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Obesity is a risk factor for breast cancer in postmenopausal women. Adipose tissue is a major source of leptin, a cytokine acting as a key regulator of energy balance. Leptin can also induce mitogenic and angiogenic effects in different cell types. New data suggested that in breast cancer cells, leptin can stimulate proliferation by activating the long form of the Ob receptor (ObR). We hypothesized that in obese women, locally elevated levels of leptin could promote the growth of primary breast tumors.

The results of our work indicated that 1) several breast cancer cell lines express the long and short form of the leptin receptor (Ob-R); 2) leptin stimulates the proliferation in different breast cancer cell lines by 30-40%; 3) in MCF-7 cells, leptin activates the ERK1/2 and STAT3 signaling pathways; 4) in MCF-7 cells, leptin interferes with the action of the antiestrogen ICI 182,780 by upregulating the activity of estrogen receptor alpha. In summary, our data suggested that higher levels of leptin could promote breast cancer cells growth and impede the efficacy of hormonal treatments.

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INTRODUCTION

Obesity in the United States has reached the alarming rate of ~60% and is considered a second, after smoking, major killer. The link between obesity and breast cancer development has been postulated but the molecular mechanisms involved are not clear (1).

Leptin, a 16 kDa protein product of the *OB* (obesity) gene is a cytokine originally discovered as a key regulator of body fat mass acting in the central nervous system (2). The abundance of leptin is greater in females than in males and is regulated by steroid hormones and growth factors, such as estradiol, insulin and insulin-like growth factor I (3-7). The levels of these substances are elevated in individuals with upper body obesity. This type of obesity correlates with increased breast cancer risk in post-menopausal women (1). In addition to its role as a regulator of appetite and metabolism, leptin can be involved in other processes, such as hematopoiesis, reproduction, and immunity (2,7) Recently, it has been demonstrated that leptin can act as a mitogen, chemoattractant, and angiogenic factor in different cell models (8-12). New data documented that human breast cancer cell lines and breast tumors may express leptin and leptin receptor (Ob-R) (8,9,13,14) In addition, leptin has been show to induce DNA synthesis and growth in MCF-7 and T47D breast cancer cell lines (8,9,14). We hypothesized that in obese women, locally elevated levels of estrogens and insulin might increase the synthesis of leptin in adipocytes and/or epithelial cells, in effect leading to increased proliferation and resistance to antiestrogen treatment.

TECHNICAL REPORT

During the reporting performance period we addressed the role of leptin in breast cancer cell proliferation and migration, as proposed in SOW. The performance period has been extended to 2 years to conclude the experiments on the effects of leptin on the efficacy of antiestrogen treatment in breast cancer cells.

1. Expression of leptin receptors in breast cancer cell lines.

At least five different isoforms of the Ob-R have been predicted to exist (15,16). The most ubiquitous are the long and short forms of Ob-R. The long Ob-R has a 302 aa cytoplasmic domain and a 841 aa extracellular domain. The short form Ob-R consists of a small fragment of the cytoplasmic domain (32-40 aa) and a full extracellular domain. Alternative splicing generates other forms that differ mostly in the length of the cytoplasmic domain (15). In addition, a presence of a soluble Ob-R has been described (17). The soluble Ob-R is generated by proteolytic cleavage of the extracellular domain and acts as leptin binding protein regulating leptin availability (6,17). The stimulation of the intracellular signal transmission by leptin

requires the ~ 45-65 kDa cytoplasmic domain. The phosphorylation of Tyrosine 985 and 1138 has been shown critical in the activation of STAT3 and ERK1/2 signaling pathways (16).

We studied the expression of ObRl and ObRs in different ER-alpha (ER)-positive and ER-negative breast cancer cell lines. As demonstrated in Fig. 1, both types of receptors are expressed in breast cancer cells. The expression of specific isoforms varies among cell lines (Fig. 1).

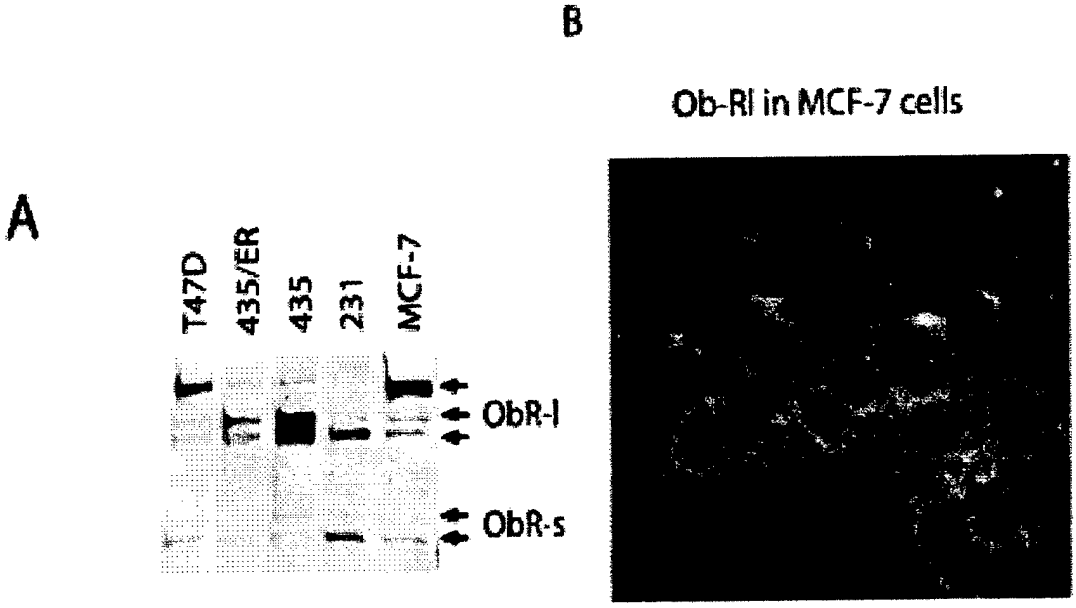

Fig. 1. The expression of Ob-R in different breast cancer cell lines. A. *The expression of Ob-R was determined in ER-positive (MCF-7, T47D, 435/ER) and ER-negative (MDA-MB-231, MDA-MB-435) cells. 70% cultures of cells were lysed using buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaF, 0.2 mM Na_3VO_4, 1% PMSF, 2 ug/ml aprotinin. The expression of Ob-R was analyzed in 50 ug of cell lysate by Western blotting with specific anti-Ob-R antibodies (Linco Research). The antibody recognizes the Ob-R long form, (ObRl) several isoforms: 150-190 kDa and Ob-R short form, (Ob-Rs) 2 isoforms: 85, 95 kDa).* ***B.*** *Localization ObRl was assessed in MCF-7 cells cultured in phenol red-free serum-free medium (SFM) for 24h. The cells were fixed in 3% paraformaldehyde, stained with the primary antibody anti-Ob-Rl C20 (Santa Cruz) 10 ug/ml, and then with fluorescein-conjugated donkey anti-goat IgG secondary Ab. The localization of Ob-Rl was studied with Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135M inverted microscope. The captured image of Ob-Rl (green fluorescence) is shown. The optical sections were taken at the central plane*

2. Effects of leptin on the growth of breast cancer cells.

Leptin has been shown to induce DNA synthesis in several cell lines (8,9,12,14). Here we tested whether leptin can stimulate cell proliferation of breast cancer cell lines. The results

indicated that leptin at a concentration 100 ng/ml moderately stimulates the growth of ER-positive MCF-7 cells and ER-negative MDA-MB-435 cells (Tab. 1). Lower doses (1 and 10 ng/ml) as well as higher dose of 1000 ng/ml were less effective (data not shown).

	Cell Number ($x10^4$)					
Day	0		2		4	
Leptin	-	+	-	+	-	+
MCF-7 cells	22±3	22±3	28±2	48±4	33±5	51±3
MDA-MB-435 cells	26±4	26±4	41±1	58±6	38±1	54±5

Tab. 1. Effects of leptin on the growth of breast cancer cells. *To analyze the growth in the presence of leptin, the cells were plated in 6-well plates at a concentration of 1.5-2.0x10^5 cells/plate in DMEM:F12 (1:1) containing 5% calf serum. The following day (day 0), the cells at approximately 50% confluence were shifted to serum-free medium (SFM) containing 100 ng/ml leptin or were left untreated (control of basal proliferation in SFM). Cell number was determined at days 0, 2, and 4. The results are average from 3 experiments +/- SD.*

3. Effects of leptin on breast cancer cell migration.

Leptin has been shown to induce invasion or migration in some cell systems (11). We investigated whether leptin can act as a chemoattractant for breast cancer cells. Using Boyden chambers we tested migration of two ER-positive cell lines, MCF-7 and T47D, and two ER-negative cell lines, MDA-MB-231 and MDA-MB-435 (Tab. 2). The results suggested that leptin has negligible effects on the migration of breast cancer cells, regardless of their differentiation status.

Cell Line	Induction of Migration (%)
MCF-7	+ 10±5
T47D	+ 7 ±2
MDA-MB-231	- 11 ±4
MDA-MB-435	+ 15±7

Tab. 2. Effects of leptin on breast cancer cell migration. *The effects of leptin were tested on breast cancer cell lines characterized by different degree of differentiation (best MCF-7>T47D>MDA231>MDA453 least). The values represent % decrease/increase relative to untreated cells and are average from 3 experiments +/- SD. The migration of cells was tested using two-well chambers divided with an 8 um porous membrane. 20,000 of cells synchronized for 24 h in SFM and suspended in 200 ul of SFM were plated into the upper chamber. The lower chamber contained 500 ul of either SFM (control of basal migration) or SFM + 100 ng/ml leptin. The cells were allowed to migrate for 24 h. Then, the cells from the upper chamber were removed and the cells that traversed the membrane were fixed and stained. The number of migrating cells was determined by direct counting under the microscope. The results are average from 3 experiments +/- SD.*

4. Leptin signaling in MCF-7 cells.

Next, we studied signaling pathways induced by leptin treatment in MCF-7 cells (Fig. 2). The results indicated that leptin can induce several common growth-associated pathways, specifically the STAT3, ERK1/2, Akt/GSK3 pathways. One of the end-points of leptin signaling was the growth suppressor protein, pRb. The phosphorylation of pRb, as seen with leptin, normally reflects inactivation of pRb and induction of cell growth.

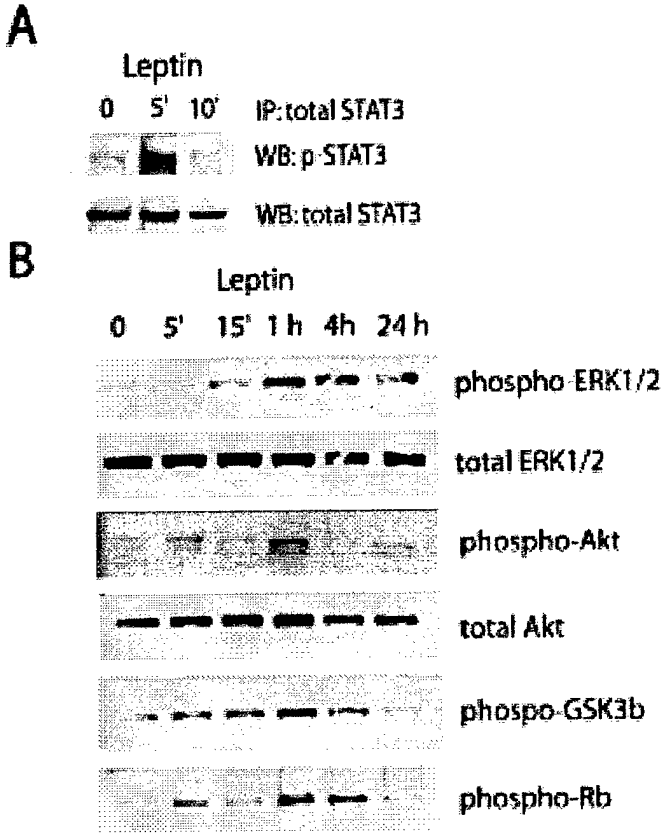

Fig. 2. Leptin signaling in MCF-7 cells. *MCF-7 cells were synchronized in phenol red-free serum free medium (SFM) for 16 h and then stimulated with 100ng/ ml leptin (R&D Systems) for 0-24 h, or left untreated.* ***A. Activation of STAT3.*** *STAT3 phosphorylation on Ser 727 was evaluated in 500 ug of proteins from treated and untreated cells. The proteins were immunoprecipitated with STAT3 Ab (Santa Cruz) (total-STAT3) and the activation of STAT was visualized with STAT3 Ser^{727} Ab (p-STAT3) (Cell Signaling).* ***B. Activation of ERK1/2, Akt, GSK3, and Rb.*** *The activation and the levels of ERK1/2, Akt, GSK3, and pRb were assessed in 50 ug of proteins using specific Abs (Cell Signaling).*

5. Effects of leptin on the efficacy of the antiestrogen ICI182,780.

Because leptin appeared to stimulate breast cancer cell proliferation, we asked whether leptin can interfere with the action of antiestrogens. Antiestrogens, such as tamoxifen and ICI 182,780 (ICI) are being used as adjuvant therapy for metastatic breast cancer as well as are now indicated for breast cancer prevention in women at high risk of breast cancer development. These antiestrogens inhibit breast cell proliferation by inactivating ER.

The treatment of MCF-7 cells with 10nM ICI for 3 days inhibited proliferation by 40%± 3(SE), while 100 ng/ml leptin increased cell growth by 37%± 2(SE). The simultaneous treatment with ICI and leptin produced only minimal growth inhibition (11%± 2 SE) (Fig. 3).

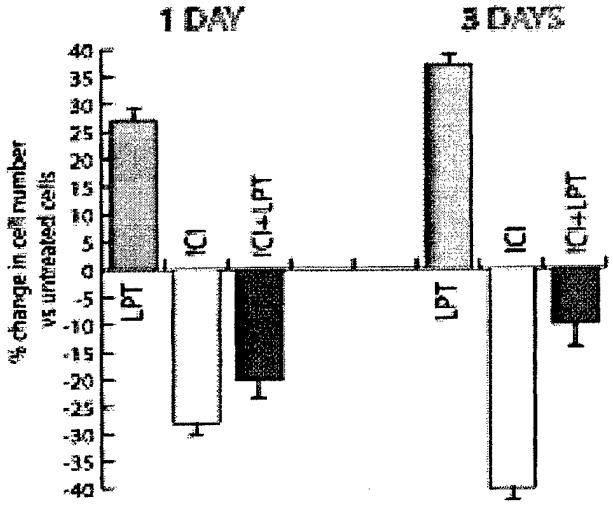

Fig. 3. Effects of leptin on cell proliferation in MCF-7 cells treated with ICI 182,780. *70 % confluent MCF-7 cells were synchronized in SFM and treated with 100 ng/ml leptin (LPT), 10 nM ICI 182,780 (ICI), or LPT+ICI for 1 and 3 days, or were left untreated. Cell number was determined by direct cell counting at the designated time points. The bars show % change in cell number (± SD) relative to untreated cells at the same day*

Next, using Western immunobloting, chromatin immunoprecipitation, and luciferase reporter assays, we studied whether leptin can counteract the effects of ICI on ER

As expected, the treatment of MCF-7 cells with ICI alone inhibited ER expression in the nuclear and cytoplasmic protein fractions (Fig. 4). The addition of leptin increased nuclear (but not cytoplasmic) ER abundance by ~ 3-fold (Fig. 4). In addition, leptin augmented by ~70% the recruitment of ER to the estrogen receptor element (ERE)-containing pS2 gene, relative to the effects obtained with ICI alone (Fig. 5). Furthermore, in cells exposed to the

combined leptin + ICI treatment, ER transcriptional activity was 2.5 greater than that observed with ICI alone (Fig. 6). These results suggest that leptin might upregulate ER activity, and thus interfere with ICI antiestrogenic action. The molecular mechanisms of this phenomenon are under investigation.

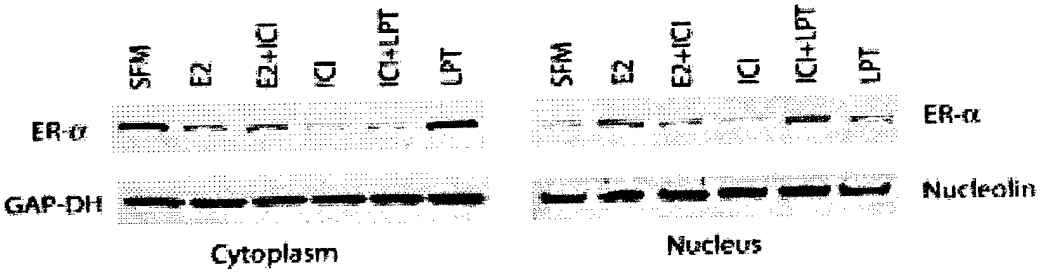

Fig. 4. Leptin increases nuclear abundance of ER in MCF-7 cells treated with ICI 182,780. *MCF-7 cells were treated with 10 nM E2 (E2), 10 nM ICI 182,780 (ICI), E2+ICI, 100 ng/ml leptin (LPT), or ICI+LPT for 24 h, or were left untreated (SFM). The expression of ER was determined by WB in cytoplasmic (C) or nuclear (N) proteins using F-10 Ab (Santa Cruz). The expression of GAP-DH (cytoplasmic enzyme) and nucleolin (nuclear protein) was assessed as a control of lysates purity.*

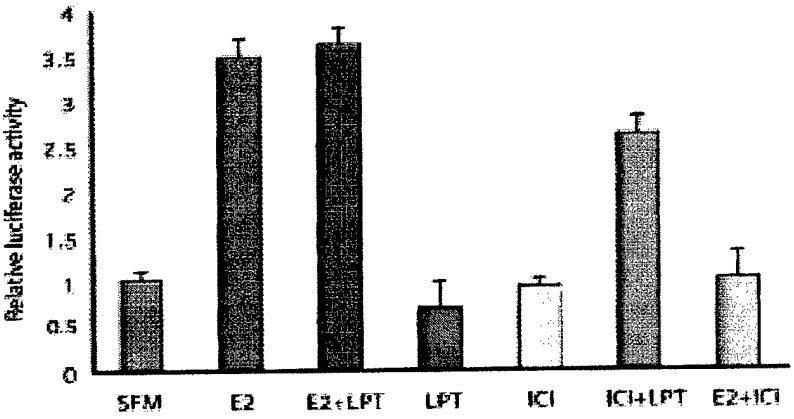

Fig. 5. Leptin interferes with ICI 182,780 inhibition of ER transcriptional activity. *MCF-7 cells grown in 24-well plates were transfected for 6 h with 0.5 ug DNA/well using Fugene 6 (Roche). All transfection mixtures contained 0.5 ug of ERE reporter plasmid ERE-TK-Luc (ERE). In addition, each of the DNA mixtures contained 50 ng of pRL-TK-Luc plasmid encoding renilla luciferase (RI Luc) to assess transfection efficiency. Upon transfection, the cells were shifted to SFM for 16 h and then treated for 24 h with 10 nM E2 (E2), 1uM ICI 182,780 (ICI), 100 ng/ml Leptin (LPT), ICI+LPT, E2+ICI, or left untreated (SFM). Luciferase activity (Luc and RI Luc) was measured in cell lysates with luminometer. Relative Luc activity in each sample was obtained upon normalization of Luc to RI-Luc values.*

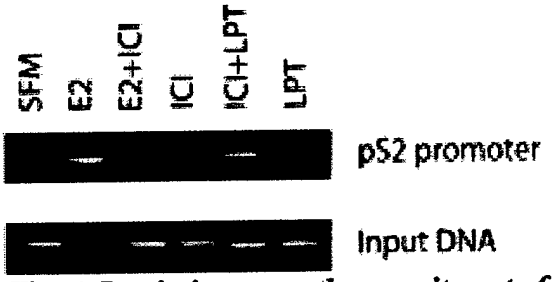

Fig. 6. Leptin increases the recruitment of ER to the pS2 gene promoter in MCF-7 treated with ICI 182,780. *The association of ER with the pS2 promoter sequences was studied with chromatin precipitation assays, as described in Ref. 18. Briefly, the cells were treated with 10 nM E2 (E2), 1uM ICI 182,780 (ICI), 500ng/ml Leptin (LPT), ICI+LPT, or E2+ICI for 60 min or left untreated (SFM). Then, the cells were cross-linked with formaldehyde, lysed, and soluble, pre-cleared chromatin was obtained. 1 ml of soluble chromatin solution was immunoprecipitated with 10 ug of anti-ERα Ab (Santa Cruz). The immune complexes were reverse cross-linked, and DNA was recovered by phenol/chloroform extraction and EtOH precipitation. 5 ul of the final DNA extract was used to detect pS2 promoter sequences containing ERE. The pS2 primers were upstream 5'-GAT TAC AGC GTG AGC CAC TG-3', downstream 5'-TGG TCA AGG TAC ATG GAA GG-3'. To determine input DNA, pS2 promoter was amplified from 5 ul of purified soluble chromatin before immunoprecipitation.*

Key Research Accomplishments:

- **Documented that several breast cancer cell lines express leptin receptors ObRl and ObRs;**
- **Demonstrated that leptin is a mitogen for breast cancer cells;**
- **Demonstrated that in MCF-7 cells leptin stimulates several growth-associated signaling pathways: Akt/GSK3, ERK1/2 and STAT3;**
- **Demonstrated that in MCF-7 cells leptin competes with the action of the antiestrogen ICI182,780 by improving the activity of ER.**

Reportable Outcomes:

1. Manuscripts, abstracts and scientific presentations:

Manuscripts:

Surmacz, E. Leptin--a growth factor in normal and malignant cells and for normal mammary development. Commentary. Women's Oncol. Rev, in press, 2003.

Sauter, E. R., Garofalo, C., Hewett, J., Morelli, C., Surmacz, E. Leptin expression in breast nipple aspirate fluid (NAF) is influenced by body mass index (BMI) in premenopausal women, in preperation.

Abstracts:

Garofalo, C., Sisci, D., Morelli, C., Surmacz, E. Leptin (obesity protein) interferes with the action of antiestrogen ICI 182,780 in MCF-7 breast cancer cells. IV International Symposium on Hormonal Carcinogenesis, Valencia, Spain, July 22-25, 2003.

Sauter ER, Garofalo C, Hewitt J, Surmacz E. Leptin expression in nipple aspirate fluid (NAF) is influenced by BMI and menopausal status. Breast Cancer Symposium, San Antonio, December 10-13, 2002.

Garofalo, C., Sauter, E., Surmacz, E. Leptin (obesity protein) in nipple aspirates and breast cancer risk. Era of Hope DOD Breast Cancer Program Meeting, Orlando, FL, September 25-28, 2002

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2003-2004 DOD Breast Cancer Research Program Concept Award "Obesity and postmenopausal breast cancer: leptin-estrogen cross-talk". E. Surmacz (PI), funded

7. Employment applied for: None

Conclusions

Leptin (obesity protein) may play a role in breast cancer development by stimulating the growth of breast cancer cells. In addition, leptin seems to counteract the action of antiestrogens, thus it may be involved in the development of antiestrogen resistance. Further studies are necessary to probe the molecular mechanism of this effect and the relevance of these findings in clinical setting.

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