Biotechnology (Uppsala, Sweden), the antiserum specific for active MAP kinase was by Promega Corp (Madison, Wi, USA). ICI 182780 was from Tocris (Bristol, UK). Polyclonal rabbit anti-IGF1-R antiserum was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antiserum specific for the non phosphorylated forms of MAPK was obtained from Transduction Laboratories (Lexington, WI, USA). Fetal calf serum (FCS) was obtained from Gibco-BRL (Grand Island, NY, USA). [^{3}H]-thymidine was from Amersham (Buckinghanshine, UK).

Subjects

The adipose tissue donor group included 9 post-menopausal women (age 64 ± 10 years; BMI: 21.9 ± 1.1 kg/m^2) and 16 men (age 63 ± 12 years; BMI: 26.3 ± 3.5 kg/m^2) undergoing surgical intervention. None of these patients suffered from endocrine malignant or chronic inflammatory diseases. This study was approved by patient's written consent and by the local Ethical Committee (CCPPRB).

Cell culture and treatment

The adipose tissue samples (25-50 g) obtained from subcutaneous fat depots were collected in

saline (NaCl 150 mM) and immediately transferred to the laboratory. After removing blood vessels and connective tissue, adipose tissue was rinsed in saline containing antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Preadipocytes were obtained by collagenase digestion as previously described [21]. Preadipocytes were plated in DMEM/F12 supplemented with streptomycin (0.1 mg/ml), penicillin (100 U/ml) and 10% FCS and maintained at 37°C under 5% CO_2, 95% air atmosphere. After plating at 2-3 x 10^4 cells cm^{-2}, cells which exhibited a fibroblast-like morphology were extensively washed and maintained in DMEM/F12 supplemented with antibiotics and 10% FCS until confluence (3-4 days after plating) and then treated with estrogens or vehicle. The effects of estrogens were essentially the same whatever the sex of the donors was. Therefore, most of the data shown herein are means of the results of pooled experiments performed in both men and women cells.

[^{3}H]-thymidine incorporation

Human preadipose cells were suspended in 12-well plates containing DMEM-Ham's supplemented with 10% FCS. During the exponential growing phase, the culture medium was replaced by DMEM-Ham's containing 2% charcoal-stripped FCS during 24 h. For the next 24 h, cells were exposed to 100 nM 17β-estradiol or to 10 µM ICI 182,780 with or without 100 nM 17β-estradiol in the presence of [^{3}H]-thymidine (1 mCi/ml). After washing 3 times with saline, cells were lysed during 5 min with 1% SDS and treated with 10% trichloroacetic acid for 45 min at 4°C. Radioactivity was counted after filtration on GF/C filters (Whatman, Clifton, NY).

Cell counting

The experimental design used was that described above. Cells were trypsinized with calcium- and magnesium-free Hank's solution containing 0.2% trypsin. Finally, cells were counted in a

hemocytometer after 24 h of treatment with 100 nM 17β-estradiol or control.

Apoptosis Assay

After 18 h in DMEM/F12 supplemented with 2% charcoal-stripped FCS, cells were cultured during 24 h in the presence of TNFα (50 ng/ml) and cycloheximide (10 µg/ml) in order to induce apoptosis of human preadipocytes as described in [22, 23]. Then after, once apoptosis was triggered, cells were maintained in the same medium during 48 h and treated or not with 17β-estradiol (100 nM) or with the known antiapoptotic factor, IGF1 (10 nM), or control. Attached cells were harvested by trypsinisation, combined with floating cells and suspended in PBS at a density of 10^6 cells/ml. Then, cells were fixed in 70% ethanol at -20°C overnight and washed twice with PBS. Cells were labeled for DNA fragmentation by TUNEL according to the instructions provided by the manufacturer. Apototic index was calculated after counting a minimum of 5000 events by flow cytometry using an EPICS flow cytometer (Coulter Electronics, Miami, USA).

IGF1-R immunoprecipitation

At confluence, the culture medium of preadipocytes was replaced by DMEM-Ham's containing 2% charcoal-stripped FCS during 24 h. For the next 24 h, 17β-estradiol (100 nM) was added to the culture medium. Preadipocytes were then lysed in a immunoprecipitation buffer containing 1% triton X-100, NaCl 150 mM, 10 mM Tris ph 7.4, EDTA 1 mM, EGTA 1 mM ph 8, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 0.5% NP-40, 1 mM sodium vanadate, 20 µg/ml AEBSF, 30 mM β-glycerophosphate, 5 µg/ml aprotinin, and 12.5 µg/ml leupeptin. After maintained in a constant agitation for 30 min at 4°C, microcentrifuge tubes were centrifugated at 100,000 x g for 10 min at 4°C. Equal amounts (200-300 µg) of the supernatant containing the "total cell lysate" were immunoprecipitated

with 5 µg of anti-IGF1-R antibody overnight at 4°C in the immunoprecipitation buffer. Immunocomplexes were captured by adding 40 µl of Protein A/G plus : Agarose for 5 h at 4°C. The pellet was collected by centrifugation at 8000g for 1 min at 4°C. After two washes in the immunoprecipitation buffer, the pellet was resuspended in 30 µl of Laemmli's buffer, boiled for 5 min and centrifuged for 5 min at 16.000 g. Immunoprecipitated proteins were then fractioned on SDS-PAGE (7%). Controls used included the omission of cell lysates or the anti-IGF1-R antibody during immunoprecipitation. Proteins were transferred to PVDF membrane and blocked in buffer A (20 mM Tris HCl, 137 mM NaCl and 0.1% Tween 20) with 2.5% gelatin during 2 h. Then membranes were incubated overnight at room temperature with the primary antibody diluted in buffer A with 2.5% gelatin (1: 300 dilution). The resulting blots were extensively washed with buffer A, and incubated with the secondary antiserum coupled to peroxidise (1: 10,000 dilution in buffer A) for 1 h at room temperature and washed in buffer A. Finally, an enhanced chemiluminescence kit was used for signal detection. Specificity of the immunoreactive proteins was verified by loss of sample immunoreactivity when incubated with the antiserum neutralized with the corresponding specific peptide.

Protein concentrations were measured according to Bradford [24] with BSA as standard.

Isolation of RNA

Total RNA was isolated from human preadipocytes according to the method of Chomczynski and Sacchi [26]. RNA recovery and quality were checked by measuring the 260/280 nm optical density ratio and by electrophoresis under denaturing conditions on 2% agarose gel.

IGF1-R mRNA expression

Total RNA (0.5 µg) was extracted and reverse transcribed as previously described [27]. Semi-

quantitative PCR method was performed using primer sets indicated in table 1. To ensure that amplification of this gene was within the exponential range, different PCR cycles (25-40 cycles) were run. Finally, 30 cycles of PCR amplification were chosen to study IGF1-R mRNA expression. The second primer set was specific for the 18S cDNA that is used as internal standard. In the same way, different PCR cycles (25-40 cycles) were run. Finally 40 cycles of PCR were found to be optimal for detection of the 18S mRNA. PCRs were performed with a thermocycler Gene Amp PCR 2400 (Perkin Elmer, USA).

PCR products were analysed on a 2 % agarose gel in 90 mM Tris-borate, 2 mM EDTA buffer (TBE) pH 8 and visualized by staining with ethidium bromide and ultraviolet transillumination. Quantification was realized with the Bio-1D software (Vilber Lourmat, Marne la Vallée, France). Controls without reverse transcriptase were systematically performed in order to detect eventual genomic DNA contaminations.

Cell cycle and apoptosis mRNA expressions

Total RNA (0.5µg) was reverse transcribed as previously described [27]. Quantitative PCR was performed using a LightCycler® instrument from Roche Diagnostics (Basel, Switzerland) with QuantiTect SYBR Green PCR Master Mix (Qiagen, Courtaboeuf, France). Primer sets used are indicated in table 1. cDNA calibrators were prepared by PCR amplification run to saturation (35 cycles) with the appropriate primers. The resulting cDNAs were purified by QIAquick PCR purification Kit (Qiagen). The samples showed a unique band in agarose electrophoresis. cDNA copies were calculated from the absorbance at 260 nm. Calibrators were defined to contain arbitrary units of human c-fos, cyclin D1, p53, bcl2, bax, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and all calculated concentrations are relative to GAPDH concentrations. In accordance with [28], the choice of

GAPDH as housekeeping gene was based on the observation that GAPDH mRNA expression is unsensitive to various hormones in human adipose cells. Separate calibration curves for human c-fos, cyclin D1, p53, bcl2, bax, and GAPDH were constructed from serial dilutions from 10^8 copies to 100 copies of cDNA calibrators. Calibration curves were log-linear over the quantification range with correlation coefficient (r^2) ≥ 0.99 and efficacity ranging from 1.8 to 2. The intra-assay variability of duplicate crossing point (Cp) values never exceeded 0.2 cycle and the inter-assay variability (CV value) ranged from 1 to 5 % CV values for the three or four runs of each transcript. Real-time PCR was performed as described in [29].

The Second Derivative Maximum Method was used to automatically determine the Cp for the individual samples. For each sample, the concentration ratio (target / GAPDH gene, used as internal standard) was calculated using the Roche Software. Fold changes in expression were determined by calculating the normalized ratio which corresponds to concentration of calibrator situation (without effector) / concentration of unknown situation (with effector) (relative quantification).

Statistical analysis

All values were expressed as means ± SEM from at least five separate experiments. Statistical analyses were performed using the non parametric paired Wilcoxon test.

Results

1- Effects of 17β-estradiol on human preadipocyte proliferation.

Cell proliferation was first studied by measuring changes in the rate of DNA synthesis ([^{3}H]-thymidine incorporation). As shown in Fig. 1a, preadipocyte exposure to the pure ERα antiestrogen (ICI 182,780 at 10 μM) alone caused a significant decrease in preadipose proliferative capacities (- 36.1 ± 5.4 %). These results suggest that ICI 182,780 may block the effect of estrogens that are produced locally by human preadipocytes.

Experiments were next performed with 17β-estradiol at 100 nM and as can be seen in the same figure, 17β-estradiol treatment of cells for 24 h resulted in an increased [^{3}H]-thymidine incorporation (+ 32 ± 8 %). This effect which was confirmed by direct cell counting (+ 28 ± 5 %) seemed to be dose-independent (data not shown). For comparison, the magnitude of the increase in [^{3}H]-thymidine incorporation caused by 10% FCS, used as a positive control, was equivalent to 2.4 ± 0.4-fold increase compared to control value.

Moreover, when 17β-estradiol was added simultaneously with ICI 182,780, this antagonist completely abolished the positive effects of estrogens on preadipocyte growth. These results suggest that, in human preadipocytes, the promoting action of estrogens requires ERα.

2- Effects of 17β-estradiol on some cell cycle gene expression in human confluent preadipose cells.

In order to delineate estrogen-target genes in human preadipocytes, the influence of 17β-

estradiol on c-fos, c-myc and, cyclin D1 mRNA expressions was measured by RT-PCR. As shown in Fig. 1b, in human preadipose cells, 17β-estradiol (100 nM) induced both c-myc and cyclin D1 mRNA expressions by 2.32 ± 0.25-fold and 2.36 ± 0.24-fold, respectively. However, under our experimental conditions, 17β-estradiol did not affect c-fos mRNA expression. These findings indicate that the cell cycle genes, c-myc and cyclin D1 seem to be nuclear targets of ER signalling in human preadipocytes.

3- Effects of 17β-estradiol on human preadipose apoptosis.

To test the influence of estrogens on cell apoptosis, we first used TUNEL assay to determine the percentage of labeled apoptotic nuclei. As shown in Fig. 2a, 17β-estradiol (100 nM) is able to reduce significantly DNA fragmentation in human preadipocytes (- 49 ± 13%) after 48 h exposure when apoptosis was induced by TNFα and cycloheximide. Under the same conditions, exposure of cells to 10 nM IGF1 (a known anti-apoptotic factor) used as control also reduced apoptosis by 43 ± 21%. However, no synergic effect on cell apoptosis was observed when both 17β-estradiol and IGF1 were simultaneously added in the culture medium (data not shown).

We next tried to better understand the molecular mechanisms whereby estrogens reduced human preadipocyte apoptosis by measuring mRNA expressions of the anti-apoptotic bcl2 gene and of the pro-apoptotic Bax and p53 genes. As shown in Fig. 2b, 17β-estradiol (100 nM) stimulated the expression of the anti-apoptotic gene Bcl2 (x 1.9 ± 0.2) after 24 h exposure. Moreover, expressions of both pro-apoptotic genes Bax and p53 were also targets for 17β-estradiol since a 38 and 49% reduction were observed in their expression, respectively. Thus, exposure to estrogens stimulated an *in vitro* anti-apoptotic response in human preadipocytes.

4- Effects of 17β-estradiol on IGF1-R expression in human preadipose cells.

As shown Fig. 3a, by using RT-PCR analysis, we found that IGF1-R mRNA expression was increased by about 5-fold after exposure to 100 nM 17β-estradiol in human preadipocytes. Moreover, these effects were reversed by simultaneous addition in the culture medium of the ER antagonist ICI 182,780 (10 μM). Immunoprecipitation experiments also showed a 3-fold induction of IGF1-R protein expression by 17β-estradiol (100 nM) in human preadipocytes (Fig. 3b-3c). These results suggest that estrogen effects on human preadipocytes could be mediated, at least in part, through modulation of IGF1-R signalling pathway.

Discussion

Adipose tissue is an important site for estrogen biosynthesis and storage [30]. Moreover, the presence of specific receptors for these hormones in both precursor and mature fat cells shows that adipose cells are targets for estrogens [10, 11]. Various observations have led to assign an important role of estrogens in adipocyte development and metabolism (For a review, [31]). For, example, the P450 aromatase- or the ERα-knockout mice displayed a significant and progressive increase in adiposity as compared with their wild type counterpart [32, 33]. More recently, it has been demonstrated that estrogen affect, both *in vivo* and *in vitro,* the expression of key lipogenic and lipolytic genes in human adipocytes [4, 5]. Finally, two studies have shown that in human adipose tissue, estrogens may increase the rate of proliferation *in vitro* [7, 8].

The present study was undertaken to get a better understanding on how estrogens modulate adipocyte proliferation *in vitro*. For this purpose, we first verified that these ovarian steroids effectively promoted preadipose growth under our experimental conditions. Next, we demonstrated that the use of the pure ERα antagonist ICI 182,780 abolished the 17β-estradiol-induced proliferation. These results are in accordance with previous studies performed in the uterus [34] and support the concept that in human preadipocytes, the promoting action of 17β–estradiol on growth requires at least in part ER. Otherwise, in agreement with investigations performed in rat adipocytes and in neuroblastoma cells [35], treatment with ICI 182,780 alone negatively affected preadipocyte cell growth. Two hypothesis can be put forward : i) since estrogens are produced locally by human preadipocytes due to *in situ* p450 aromatase expression [30], ICI 182,780 could block the paracrine and/or paracrine action of estrogen on cell growth ; ii) ICI 182,780 is described as a transcriptional inhibitor for certain cell

cycle genes and this effect is mediated *via* the ERβ subtype [36, 37]. Different ERβ isoforms have been recently characterized in human preadipocytes and their expression generally declined during adipocyte differentiation [38].

Cell proliferation is the result of the balance between cell division and apoptosis. In order to explain the stimulatory effect of estrogens on human preadipocyte number, we have tested the expression of some specific cell cycle and apoptotic genes that could be under the control of estrogens. In different tissue and cell types, estrogens are able to induce G1-S phase cell cycle progression through increased expression of cyclin D1 and of some proto-oncogenes such as c-fos and c-myc [39, 40]. In our study, we have shown that estrogens induce c-myc and cyclin D1 mRNA expressions but not c-fos mRNA expression. This suggests that the stimulatory effect of this hormone on human preadipocyte growth is due to a direct activation of G1-S phase cell cycle progression. Concerning the apoptotic process, as previously reported in other cell types [41, 42], we have also observed that estrogens were able to repress DNA fragmentation. This inhibitory effect was accompagnied by changes in the expression profile of some pro- and anti-apoptotic genes : the pro-apoptotic Bax and p53 mRNA expressions were reduced while the anti-apoptotic Bcl2 mRNA expression was increased. Estrogens mediated their action by a wide variety of signalling pathways (For a review, [43]). Among these, the p42/p44 MAP- and the PI3- kinases played a crucial role in the regulation of both adipose growth and apoptosis processes. For example, previous results in our laboratory showed that in rat preadipocytes, the mitogenic actions of ovarian hormones were associated to the activation of the p42/p44 MAP kinase pathway [44]. Additional experiments are currently in progress in order to delineate the pathway by which estrogens transduce their effects on human preadipocytes. Our preliminary experiments failed to show any effect of cell-membrane permeable 17β–estradiol or impermeable estrogen (estradiol conjugated to BSA) on p42/p44 MAP kinase activation (data not shown).

It is now well established that the regulation of cell growth by estrogens is mediated through changes in the expression of some growth factors and/or their receptors. Among these factors, cross-talk between IGF1 and ER signallings has been extensively described in various cell types and tissues [34, 45, 46]. For example, it has been recently demonstrated that estrogens cause cell proliferation in the uterine epithelium of mice and humans through ERα [47]. In this work, they also showed that this signalling pathway is mediated by IGF1-R whose activation leads to the stimulation of the PI3 kinase pathway leading to cyclin D1 nuclear accumulation and engagement with the canonical cell cycle machinery. Moreover, in adipose tissue, IGF1 is considered as an important mitogenic factor. Indeed, previous experiments performed in our laboratory with anti-IGF1 antiserum in the culture medium revealed that depletion of exogenous and endogenous IGF1 resulted in a significant decrease of cell growth in rat and human preadipocyte [12, 29]. In the present paper, we demonstrate that 17β-estradiol increases significantly IGF1-R expression in human preadipocytes. So, in addition to breast cancer cells and uterus [34, 45, 46], adipose tissue is another example of tissue where expression of IGF1-R is under the control of estrogens. ER-mediated transactivation is a complex process. Estrogens can modulate gene transcription by direct binding of the ER to classical estrogen response element [48]. However, some reports have suggested that transcriptionnal stimulation triggered by the steroid receptors could also involve cooperativity between ER and adjacent promoter elements like AP-1 or SP1 sites [49, 50]. This is the case for c-fos gene which expression is dependent on the formation of a transcriptionally active ER/SP1 complex binding to an SP1 site [50]. The presence of various SP1 consensus sequences in the promoter of IGFI-R gene could explain the regulation of this gene by estrogens [51].

Different studies also indicate that IGF1, in an auto-/paracrine manner, can rescue 3T3-L1 preadipocytes from apoptosis [52, 53] and human preadipocytes as described in this paper and by others

[23]. Indeed, it has been demonstrated in human adipose tissue that IGF1 induces the expression of the anti-apoptotic proteins, bcl-xl and that this protective effect is mediated by PI3 kinase pathway but not by the p38 or the p42/p44 MAP kinases [23, 52]. Experiments are currently in progress in our laboratory in order to determine the part played by IGF1 signalling in estrogen action in human preadipocytes and more precisely the role played by the PI3 kinase pathway.

In summary, we have demonstrated that estrogens exert direct regulatory effects on proliferation in human preadipocytes. Taken together, our results not only underline the concept of a cross-talk between IGF1- and ER- signalling pathways but also indicate that the local estrogen production by adipose tissue may act directly or indirectly *via* IGF1-R on the regulation of human adipose tissue development.

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Legends

Figure 1 : Influence of 17β-estradiol on human preadipocyte proliferation.

(A) Effect of 17β-estradiol on DNA synthesis.

Growing human preadipocytes were exposed to 0 nM (cont) or 100 nM 17β-estradiol (E2) concentrations or or to the estrogen antagonist ICI 182,780 (100 nM) or to 10% FCS used as positive control in the presence of [^{3}H] thymidine as described under "Materials and Methods". Assays were performed in triplicates for each sample. Results are means ± SEM of five experiments and are normalized as percentages of the control value (without estradiol) : 1705 ± 320 cpm / 24h.

* $p < 0.03$, ** $p < 0.01$, Wilcoxon test.

(B) Effect of 17β-estradiol on c-fos, c-myc, cyclin D1 mRNA expressions.

Human confluent preadipocytes were deprivated for 18 h and then incubated in the absence (cont) or presence of 17β-estradiol (100 nM) for the indicated times. Total RNA was extracted and subjected to real time RT-PCR to determine c-fos mRNA level as described under "Materials and Methods". Results are expressed as arbritrary units. Each bar represents the mean ± SEM of three to four separate experiments.

* $p < 0.01$, ** $p < 0.008$ compared with control values, ns = non significant compared with control values, Wilcoxon test.

Figure 2 : Influence of 17β-estradiol on human preadipocyte apoptosis.

(A) Effects of 17β-estradiol on DNA fragmentation.

After 18h in DMEM/F12 supplemented with 2% charcoal-stripped FCS, human preadipocytes were cultured during 24h in the presence of TNFα (50 ng/ml) and cycloheximide (10 µg/ml) in order to induce apoptosis. Once apoptosis was triggered, cells were maintained in the same medium during 48 h and treated with or without 17β-estradiol (100 nM) or IGF1 (10 nM). Cells were analysed by TUNEL staining and flow cytometer. Results are expressed as arbritrary units. Each bar represents the mean ± SEM of five to seven separate experiments.

** $p < 0.03$ compared with control values, Wilcoxon test.

(B) Effects of 17β-estradiol on p53, bax and bcl2 mRNA expressions.

Human confluent preadipocytes were deprivated for 18 h in serum-free F12 medium and then incubated in the absence (cont) or presence of 17β-estradiol (100 nM) for the indicated times. Total RNA was extracted and subjected to real time RT-PCR to determine c-fos mRNA level as described under "Materials and Methods". Results are expressed as arbritrary units. Each bar represents the mean ± SEM of five to seven separate experiments.

* $p < 0.03$ compared with control values, Wilcoxon test.

Figure 3 : Influence of 17β-estradiol on IGF1-R expression in human preadipocytes .

Human confluent preadipocytes were deprivated for 18 h in serum-free F12 medium and then incubated in the absence (cont) or presence of 17β-estradiol (100 nM) for 24 h.

A: Effects of 17β-estradiol on IGF1-R mRNA expression.

Results are expressed as arbritrary units. Each bar represents the mean ± SEM of five to seven separate

experiments.

**p < 0.03 compared with control values, ns = non significant compared with control values, Wilcoxon test.

B: Representative Western Blot of the effects of 17β-estradiol on IGF1-R expression.

Cell lysates were subjected to SDS-PAGE, immunoblotted and quantified as described under "Materials and Methods".

C: Densitometric analysis of the effects of 17β-estradiol on IGF1-R protein expression.

Results are expressed as arbitrary units. Each bar represents the mean ± SEM of four to five separate experiments.

* p < 0.05 compared with control values, Wilcoxon test.

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Figure 1A

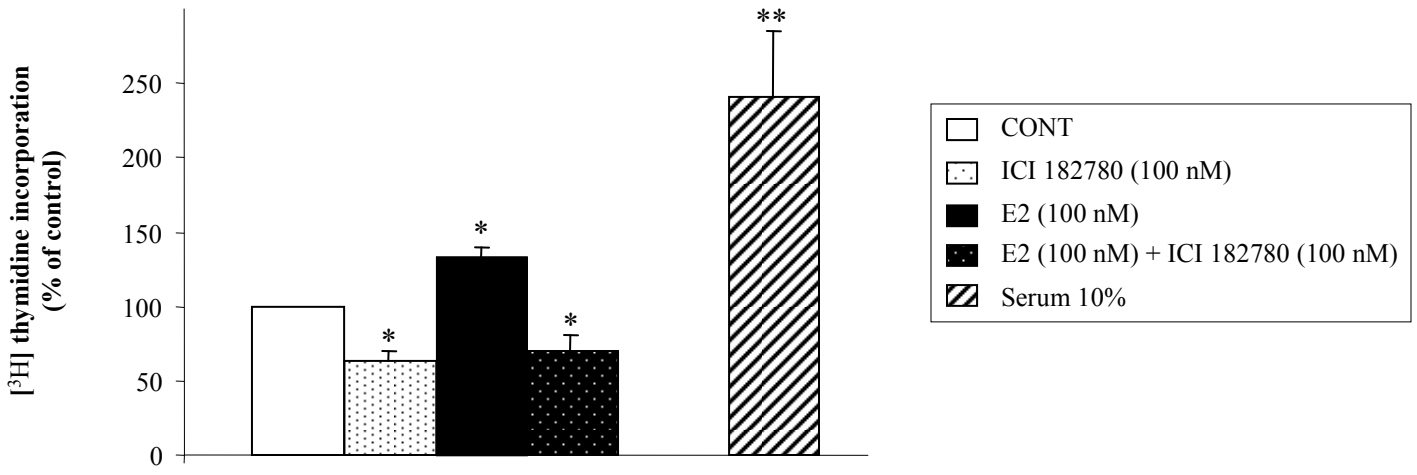
[3H] thymidine incorporation
(% of control)
0
50
100
150
200
250
*
*
*
**
CONT
ICI 182780 (100 nM)
E2 (100 nM)
E2 (100 nM) + ICI 182780 (100 nM)
Serum 10%

Figure 1B

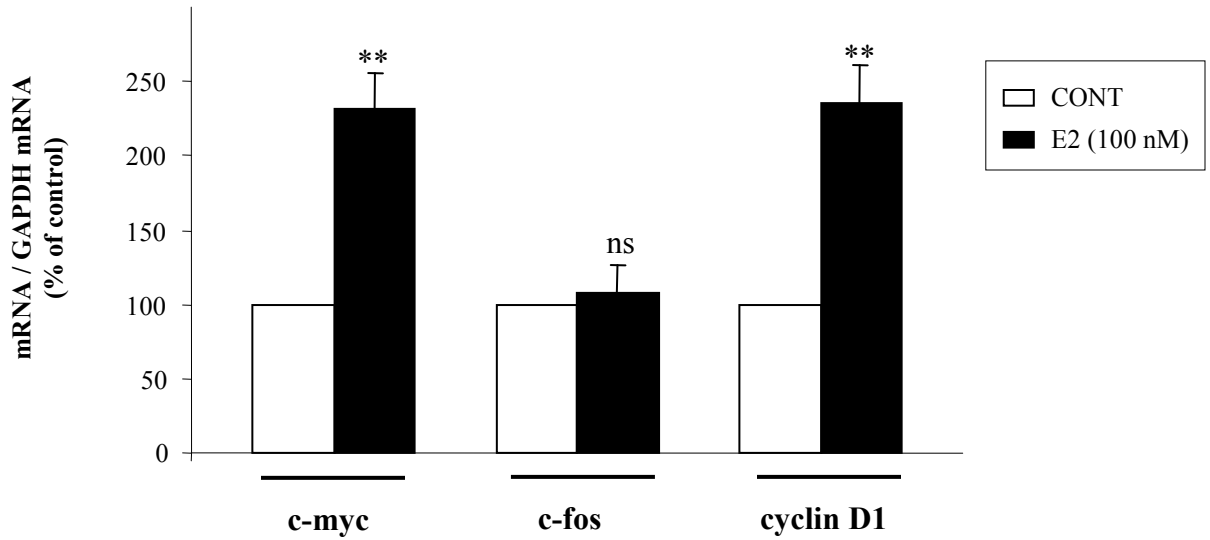
mRNA / GAPDH mRNA
(% of control)
250
200
150
100
50
0
**
ns
**
CONT
E2 (100 nM)
c-myc
c-fos
cyclin D1

Figure 2A

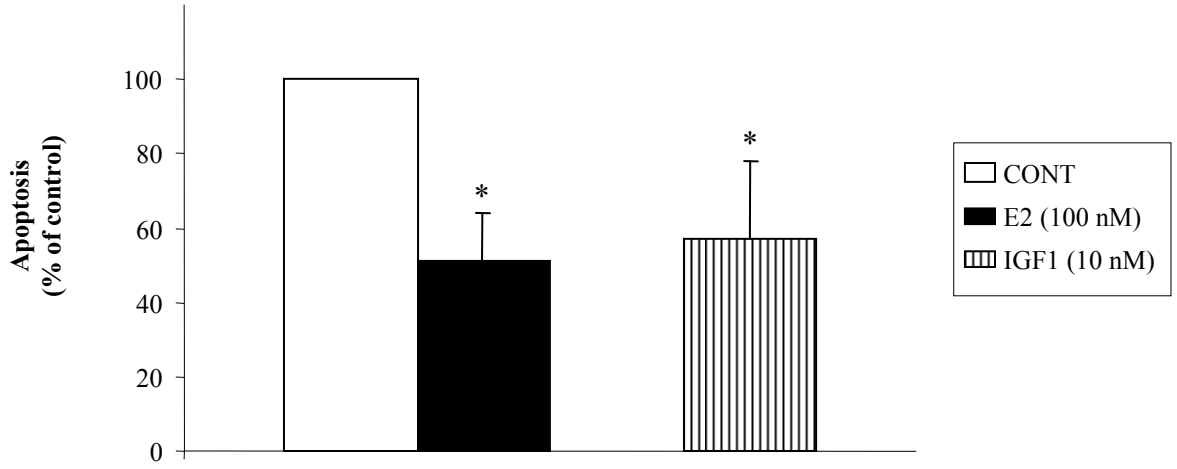

Figure 2B

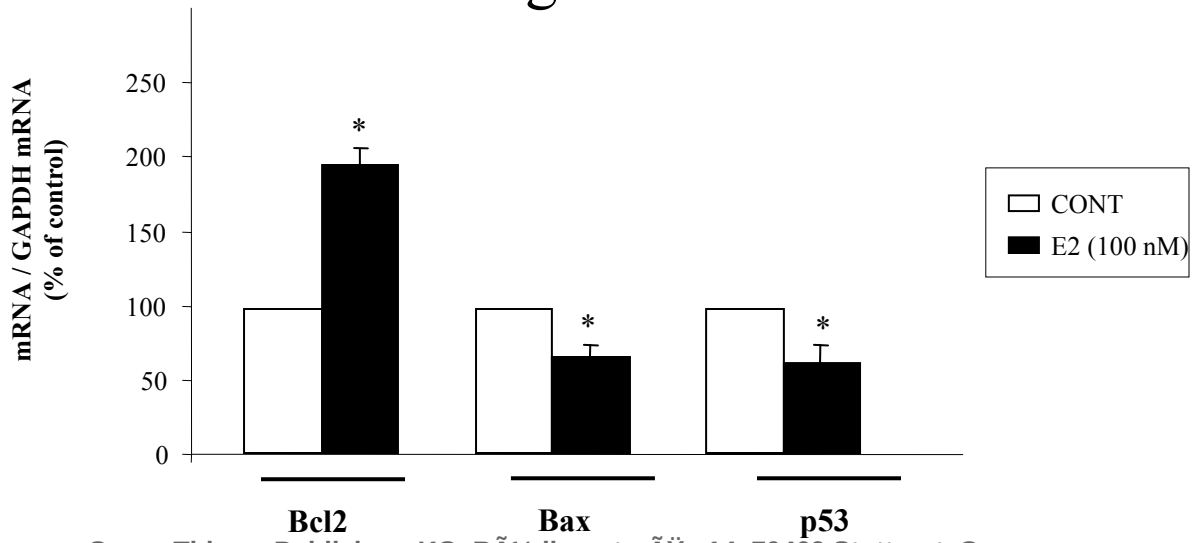

Figure 3A

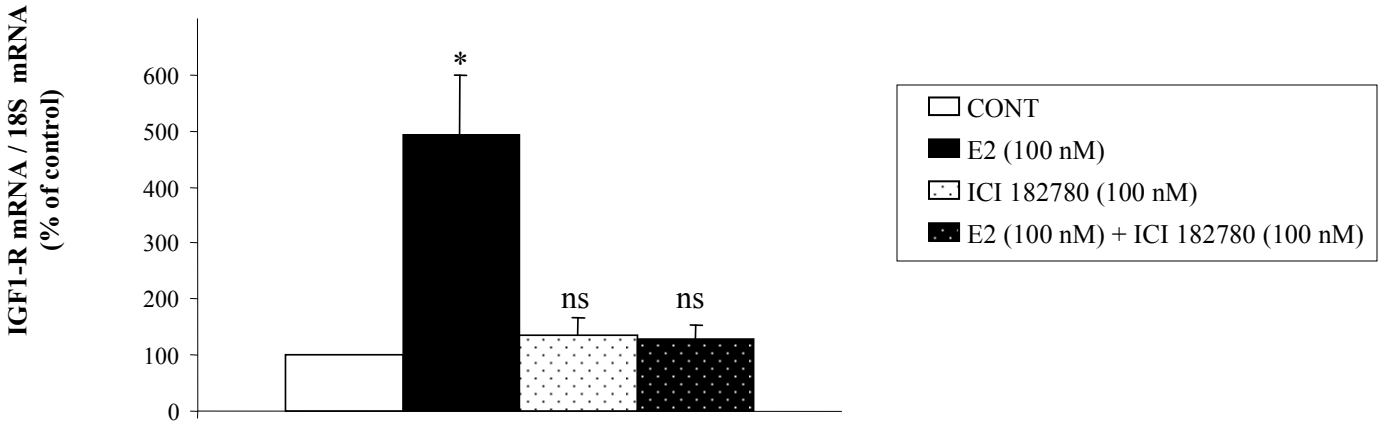

Figure 3B

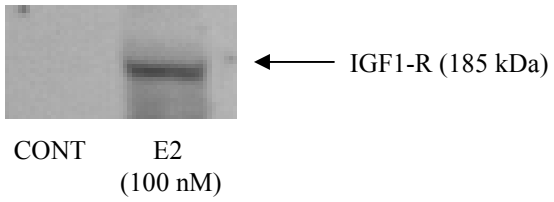

Figure 3C

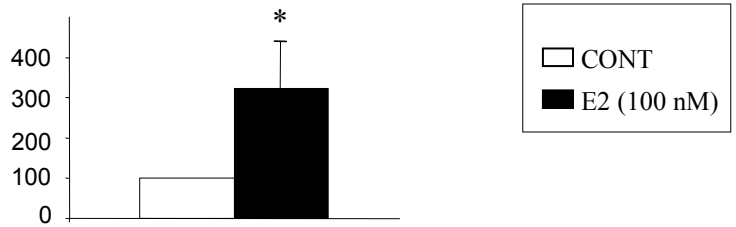

Table 1 : Primer pairs used for RT-PCR

Primer sets	Sequence	PCR product (bp)
Cyclin D1 Sense Antisense	5’ GAG TGG CGG CAG CGG C 3’ 5’ CAG TTC GCG GCT CAG CTG TT 3’	69
c-fos Sense Antisense	5’ GCT ATG CTC TTC ACC TAT 3’ 5’ GTC ATT GTC ATT ATC AGC 3’	111
c-myc Sense Antisense	5’ GAC GCG GGG AGG CTA TTC TG 3’ 5’ GAC TCG TAG AAA TAC GGC TGC ACC GAGTC 3’	236
IGF1-R Sense Antisense	5’ AAT AAC ATT GCT TCA GAG CTG 3’ 5’ GAT GGT GCC GTC GGC ATA CTT 3’	933
18 S Sense Antisense	5’ ATT CCA GCT CCA ATA GCG 3’ 5’ CAC TCA GCT AAG AGC ATG G 3’	172
GAPDH Sense Antisense	5’ ACC CAC TCC TCC ACC TTT G 3’ 5’ CTC TTG TGC TCT TGC TGG G 3’	178
p53 Sense Antisense	5’ ACT AAG CGA GCA CTG CCC AA 3’ 5’ ATG GCG GGA GGT AGA CTG AC 3’	231
bcl2 Sense Antisense	5’ ATG TGT GTG GAC AGC GTC AAC C 3’ 5’ TGA GCA GAG TCT TCA GAG ACA GCC 3’	196
Bax Sense Antisense	5’ CAA ACT GGT GCT CAA GGC C 3’ 5’ GCA CTC CCG CCA CAA AGA T 3’	188