Featured Article

Leptin Interferes with the Effects of the Antiestrogen ICI 182,780 in MCF-7 Breast Cancer Cells

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ABSTRACT

Purpose: **Obesity is a risk factor for breast cancer development in postmenopausal women and correlates with shorter disease-free and overall survival in breast cancer patients, regardless of menopausal status. Adipose tissue is a major source of leptin, a cytokine regulating energy balance and controlling different processes in peripheral tissues, including breast cancer cell growth. Here, we investigated whether leptin can counteract antitumorigenic activities of the antiestrogen ICI 182,780 in breast cancer cells.**

Experimental Design: **Mitogenic response to leptin and the effects of leptin on ICI 182,780-dependent growth inhibition were studied in MCF-7 estrogen receptor α-positive breast cancer cells. The expression of leptin receptor and the activation of signaling pathways were studied by Western immunoblotting. The interference of leptin with ICI 182,780-induced estrogen receptor α degradation was probed by Western immunoblotting, fluorescence microscopy, and pulse-chase experiments. Leptin effects on estrogen receptor α–dependent transcription in the presence and absence of ICI 182,780 were studied by luciferase reporter assays and chromatin immunoprecipitation.**

Results: **MCF-7 cells were found to express the leptin receptor and respond to leptin with cell growth and activation the signal transducers and activators of transcription 3, extracellular signal-regulated kinase-1/2, and Akt/GSK3/pRb pathways. The exposure of cells to 10 nmol/L ICI 182,780 blocked cell proliferation, induced rapid estrogen receptor α degradation, inhibited nuclear estrogen receptor α expression, and reduced estrogen receptor α–dependent transcription from estrogen response element–containing promoters. All of these effects of ICI 182,780 were significantly attenuated by simultaneous treatment of cells with 100 ng/mL leptin.**

Conclusions: **Leptin interferes with the effects of ICI 182,780 on estrogen receptor α in breast cancer cells. Thus, high leptin levels in obese breast cancer patients might contribute to the development of antiestrogen resistance.**

INTRODUCTION

Numerous epidemiologic studies documented that obesity is a risk factor for postmenopausal breast cancer (1–4). Furthermore, increased body weight and body mass index have been associated with shorter disease-free and overall survival in breast cancer patients, regardless of age and menopausal status (4). Some studies also suggested that obesity can reduce the efficacy of anti-breast cancer chemotherapy (5). In animal models, high adiposity has been linked with increased incidence of spontaneous and chemically induced mammary tumors (6–9).

Human obesity is associated with increased levels of leptin, a M_r 16,000 circulating hormone controlling food intake and energy balance by providing signals to the hypothalamus (10). In addition to its central nervous system activities, leptin regulates multiple processes in peripheral tissues, including hematopoiesis, immune responses, puberty, pregnancy, and lactation (10–14). In cellular models, leptin has been shown to activate proliferation, angiogenesis, motility, and invasion (10, 15–22). The major source of leptin is adipose tissue; however, leptin can be produced by other organs, including the mammary gland (10–12).

The activities of leptin are mediated through the transmembrane leptin receptor (ObR; ref. 23). In human tissues, at least four isoforms of ObR with different COOH-terminal cytoplasmic domains have been described previously (24). The full (long) form of ObR (ObR_l) is 1165 amino acids long (M_r ~150,000–190,000) and contains extracellular, transmembrane, and intracellular domains. The extracellular domain binds ligand, whereas intracellular tail recruits and activates signaling substrates. Only ObR_l has a full signaling potential, whereas the shorter ObR isoforms have diminished or abolished signaling capability (25–28). The signaling pathways known to be activated by ObR_l include the classic cytokine JAK2/signal transducers and activators of transcription 3 (STAT3) pathway; the Ras/extracellular signal-regulated kinase (ERK) signaling cascade; the kinases phosphatidylinositol 3′-kinase, Akt, p38, and protein kinase C; nitric oxide; and phospholipase Cγ (25–27). Ultimately, induction of ObR_l can activate the expression of several genes involved in cell proliferation, including c-*fos*, c-*jun*, *jun*B, *egr-1*, and *socs3* (25, 26).

Although leptin is necessary for normal mammary gland development in rodents and humans (11, 12, 18, 29), recent studies suggested that the hormone might be involved in mam-

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mary carcinogenesis (18–22, 30, 31). Notably, leptin (31) and ObR (20) have been detected in human breast cancer specimens. In breast cancer cell lines T47D and MCF-7, leptin has been shown to stimulate DNA synthesis and cell growth acting through the STAT3 and ERK1/2 signaling pathways (18–20, 22). Leptin has also been shown to induce transformation (anchorage-independent growth) of cancer but not normal breast epithelial cells (18). Finally, leptin-deficient mice have decreased incidence of spontaneous and oncogene-induced mammary tumors (30).

The possible impact of leptin produced in mammary tissue on breast cancer development is yet unknown. The role of circulating leptin remains unclear, with clinical studies reporting positive (32), negative (33), or no association (34) of serum leptin levels with breast cancer.

In addition to leptin, adipose tissue is a source of estrogens produced, via aromatase conversion, from androstenedione in postmenopausal women (35). Recent studies suggest that leptin and estrogen systems are involved in functional cross-talk. For instance, leptin has been shown to modulate, either positively (36–38) or negatively (39, 40), aromatase activity. Reciprocally, 17-β-estradiol (E_2) has been found to up-regulate leptin mRNA and protein synthesis in adipocytes (41). E_2 can also modulate ObR expression (42), possibly through the putative estrogen-responsive element in the *ObR* gene promoter (43).

In this study, we explored a new aspect of leptin/estrogen cross-talk. Specifically, we asked whether leptin can interfere with antitumorigenic effects of the antiestrogen ICI 182,780. ICI 182,780 [Faslodex (fulvestrant); AstraZeneca, Macclestield, United Kingdom], which induces estrogen receptor α degradation through ubiquitin-mediated mechanism (44–47), is currently used for treatment of hormone receptor–positive metastatic breast cancer in post-menopausal women with disease progression following other hormonal therapy (44).

MATERIALS AND METHODS

Cell Lines and Cell Culture. MCF-7, T47D, MDA-MB-231, and MDA-MB-435 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium:Ham's F-12 containing 5% calf serum. In the experiments requiring E_2- and serum-free conditions, the cells were cultured in phenol red-free serum-free medium (48, 49).

Cell Growth. MCF-7 cells were plated in 35-mm plates at a concentration of 1.5 to 2.0 × 105 cells/plate in Dulbecco's modified Eagle's medium:Ham's F-12 (1:1) containing 5% calf serum. The following day (day 0), the cells at approximately 70% confluence were shifted to serum-free medium and treated with 10 nmol/L E_2 (Sigma. St. Louis, MO), 10 nmol/L ICI 182,780 (Tocris Cookson, Ellisville, MI), 100 ng/mL leptin (R&D Systems, Minneapolis, MN), or 10 nmol/L ICI 182,780 + 100 ng/mL leptin, singly or in combination. Cell number was determined by direct cell counting at days 0, 1, and 3. The number of cells at day 0 was taken as 100%, and the relative values at days 1 and 3 were calculated for each treatment.

Fluorescence Microscopy. Fifty percent confluent MCF-7 cells grown on coverslips were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS, and incubated for 1 hour with 2 μg/mL estrogen receptor α Ab H-184 (Santa Cruz Biotechnology, Santa Cruz, CA). Next, the cells were washed three times with PBS, and incubated with the rhodamine-conjugated goat antirabbit immunoglobulin G (Calbiochem, San Diego, CA) used as a secondary Ab. After this step, the slides were covered with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) to allow visualization of cellular nuclei. Nuclear abundance of estrogen receptor α under different conditions was assessed using Zeiss Axiovert zoom microscope with magnification ×100.

Immunoprecipitation and Western Blotting. The expression of ObR, activation of leptin signaling pathways, and the abundance of estrogen receptor α were assessed by Western blotting or immunoprecipitation followed by Western blotting using total protein lysates or fractionated proteins, where appropriate. Total cell proteins were obtained using RIPA buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS. Cytoplasmic proteins were obtained using the lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, EGTA, 10 mmol/L (pH 7.5), glycerol 10%, and inhibitors (0.1 mmol/L Na_3VO_4, 1% phenylmethylsulfonyl fluoride, and 20 mg/mL aprotinin). After the collection of cytoplasmic proteins, the nuclei were lysed with the nuclear buffer containing 20 mmol/L HEPES (pH 8), 0.1 mmol/L EDTA, 5 mmol/L $MgCl_2$, 0.5 mol/L NaCl, 20% glycerol, 1% Nonidet P40, and inhibitors (as above). For Western blotting, 50 mg of protein lysates were separated on a 4 to 15% polyacrylamide denaturing gel (PAGE), and proteins of interest were detected with specific antibodies (Abs) and visualized by ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ). The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

For immunoprecipitations, 500 μg of protein lysates were incubated with primary Abs at 4°C or 18 hours in HNTG buffer [20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol, and 0.1 mmol/L Na_3VO_4], and then the antigen/Ab complexes were precipitated with Protein A agarose (Calbiochem) for pAbs or Protein G for mAbs (Calbiochem) at for 2 hours in HNTG buffer. In control samples, primary immunoprecipitating Abs were replaced with normal rabbit immunoglobulin G (Santa Cruz Biotechnology). The immunoprecipitated proteins were washed three times with HNTG buffer, separated on PAGE, and processed by Western blotting.

Antibodies for Western Blotting and Immunoprecipitation. ObR expression was studied by Western blotting with the anti-ObR H300 pAb (Santa Cruz Biotechnology). Estrogen receptor α was assessed by Western blotting with the anti-estrogen receptor α F-10 mAb (Santa Cruz Biotechnology). Ubiquitination of estrogen receptor α was assessed by immunoprecipitation/Western blotting in 500 μg of total proteins. In this assay, estrogen receptor α was immunoprecipitated with the anti-estrogen receptor α F10 mAb, and ubiquitination was detected by Western blotting with the anti-ubiquitin P4D1 mAb (Santa Cruz Biotechnology). The expression of STAT3 was probed in 500 μg of total proteins by immunoprecipitation and Western blotting with the anti-STAT 3 pAb (Santa Cruz Biotechnology). The activation of STAT 3 was measured in STAT3 immunoprecipitations with the anti-STAT3 Ser^{727} pAb (UBI, Lake Placid, NY) and the anti-STAT Tyr^{705} pAb (Cell Signaling, Beverly, MA). The following Abs were used to study other elements of leptin signaling by Western blotting: anti-phospho-ERK1/2 Thr^{202}/Tyr^{204} mAb (Cell Signaling); anti-p44/42 MAP kinase pAb (Cell Signaling); anti-phospho-Akt Ser^{473} pAb (Cell Signaling); anti-Akt pAb (Cell Signaling); anti-phospho-GSK3β pAb (Cell Signaling); and anti-phospho-pRB pAb (Cell Signaling). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and nucleolin was assessed by Western blotting as controls of loading and purity of lysates with the anti-GAPDH mAb (Research Diagnostics Inc., Flanders, NJ) and the anti-nucleolin C23 mAb (Santa Cruz Biotechnology), respectively. The expression of β-catenin was probed with the anti-β-catenin mAb (BD Transduction Laboratories, San Jose, CA). All Abs were used at concentrations recommended by the manufacturers.

Estrogen Response Element Reporter Assays. MCF-7 cells were grown in 24-well plates. At 70% confluence, the cultures were transfected for 6 hours with 0.5 μg DNA/well using Fugene 6 (DNA:Fugene 3:1; Roche, Gipf-Oberfrick, Switzerland). All transfection mixtures contained 0.5 μg of the reporter plasmid, estrogen response element-Luc, encoding the firefly luciferase (Luc) cDNA under the control of the TK promoter and three estrogen response element sequences. In addition, to test transfection efficiency, each DNA mixture contained 50 ng of pRL-TKLuc, a plasmid encoding renilla luciferase (RI Luc; Promega, Madison, WI). Upon transfection, the cells were shifted to serum-free medium for 16 hours and then treated with 10 nmol/L E_2, 10 nmol/L ICI 182,780, 100 ng/mL leptin, and ICI 182,780 + leptin for 24 hours. Untreated cells in serum-free medium served as controls. Luciferase activity (Luc and RI Luc) in cell lysates was measured using Dual Luciferase Assay System (Promega) following the manufacturer's instructions. The values obtained for Luc were normalized to that of RI Luc to generate relative Luc units.

Chromatin Immunoprecipitation. We followed the chromatin immunoprecipitation methodology described by Shang *et al.* (50) with minor modifications. MCF-7 were grown in 100-mm plates. Ninety percent confluent cultures were shifted to serum-free medium for 24 hours and then treated for 4 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780, 100 ng/mL leptin, 10 nmol/L E_2 + 10 nmol/L ICI 182,780, or 100 ng/mL leptin + 10 nmol/L ICI 182,780, or left untreated in serum-free medium. After treatment, the cells were washed twice with PBS and cross-linked with 1% formaldehyde at 37°C for 10 minutes. Next, the cells were washed twice with PBS at 4°C, collected, and resuspended in 200 mL of lysis buffer [1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-Cl (pH 8.1)] and left on ice for 10 minutes. Then, the cells were sonicated four times for 10 seconds at 40% maximal power (Fisher Sonic Dismembrator, Pittsburgh, PA), and insoluble material was collected by centrifugation at 4°C for 10 minutes at 14,000 rpm. Supernatants were diluted in 1.3 mL of immunoprecipitation buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-Cl (pH 8.1), and 16.7 mmol/L NaCl] and precleared with 80 mL of sonicated salmon sperm DNA/protein A agarose (UBI) for 1 hour at 4°C. The precleared chromatin was immunoprecipitated with either the anti-estrogen receptor α mAb F-10 (Santa Cruz Biotechnology) or the anti-polymerase II CTD4H8 mAb (UBI) for 12 hours. After that, 60 mL of salmon sperm DNA/protein A agarose were added, and precipitation continued for 4 hours at 4°C. After pelleting, the precipitates were washed sequentially for 5 minutes with the following buffers: wash A [0.1% SDS, 1% Trition X-100, 2 mmol/L EDTA, 20 mmol/L Tris-Cl (pH 8.1), and 150 mmol/L NaCl], wash B [0.1% SDS, 1% Trition X-100, 2 mmol/L EDTA, 20 mmol/L Tris-Cl (pH 8.1), and 500 mmol/L NaCl], and wash C [0.25 mol/L LiCl, 1% Nonidet P40, 1% sodium deoxycholate, 1 mmol/L EDTA, and 10 mmol/L Tris-Cl (pH 8.1)]. The precipitates were then washed twice with 10 mmol/L Tris and 1 mmol/L EDTA. The immune complexes were eluted with the buffer containing 1% SDS and 0.1 mol/L $NaHCO_3$. The eluates were reverse cross-linked by heating at 65°C for 12 hours and digested with 0.5 mg/mL proteinase K at 45°C for 1 hour. DNA was obtained by phenol and phenol/chloroform extractions. Two mL of 10 mg/mL yeast tRNA were added to each sample, and DNA was precipitated with ethanol for 12 hours at 4°C and resuspended in 20 mL of 10 mmol/L Tris and 1 mmol/L EDTA. Four mL of each sample were used for PCR with pS2 promoter sequences containing estrogen response element: upstream, 5′-TGG CCA GGC TAG TCT CAA AC-3′; and downstream, 5′-CTT AAT CCA GGT CCT ACT CAT A-3′. The PCR conditions were: 30 seconds at 94°C, 50 seconds at 60°C, and 2 minutes at 72°C. The amplification products obtained in 35 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. The intensity of bands was measured by laser scanning.

Pulse-Chase Labeling. Seventy percent of cultures were shifted to methionine- and cysteine-free Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) for 16 hours and then metabolically labeled with 100 mCi/mL ^{35}S (Express protein labeling mix; Perkin-Elmer, Fremont, CA) for 1 hour. After that, the labeling medium was replaced with serum-free medium containing 10 nmol/L ICI 182,780 or 10 nmol/L ICI 182,780 + 100 ng/mL leptin, and the cultures were grown for 1, 2, 4, 6, and 8 hours. Untreated cells in serum-free medium served as controls. At specific time points, the cells were lysed in RIPA buffer, and 500 mg of proteins were precipitated with the anti-estrogen receptor α F10 mAb. The estrogen receptor α immunoprecipitations were separated by SDS-PAGE, and labeled estrogen receptor α was identified by autoradiography.

Statistical Analysis. Data were analyzed with Student's *t* test, where appropriate. Means ± SE are shown.

RESULTS

ObR_l Is Expressed in Estrogen Receptor α–Positive Breast Cancer Cell Lines. To study possible effects of leptin on ICI 182,780 action, we first assessed the expression of ObR_l, a signaling form of ObR, in different breast cancer cell lines. Several ObR isoforms (M_r ~190,000–150,000) were detected in estrogen receptor α–positive and estrogen receptor α–negative breast cancer cells by Western blotting (Fig. 1). Notably, the greatest expression of ObR_l M_r 190,000 was found in estrogen receptor α–positive cell lines, MCF-7 and T47D. The shorter isoforms of ObR were abundant in estrogen receptor α–negative cells (Fig. 1). For additional experiments, we selected MCF-7

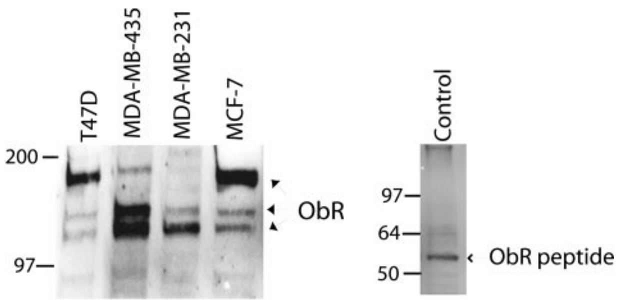

Fig. 1 ObR is expressed in breast cancer cell lines. *Left panel*. The expression of ObR was determined by Western blotting in 50 μg of cytoplasmic protein lysates obtained from proliferating estrogen receptor α–positive (*MCF-7* and *T47D*) and estrogen receptor α–negative (*MDA-MB-231* and *MDA-MB-435*) cells. The ObR Ab used for Western blotting recognizes a common domain in ObR, revealing several isoforms of ObR (M_r 150,000–190,000) indicated by *arrows*. The M_r 190,000 isoform represents ObR_l, which is highly expressed in MCF-7 and T47D cells. *Right panel*. The specificity of the ObR Ab was tested using 250 ng of a M_r 60,000 ObR-tagged fusion protein (amino acids 541–840 of human ObR) provided as a positive control by the manufacturer of ObR Abs (Santa Cruz Biotechnology). The molecular weight markers are indicated on the *left* of both panels.

cells because they are E_2- and ICI 182,780-responsive and express high levels of ObR_l

Leptin Induces Multiple Signaling Pathways in MCF-7 Cells. We examined leptin effects on the activation of different ObR_l signaling pathways in MCF-7 cells. In addition to ObR_l pathways known to be induced in breast cancer cells, i.e., STAT3 and ERK1/2 (18–20), we studied whether leptin can activate Akt/GSK3 antiapoptotic signaling and whether it can phosphorylate (and thereby block) a key cell cycle inhibitor, pRb.

The stimulation of ObR_l by leptin was assessed at different time points, from 5 minutes to 24 hours. We used leptin at a concentration of 100 ng/mL, which in our preliminary dose-response experiments proved to exert maximal mitogenic effects (data not shown). The stimulation of MCF-7 cells with leptin induced multiple signaling elements, including STAT3, ERK1/2, Akt, and GSK3β (Fig. 2). The phosphorylation of STAT3 on Tyr^{705} and on Ser^{727}, reflecting STAT3 activation, was maximal at 5 minutes of leptin treatment and then declined to basal levels (Fig. 2*A*). The stimulation of ERK1/2 become detectable at 15 minutes, was maximal at 1 hour, and persisted up to 24 hours. The activation of Akt appeared at 15 minutes, was maximal at 1 hour, and was reduced to basal levels at 4 hours. GSK3β, a downstream effector of Akt and other kinases was induced at 5 minutes, reached the maximal activation at 1 hour, and then declined to basal levels at 24 hours (Fig. 2*B*). These leptin effects coincided with the phosphorylation of pRb on Ser^{795} (maximum at 1–4 hours; Fig. 2*B*).

Leptin Stimulates the Proliferation of MCF-7 Cells and Interferes with ICI 182,780-Dependent Growth Inhibition. The mitogenic effects of leptin at doses 1 to 1000 ng/mL were studied in MCF-7 cells at 1 and 3 days of treatment. Confirming the results of other investigators (18, 20), we found that the highest proliferation rates were induced with 100 ng/mL leptin, whereas lower leptin concentrations (1 and 10 ng/mL) were less mitogenic (data not shown). Increasing the dose over 100 ng/mL did not improve growth response (data not shown).

At days 1 and 3, 100 ng/mL leptin increased cell growth over that seen in serum-free medium by 20 and 38%, respectively (Fig. 3). Leptin did not affect cell proliferation in the presence of E_2 at any time point. However, leptin consistently counteracted cytostatic effects of ICI 182,780. Specifically, at day 1 and 3, the addition of leptin to ICI 182,780-treated cells increased proliferation by ~30 and ~45%, respectively (Fig. 3). In these studies, ICI 182,780 was used at a concentration of 10 nmol/L, which is cytostatic but not cytotoxic for MCF-7 cells, as demonstrated by us previously (48).

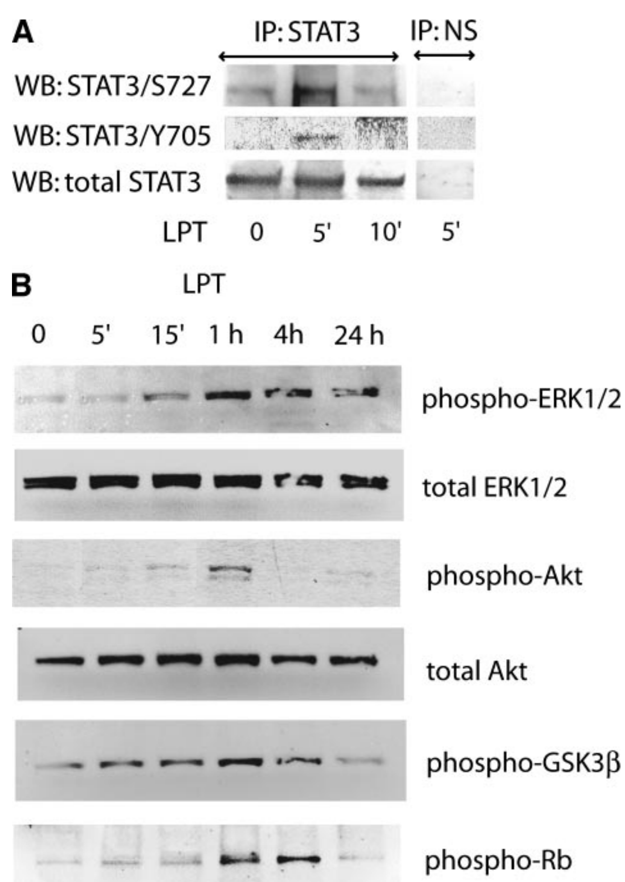

Fig. 2 Leptin activates multiple signaling pathways in MCF-7 cells. *A*, activation of STAT3. MCF-7 cells were synchronized in serum-free medium for 16 hours and then stimulated with 100 ng/mL leptin (*LPT*) for 5 and 10 minutes or left untreated in serum-free medium. STAT3 was immunoprecipitated (*IP*) with the anti-STAT3 pAb (Santa Cruz Biotechnology) from 500 μg of total protein lysates, and the activation of STAT3 was visualized with the STAT3 Ser^{727} pAb (*STAT3/S727*; UBI) and then after stripping of the membrane with the anti-$STAT3^{Tyr705}$ pAb (*STAT3/Y705*; Cell Signaling). In control experiments, the proteins were precipitated with control rabbit immunoglobulin G and processed for Western blotting (*WB*), as described above. *B*, activation of ERK1/2, Akt, GSK3, and Rb. MCF-7 cells were synchronized in serum-free medium for 16 hours and then stimulated with 100 ng/mL leptin (*LPT*) for 5 minutes to 24 hours or left untreated in serum-free medium. The activation (*phospho*) and levels of ERK1/2, Akt, GSK3β, and pRb were assessed by Western blotting in 50 μg of proteins using specific Abs.

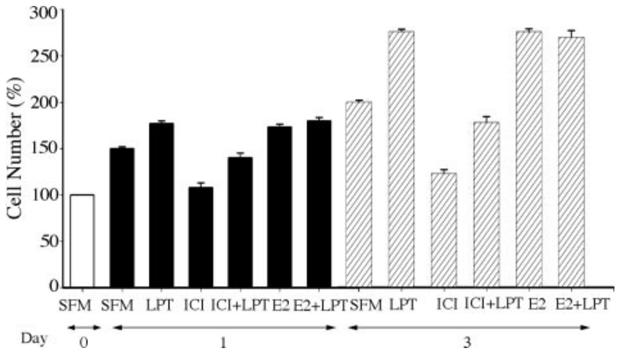

Fig. 3 Leptin stimulates the growth of MCF-7 cells and counteracts the effects of ICI 182,780. Seventy percent confluent MCF-7 cells were synchronized in serum-free medium for 16 hours and treated with 100 ng/mL leptin (*LPT*), 10 nmol/L ICI 182,780 (*ICI*), leptin + ICI 182,780 (*LPT+ICI*), 10 nmol/L E_2, or E_2 + ICI 182,780 (*E2+ICI*) for 1 and 3 days or were left untreated (*SFM*). Cell number was determined by direct cell counting. Please note that MCF-7 cells grow in serum-free medium due to activation of autocrine pathways, as described by us previously (57, 58). Cell number at day 0 in serum-free medium was taken as 100%. The experiments were performed at least four times. The *bars* demonstrate relative cell number (±*SEM*) at different time points. The differences between serum-free medium and leptin values and between leptin and ICI 182,780 + leptin values were statistically significant at days 1 and 3 ($P < 0.05$).

Effects of Leptin on the Nuclear Abundance of Estrogen Receptor α in ICI 182,780-Treated MCF-7 Cells. To study the mechanism of leptin interference with ICI 182,780, we assessed the abundance of cytoplasmic and nuclear estrogen receptor α in MCF-7 cells treated with E_2, E_2 + ICI 182,780, ICI 182,780, ICI 182,780 + leptin, and leptin alone (Fig. 4). As expected, E_2 significantly (by ~50%) decreased the cytoplasmic expression of estrogen receptor α and increased (by ~150%) its nuclear levels, relative to estrogen receptor α under serum-free medium conditions. Also predictably, ICI 182,780 treatment induced the degradation of estrogen receptor α, resulting in reduced estrogen receptor α abundance in the cytoplasm and nucleus (~85 and 70%, respectively). These effects of ICI 182,780 were partially reversed in the presence of E_2 (Fig. 4). The addition of leptin to ICI 182,780-treated cells significantly improved nuclear estrogen receptor α expression but had only minimal effects on the cytoplasmic estrogen receptor α levels. Leptin alone had no significant effects on estrogen receptor α expression in the cytoplasmic and nuclear compartments (Fig. 4).

The above observations were confirmed by fluorescence microscopy of estrogen receptor α in MCF-7 cells treated with ICI 182,780 in the presence or absence of leptin. estrogen receptor α accumulated in the nucleus upon E_2 stimulation, whereas a 24-hour treatment with ICI 182,780 dramatically reduced nuclear estrogen receptor α expression. The effect of ICI 182,780 was prevented by the addition of leptin (Fig. 4*B*).

Leptin Increases Estrogen Receptor α Recruitment to the pS2 Promoter in ICI 182,780-Treated MCF-7 Cells. The function of nuclear estrogen receptor α under different conditions was addressed with chromatin immunoprecipitation assay (Fig. 5). We found that the stimulation of MCF-7 cells with E_2 increased (~5-fold) the recruitment of estrogen receptor α to the classical E_2-responsive estrogen response element–containing pS2 gene promoter. This effect coincided with the greater association of polymerase II to the pS2 regulatory sequences (Fig. 5). In the presence of ICI 182,780, the recruitment of estrogen receptor α to the pS2 promoter was similar to that seen in untreated cells, and the recruitment of polymerase II was completely blocked. The addition of leptin counteracted the inhibitory action of ICI 182,780, resulting in a greater association of polymerase II (increased by ~2-fold) and estrogen receptor α (~3-fold) to the pS2 promoter. Leptin alone did not stimulate the recruitment of either estrogen receptor α or polymerase II to the pS2 promoter (Fig. 5).

Effects of Leptin on Estrogen Receptor α Transcriptional Activity in ICI 182,780-Treated MCF-7 Cells. We validated the information obtained with chromatin immunoprecipitation assays using estrogen response element–luciferase reporter system (Fig. 6). In control experiments, E_2 significantly (by ~250%) stimulated estrogen response element-dependent transcription above the basal level, whereas the addition of ICI 182,780 to E_2 abolished this effect (Fig. 6). Leptin alone did not activate estrogen response element transcription above that seen under serum-free medium conditions. Similarly, leptin did not improve E_2-dependent estrogen response element activation. In the presence of ICI 182,780, estrogen response element activity decreased ~60% below basal levels. In contrast, in ICI 182,780 + leptin cotreated cells, estrogen response element activity was increased ~50% above the level recorded in untreated cells (Fig. 6).

Leptin Increases Estrogen Receptor α Half-Life and Reduces Estrogen Receptor α Ubiquitination in ICI 182,780-Treated MCF-7 Cells. ICI 182,780 is known to induce rapid degradation of estrogen receptor α in MCF-7 (46, 51). We probed the possibility that leptin treatment competes with ICI 182,780 action and increases estrogen receptor α stability. Using pulse-chase assay, we confirmed previous observations that estrogen receptor α half-life in untreated cells is ~4 hours, and in ICI 182,780-treated cells, ~1.5 hours (refs. 47 and 51; Fig. 7*A*). The addition of leptin increased estrogen receptor α half-life to ~2.5 hours (Fig. 7*A*).

Next, we addressed the mechanism by which leptin might decrease estrogen receptor α turnover. Because ICI 182,780- and E_2-dependent degradation of estrogen receptor α occurs through the ubiquitin-proteasome pathway (46, 47), we studied the effects of leptin on estrogen receptor α ubiquitination (Fig. 7*B*). The ubiquitination of estrogen receptor α was undetectable in untreated cells, whereas it was increased when the cells were treated for 1 hour with ICI 182,780 or E_2. The addition of leptin greatly reduced estrogen receptor α ubiquitination in ICI 182,780-treated cells (Fig. 7*B*). Leptin alone did not induce estrogen receptor α ubiquitination. However, estrogen receptor α ubiquitination was still observed when ICI 182,780 was challenged with E_2 (data not shown).

The above treatments had no effects on the expression and ubiquitination of β-catenin, a known target of proteasome (ref. 52; Fig. 7*B*; data not shown).

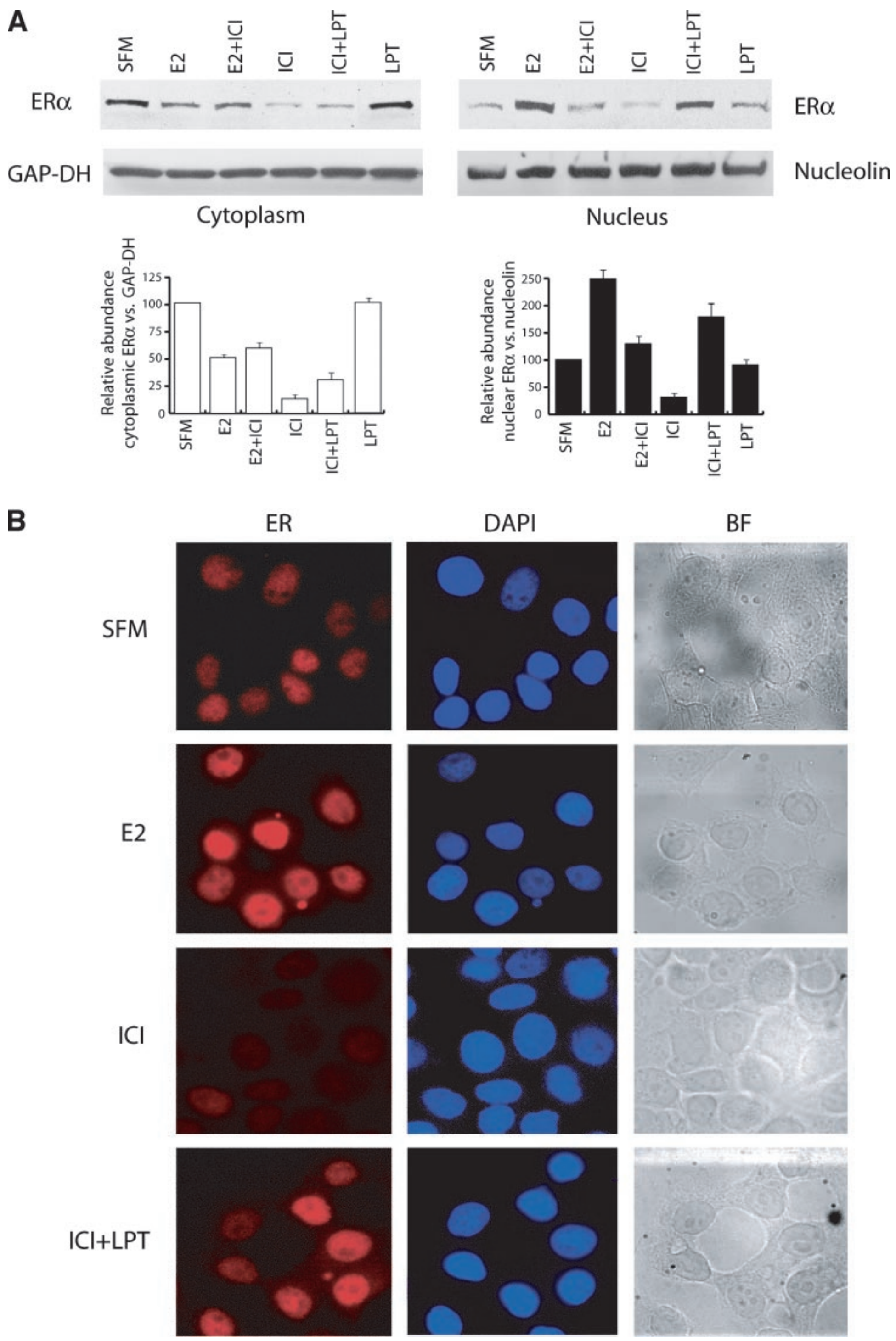

Fig. 4 Leptin increases nuclear abundance of estrogen receptor α (*ER*α) in ICI 182,780-treated MCF-7 cells. *A*, effects of leptin on subcellular estrogen receptor α expression. MCF-7 cells were treated with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), E_2 + ICI 182,780 (*E2+ICI*), 100 ng/mL leptin (*LPT*), or ICI 182,780 + leptin (*ICI+LPT*) for 24 hours or were left untreated (*SFM*). The expression of estrogen receptor α was determined by Western blotting in 50 μg of cytoplasmic or nuclear proteins. The expression of GAPDH (cytoplasmic enzyme) and nucleolin (nuclear protein) was assessed as a control of protein loading. The experiments were performed three times. The *bars* represent mean levels of estrogen receptor α expression (±*SEM*). The differences between ICI 182,780 and ICI 182,780 + leptin values were statistically significant ($P < 0.05$). *B*, fluorescence microscopy. The expression of estrogen receptor α (*ER*) was assessed by immunostaining and fluorescence microscopy in MCF-7 cells treated for 24 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), or 10 nmol/L ICI 182,780 + 100 ng/mL leptin (*ICI+LPT*) or left untreated in SFM. 4′,6-Diamidino-2-phenylindole (*DAPI*) staining and bright field (*BF*) of the same fields is shown to visualize cell nuclei and general morphology. Magnification, ×100.

DISCUSSION

Obesity is a risk factor for the development of breast cancer in postmenopausal women (1–4) and for tumor recurrence in all breast cancer patients, regardless of age and menopausal status (4). However, molecular mechanisms by which excessive fat accumulation could promote mammary carcinogenesis remain unknown. One possibility is that the process is mediated by elevated estrogen levels produced by adipose tissue in postmenopausal women (35, 53). In addition, it has been suggested that the development and progression of breast cancer could be stimulated by mitogenic and transforming activity of leptin (18), the levels of which rise proportionally to body mass index and

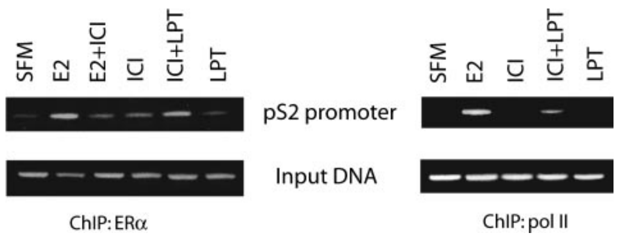

Fig. 5 Leptin increases estrogen receptor α recruitment to pS2 promoter in ICI 182,780-treated MCF-7 cells. The cells were treated for 4 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), 100 ng/mL leptin (*LPT*), ICI 182,780 + leptin (*ICI+LPT*), E_2 + ICI 182,780 (*E2+ICI*) or left untreated (*SFM*). The cells were then cross-linked with formaldehyde and lysed, and soluble, precleared chromatin was obtained. The soluble chromatin was immunoprecipitated with either the anti-estrogen receptor α F-10 mAb (Santa Cruz Biotechnology; *ChIP: ERα*) or the anti-polymerase II CTD4H8 mAb (UBI; *ChIP:pol II*). The estrogen receptor α and polymerase II immunocomplexes were reverse cross-linked, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The pS2 promoter sequences containing estrogen response element were detected by PCR with specific primers, as detailed in Materials and Methods. To control input DNA, pS2 promoter was amplified from 30 μL of initial preparations of soluble chromatin (before immunoprecipitations).

are generally higher in women than in men (10). Furthermore, because estrogen receptor α and ObR have been found coexpressed in malignant mammary tissue and breast cancer cell lines (18–20), it is also possible, that both signaling systems are involved in a functional cross-talk contributing to carcinogenesis. However, leptin/E_2 interactions and their possible role in breast cancer have not been extensively studied. In this work, we investigated whether the presence of leptin could compete with antiestrogenic effects produced by ICI 182,780.

First, we provided evidence that estrogen receptor α–positive breast cancer cells MCF-7 and T47D express higher levels of ObRl than estrogen receptor α–negative cell lines MDA-MB-231 and MDA-MB-435. These results confirmed the data of other investigators who demonstrated ObR_l expression in T47D and MCF-7 cells (18–20) but lack of ObR_l mRNA in MDA-MB-231 cells (19). As a cellular model of this study, we selected estrogen receptor α–positive and ICI 182,780-sensitive MCF-7 breast cancer cells. We demonstrated that MCF-7 cells respond to leptin stimulation with the activation of several signaling intermediates, including the STAT3, ERK1/2, and Akt pathways (Figs. 1 and 2). In MCF-7 cells, leptin was also able to inactivate the cell cycle inhibitor pRb and stimulate cell growth (Fig. 2). These results extend the observations of Xu *et al.* (18), Dieudonne *et al.* (19), Laud *et al.* (20), and Okamura *et al.* (22), who described leptin-dependent proliferation and leptin-induced STAT3 and ERK1/2 signaling in different estrogen receptor α–positive breast cancer cell lines. The maximal mitogenic concentrations of leptin used in our and other studies (100 ng/mL) are in the range of serum leptin levels found in obese and morbidly obese (body mass index > 40) individuals (54, 55).

The growth of estrogen receptor α–positive breast cancer cells can be effectively inhibited by ICI 182,780, which induces rapid proteasome-mediated degradation of estrogen receptor α (44–46). We report here, for the first time, that antiestrogenic action of ICI 182,780 can be significantly reduced in the presence of leptin. Specifically, in ICI 182,780-treated MCF-7 cells, leptin increased estrogen receptor α half-life and decreased estrogen receptor α ubiquitination. These effects coincided with elevated nuclear estrogen receptor α expression, increased estrogen receptor α recruitment to the E_2-sensitive gene promoter, and increased estrogen response element–dependent transcription. Leptin also counteracted cytostatic effects of ICI 182,780, resulting in increased cell proliferation (Figs. 3–7).

Interestingly, the mechanism by which leptin competes with ICI 182,780 appears to be different from that exerted by E_2. For instance, estrogen receptor α is still ubiquitinated in ICI 182,780 + E_2-treated cells, whereas it is not ubiquitinated in ICI 182,780 + leptin-treated cells (Fig. 7*B*; data not shown). Similarly, the abundance of nuclear estrogen receptor α is higher under ICI 182,780 + leptin conditions than that seen in ICI 182,780 + E_2-treated cells (Fig. 4*A*). In part, this phenomenon could be explained by the recent discovery that estrogen receptor α turnover is differentially regulated depending on whether the receptor is unliganded, agonist bound, or antagonist bound and whether other cellular pathways (*e.g.,* MAP kinases) are induced by cell surface receptors (46). Thus, it is possible that leptin can exert its action only on ICI 182,780-dependent estrogen receptor α processing. Indeed, in different assays, we did not observe any effects of leptin on basal or E_2-induced activity

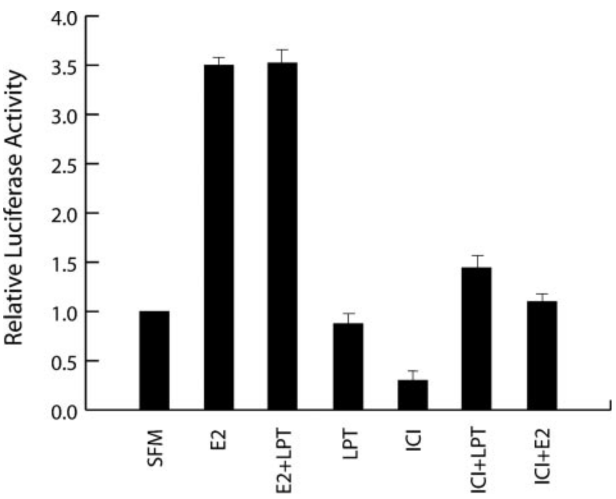

Fig. 6 Leptin increases estrogen receptor α transcription at estrogen response element promoters in ICI 182,780-treated cells. MCF-7 cells grown in 24-well plates were transfected for 6 hours with 0.5 mg of DNA per well using Fugene 6. All transfection mixtures contained 0.5 mg of estrogen response element reporter plasmid estrogen response element-TK-Luc. In addition, each of the DNA mixtures contained 50 ng of pRL-TK-Luc plasmid encoding renilla luciferase to assess transfection efficiency. Upon transfection, the cells were shifted to SFM for 16 hours and then treated for 24 hours with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), 100 ng/mL leptin (*LPT*), ICI 182,780 + leptin (*ICI+LPT*), E_2 + ICI 182,780 (*E2+ICI*) or left untreated (*SFM*). Luciferase activity (Luc and RI Luc) was measured in cell lysates with a luminometer. Relative Luc activity in each sample was obtained upon normalization of Luc to RI-Luc values. The mean relative Luc activity (±*SEM*) obtained in five experiments is shown. The differences between leptin and ICI 182,780 values and between ICI 182,780 and leptin + ICI 182,780 values were statistically significant ($P < 0.05$).

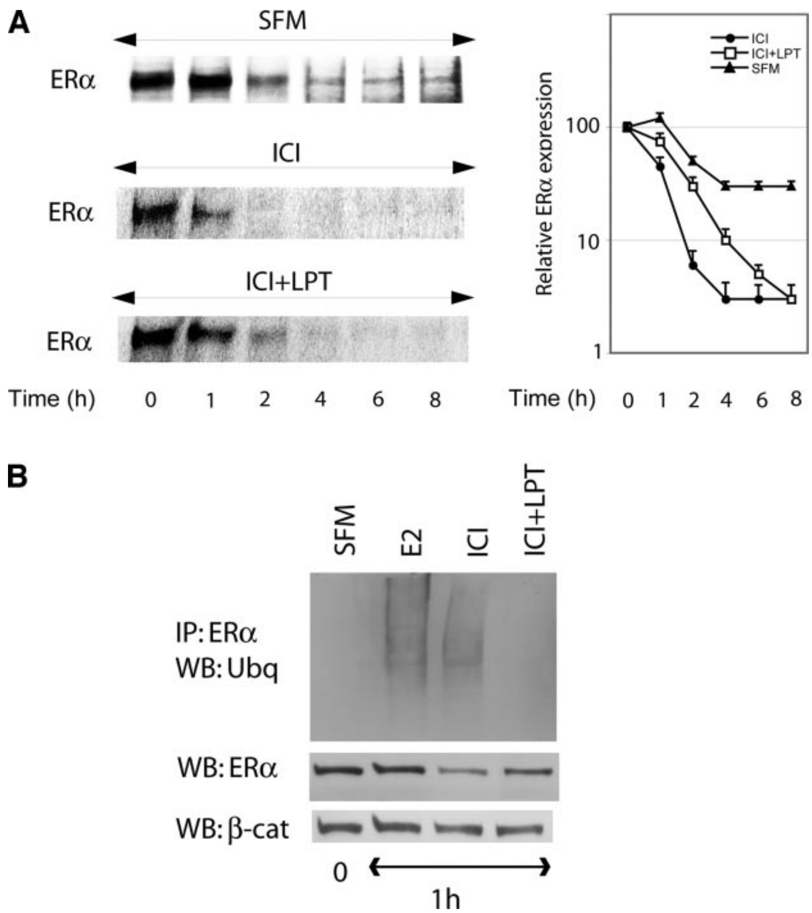

Fig. 7 Leptin effects of estrogen receptor α half-life and ubiquitination in ICI 182,780-treated MCF-7 cells. *A*, effects of leptin on estrogen receptor α half-life. The half-life of estrogen receptor α was determined by ^{35}S pulse-chase labeling, as described in Materials and Methods. The abundance of estrogen receptor α was analyzed at different time points (0, 2, 4, 6, and 8 hours) in untreated cells (*SFM*) and in cells treated with 10 nmol/L ICI 182,780 and 10 nmol/L ICI 182,780 +100 ng/mL leptin (*ICI+LPT*). The expression of estrogen receptor α at time 0 was assigned a value of 100. The relative estrogen receptor α expression (±*SEM*) under different experimental conditions is presented in the graph. This experiment was repeated three times. The differences between ICI 182,780 and ICI 182,780 + leptin values were statistically significant ($P < 0.05$) at 1, 2, and 4 hours. *B*, effects of leptin on estrogen receptor α ubiquitination. MCF-7 cells were treated for 1 hour with 10 nmol/L E_2, 10 nmol/L ICI 182,780 (*ICI*), or 10 nmol/L ICI 182,780 + 100 ng/mL leptin (*ICI+LPT*) or left untreated in SFM. Estrogen receptor α was immunoprecipitated (*IP*) from 500 μg of total protein lysates, and its levels and ubiquitination were evaluated by Western blotting (*WB*) with specific Abs, as described in Materials and Methods. The expression of β-catenin (β-*cat*) in 50 μg of total protein lysates is shown as a control of protein loading.

of estrogen receptor α. These data suggest that in our cell model, leptin did not modulate the synthesis of endogenous E_2. This latter point is worth discussion because leptin has been suggested as a potential modulator of E_2 production (36–40). In some cell models (36, 37), including breast cancer cells (38), leptin has been shown to stimulate the aromatase gene promoter and aromatase activity. Furthermore, pharmacologic doses of leptin (1000 ng/mL) apparently activate estrogen response element promoters, presumably through the stimulation of E_2 synthesis (56), however, increased E_2 expression has not been formally shown in this setting. Our data included in Figs. 4*A*, 5, and 6 suggest that the exogenous E_2 levels were similar in untreated and leptin-induced MCF-7 cell cultures.

In summary, we demonstrated that leptin interferes with the action of ICI 182,780 in MCF-7 cells. Our results suggest that the mechanism of this phenomenon involves increased nuclear expression and activity of estrogen receptor α but is independent of E_2. Future studies should explore whether obesity might impede the benefits of ICI 182,780 therapy in breast cancer patients.

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