

Zeranol enhances leptin-induced proliferation in primary cultured human breast cancer epithelial cells

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Abstract. Breast cancer is the leading type of cancer in women in the United States. One of the known risk factors of breast cancer is obesity. Leptin is a product of the obese (*ob*) gene and plays an important role in breast cancer development. Its expression is up-regulated in obesity and it promotes breast cancer cell growth. Exposure to environmental estrogenic disruptors has been found to be directly related to the increase in the incidence of breast cancer. Zeranol (Z) is a non-steroidal anabolic growth promoter with potent estrogenic activity that is widely used in the US beef industry. The objective of this study was to determine the mechanisms of Z- and leptin-induced proliferation of primary cultured human breast cancer epithelial cells (HBCECs). A cell proliferation assay was used to determine the extent to which Z is capable of enhancing the mitogenic activity of leptin in HBCECs. RT-PCR was used to explore the possible mechanisms by quantifying the transcription of cyclin D1 and ObR genes. Our results demonstrated that when the HBCECs were pre-treated with 3 nM leptin for 24 h, the sensitivity to Z exposure greatly enhanced the mitogenic action of leptin. The experimental data observed show that there is interaction between leptin and Z in HBCEC growth.

Introduction

Breast cancer is the most commonly diagnosed type of cancer among women and was the second leading cause of mortality among women in 2008 (1). One of the currently known risk factors of breast cancer is obesity, which has become a major public health concern (2). The incidence of breast cancer is increased with obesity, and morbidity is also increased in obese cancer patients as compared to cancer patients with

normal or low weight (3,4). The relationship between breast cancer and obesity has been studied for many years (2).

Leptin, a product of the obese (*ob*) gene, has been studied since its discovery in 1994 (5). It is a 167-amino acid protein with a molecular mass of 16 kDa. Besides its involvement in appetite regulation and energy balance (6), leptin has a number of other regulatory functions. They include ensuring normal mammary gland development, bone development, fetal development, sex maturation, angiogenesis, lactation, hematopoiesis and immune responses (7).

Leptin promotes breast cancer cell growth by signaling through its receptors and by directly affecting the estrogen receptor (ER) pathway (3,7). The leptin receptor (ObR), localized in the cell membrane of various tissues including breast cancer tissue (6), has been identified as having one long isoform and five short isoforms (2). Both the short and long isoforms are expressed in normal mammary epithelial cells during pregnancy and lactation (2,3). Such expression suggests an autocrine action of leptin in the regulation of mammary gland growth and development. Furthermore, since it is commonly expressed in breast cancer, this gene could serve as a breast cancer molecular biomarker or a prognostic or diagnostic indicator (8).

Similar to other growth factors and cytokines, leptin and its receptor are present in human serum and many organs, and play a role in human cancer development (7). Since leptin was found to be associated with several types of cancer (9), researchers attempted to find the relationship and mechanisms of leptin action in prostate cancer (10), gastric cancer (11), oral cancer (12), esophageal adenocarcinoma (13), hepatocellular carcinoma (14), gallbladder cancer (15) and breast cancer (16-22). Ishikawa *et al* found that leptin was overexpressed in breast cancer cells (23) and concluded that high leptin levels in obese breast cancer patients may play a role in the development of antiestrogen resistance (21,23). Leptin is not expressed in normal breast tissue, but exists near malignant breast lesions (8), while its receptors were detectable in breast cancer cells, but undetectable in normal mammary epithelial cells (23). In addition to its mitogenic effects, leptin promotes the proliferation of the human breast cancer cell line T47-D (9). A high level of leptin may contribute to the development of a more aggressive malignant phenotype (24). The power of leptin to stimulate human MCF-7 cell growth and its ability to

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counteract the effects of ICI 182,780, which is a pure estrogen antagonist approved for the treatment of breast cancer patients who fail to respond to tamoxifen therapy, strongly suggests that leptin acts as a paracrine/endocrine growth factor towards mammary epithelial cells (21,22).

Estrogen has been regarded as a positive regulator of leptin production (25). Serum leptin levels in breast cancer patients are significantly higher than levels in a control group (26). Thorn *et al* found that estrogen modulates ObR expression in some estrogen-responsive tissues (25,27). Zearalenone, a stable natural product that mimics estrogen activity, is a carcinogen that is regarded as hazardous to human health (28). Zeranol (Z), produced from Zearalenone, is a non-steroidal anabolic growth promoter with potent estrogenic activity approved by the FDA that is widely used to stimulate cattle growth in the US beef industry (29). Both Zearalenone and Z bind to the active site of human ER α and ER β in a similar manner to 17 β -estradiol (30). As a food contaminant, Z is very hard to avoid (28). Researchers have reported that Z does not affect the serum concentration of leptin in growing wethers (31). At a low concentration, Z increases ER α -positive cell growth, but at higher concentrations it reduces the proliferation of both ER α -positive and ER α -negative cell lines (32). Moreover, our previous data showed that Z transforms the human normal breast epithelial cell line MCF-10A and increases primary cultured human normal and cancerous breast epithelial cell growth in a dose-dependent manner (29). It also down-regulates expression of the estrogen-regulated human breast cancer candidate suppressor gene protein tyrosine phosphatase γ (PTP γ) (33). Furthermore, we found that implantation of Z in beef heifers greatly induces pre-adipocyte growth by up-regulating cyclin D1 and down-regulating P53 expression (34). We also found that 2.5% Z containing sera harvested from 60-day post-Z-implanted beef cattle (72 mg Z pellets) was capable of transforming the human normal breast epithelial cell line MCF-10A to neoplastic breast cancer cells within a 21-day culture. The objective of this study was to investigate the interactions of leptin and Z in primary cultured human breast cancer epithelial cells.

Materials and methods

Reagents. Recombinant human leptin was purchased from R&D Systems (Minneapolis, MN, USA) and was prepared as a stock solution of 1 mg/ml in sterile 20 mM Tris/HCl (pH 8.0) then stored at -20°C. Z was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and was prepared as a 50% stock solution in dimethyl sulfoxide (DMSO).

Human breast normal and cancer tissues. Human breast normal and cancer tissues were obtained through the Tissue Procurement Program of the Ohio State University Comprehensive Cancer Center Hospital (Columbus, OH, USA). At the time of procurement, the tissue samples were placed in Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM/F12) without phenol red (Sigma Chemical Co.) and stored at 4°C before transfer to the laboratory.

Primary cultured human breast normal and cancer epithelial cell isolation. Fourteen breast normal and cancer tissues

(six matched tissues; both the normal and cancer tissues were from the same patients) from 11 patients were sterilized in 70% ethanol for 30 sec and then washed three times with fresh DMEM/F12. The breast normal and cancer tissues were minced and then digested using digestion buffer, which consisted of phenol red-free high calcium DMEM/F12 (1.05 mM CaCl₂) with 2% bovine serum albumin (BSA; Invitrogen, Carlsbad, CA, USA) containing 10 ng/ml Cholera toxin (Sigma Chemical Co.), 6,300 U/ml Collagenase (Invitrogen) and 100 U/ml Hyaluronidase (Calbiochem, Gibbstown, NJ, USA). After the mixture was incubated at 37°C overnight, the solution was transferred to a 45-ml tube and centrifuged in 1,200 rpm for 5 min. The upper layer containing oil and fat tissue was discarded and the middle and lower layers were centrifuged again. The middle layer containing stromal cells was transferred to another 45-ml tube and centrifuged. The lower layer containing pellet was washed with DMEM/F12 medium with antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate and 0.25 μ g/ml amphotericin B) (Gibco BRL, Bethesda, MD, USA) and centrifuged again. This wash procedure was repeated three times. The final pellet in the tube contained primary human breast cancer epithelial cells and very few stromal cells. The pellet was re-suspended in 10 ml low calcium (0.04 mM CaCl₂) DMEM/F12 medium supplemented with 10% of low calcium fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) and then transferred into a 75-cm² flask for culturing.

Cell culture. The isolated primary cultured human breast normal epithelial cells (HBNECs) and human breast cancer epithelial cells (HBCECs) were cultured in a 75-cm² culture flask in a humidified incubator (5% CO₂, 95% air, 37°C) with 10 ml low-calcium (0.04 mM CaCl₂) DMEM/F12 mixture (Atlanta Biologicals) supplemented with 10% of Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA) treated with FBS (Gibco Cell Culture™, Grand Island, NY, USA). The low calcium DMEM/F12 medium, which guaranteed the purity of HBNECs and HBCECs, was changed every 2 days (29). When the cells reached 85-90% confluence, they were washed with 10 ml of calcium- and magnesium-free phosphate buffered saline (PBS; pH 7.3) and then dissociated with 3 ml of 0.25% trypsin-5.3 mM EDTA (Gibco Cell Culture™) for 10 min at 37°C. The trypsinization was stopped by the addition of 10 ml DMEM/F12 medium with 10% FBS. After centrifugation for 5 min, the cells were re-suspended in low-calcium DMEM/F12 medium with 10% low-calcium FBS, and sub-cultured into 75-cm² culture flasks at a ratio of 1:5 flasks. All experiments were conducted on primary cultured HBNECs and HBCECs not generated beyond the fourth passage.

Cell proliferation (MTT) assay. Medium (100 μ l) containing 4,000 HBNECs and HBCECs was seeded in each well of 96-well plates in low-calcium DMEM/F12 medium and incubated at 37°C for 24 h. The following day, the medium was replaced by 100 μ l low-calcium DMEM/F12 supplemented with 0.2% BSA, and was incubated at 37°C for another 24 h. The HBCECs were treated with 1.5, 3, 6 and 12 nM leptin and 5, 10 and 20 nM Z for 24 h, while the control group was treated with 0.1% DMSO. The proliferation of HBNECs and

HBCECs was measured by adding 20 μ l of a fresh mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) (20:1) solution (Promega, Madison, WI, USA) to each well. After incubation for 1-3 h at 37°C, the OD values were measured using a kinetic microplate reader (Molecular Devices Cooperation, Menlo Park, CA, USA) at 490 nm. Cell growth was also measured and the average sensitive dose of leptin was calculated.

In an experiment investigating the sensitivity of leptin-induced HBCECs to Z, 4,000 HBCECs were planted in each well of 96-well plates containing 100 μ l low-calcium DMEM/F12 medium and incubated in 37°C for 24 h. The following day, the medium was changed to 100 μ l low-calcium DMEM/F12 supplemented with 0.2% BSA, and the HBCECs were incubated at 37°C for another 24 h. The cells were treated in five groups: i) control group (CT), cells treated with 0.1% DMSO; ii) leptin treatment group (lp), cells only treated with 3 nM leptin for 48 h; iii) pre-treatment with leptin group (pre-lp), cells treated with 3 nM leptin for 24 h and with 1.25, 2.5 or 5 nM Z in the following 24 h; iv) post-treatment with leptin group (post-lp), cells initially treated with 1.25, 2.5 or 5 nM Z for 24 h and then with 3 nM leptin in the following 24 h; and v) Z treatment group (Z), cells only treated with 1.25, 2.5 or 5 nM Z for 48 h. All media were changed every 24 h. After 48 h of treatment, cell growth was measured by the methods described above.

Cell treatment for RNA and PCR analyses. A total of 10⁵ viable HBCECs/well were seeded in 6-well plates in 5 ml low-calcium DMEM/F12 supplemented with 10% Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA)-treated FBS (Gibco Cell Culture™) medium. Twenty-four hours later, the medium was replaced with low-calcium DMEM/F12 supplemented with 10% dextran coated charcoal (DCC) stripped FBS, and the cells were cultured overnight. After 24 h, the medium was changed and treatment was administered to five groups: i) CT group, cells treated with 0.1% DMSO; ii) lp group, cells treated with 3 nM leptin for 48 h; iii) Z group, cells treated with 5 nM Z for 48 h; iv) post-lp group, cells treated with 5 nM zeranol for 24 h and with 3 nM leptin in the following 24 h; and v) pre-lp group, cells treated with 3 nM leptin for 24 h and with 5 nM Z in the following 24 h. All media were changed every 24 h.

RNA isolation and cDNA synthesis. After the HBCECs had been treated for 48 h, total RNA was isolated in 1 ml TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was measured by the DU-70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). RNA (1 μ g) from the cultured cells was reverse transcribed with 200 units of M-MLV reverse transcriptase (Invitrogen) at 37°C for 50 min, then at 70°C for 15 min in the presence of 1 μ l 10 mM dNTP (10 mM each dATP, dGTP, dCTP and dTTP at a neutral pH) (Invitrogen), 1 μ l 50 μ M Random hexamer (Amersham, Piscataway, NJ, USA), RNase Inhibitor (Invitrogen), 10 μ l 5X First Strand buffer, 5 μ l 0.1 M DTT and 1 μ l RNase Inhibitor (Invitrogen) in a total volume of 50 μ l in a gradient mastercycle (Eppendorf®, Westbury, NY, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR conditions were optimized for every primer pair and performed with a thermocycler Gene Amp PCR (Eppendorf®). A volume of 2 μ l of the newly synthesized cDNA was used as templates for RT-PCR. MgCl₂ concentration, annealing temperature and cycle number for the amplification of each of the PCR products (ER α , cyclin D1 and ObR) were optimized. Under optimal conditions, 1 unit of platinum Taq DNA polymerase (Gibco BRL) was added for a total volume of 25 μ l.

Primers for cyclin D1 were 5'-GCT CCT GTG CTG CGA AGT GG-3' (sense) and 5'-TGG AGG CGT CGG TGT AGA TG-3' (antisense; product size 372 bp). PCR conditions were: denaturing at 95°C for 5 min, followed by 27 cycles at 94°C for 45 sec, 54°C for 45 sec, 72°C for 60 sec, and lastly extension at 72°C for 10 min. Primers for ObR common domain were 5'-CAT TTT ATC CCC ATT GAG AAG TA-3' (sense) and 5'-CTG AAA ATT AAG TCC TTG TGC CCA G-3' (antisense; product size 273 bp). PCR conditions were: 95°C for 5 min, followed by 30 cycles at 95°C for 40 sec, 60°C for 50 sec, 72°C for 50 sec, and lastly extension at 72°C for 10 min. Primers for ER α were 5'-TGG CCC AGC TCC TCC TCA T-3' (sense) and 5'-AGT GGC TTT GGT CCG TCT CCT C-3' (antisense; product size 107 bp). PCR conditions were: 95°C for 5 min, followed by 31 cycles at 95°C for 60 sec, 60°C for 45 sec, 72°C for 60 sec, and lastly extension at 72°C for 10 min. cDNA from MDA-MB-231 and MCF-7 cells was used as a positive and negative control to ER α expression. Primers for 36B4, an internal control, were 5'-AAA CTG CTG CCT CAT ATC CG-3' (sense) and 5'-TTT CAG CAA GTG GGA AGG TG-3' (antisense; product size 563 bp). PCR conditions were 95°C for 5 min, followed by 24 cycles at 95°C for 60 sec, 63°C for 60 sec, 72°C for 60 sec, and lastly extension at 72°C for 10 min. 36B4 is internal control whose RNA is unmodified by treatment. MCF-7 and MDA-MB-231 cells were used as positive and negative controls, respectively, to the ER α gene.

The final RT-PCR products (10 μ l) mixed with 1 μ l 10X loading buffer were separated on 1.5% agarose gel and visualized by staining with ethidium bromide. Electronic images were captured under a FUJIFILM LAS-3000 image system (Fujifilm Medical Systems USA, Inc., Stamford, CT, USA). The densities of specific band were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The results were presented as the ratio of cyclin D1 to 36B4 and ObR to 36B4.

Statistical analysis. The results of the cell proliferation assay are presented compared to the control group as the mean \pm standard deviation (SD) of four replicate culture wells. The results of mRNA expression are presented compared to the control group as the mean \pm SD of three replicates. Analysis was performed using Minitab 15 software (Minitab Inc., PA, USA). Statistical difference was determined using two sample t-test analysis for independent samples. P-values of <0.05 were considered to represent a statistically significant difference.

Results

Leptin and Z significantly increase HBNEC and HBCEC proliferation. Leptin significantly increased the growth of all the human breast epithelial cells isolated from 14 normal or cancer breast tissues (data not shown, P<0.05). As shown in

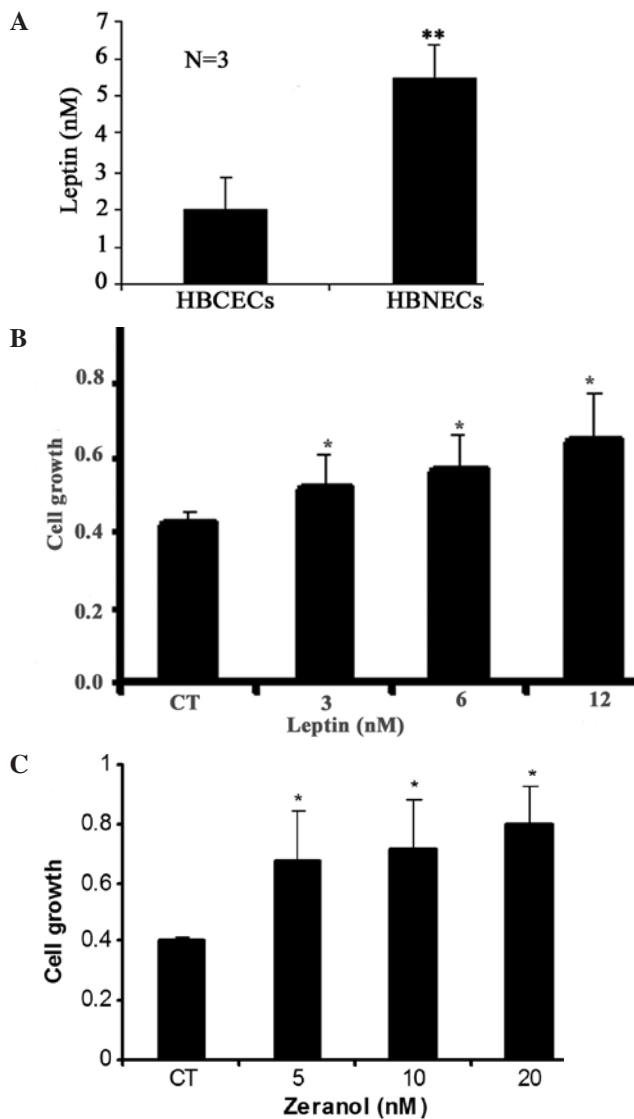


Figure 1. (A) Effective dose of leptin on primary cultured HBCEC and HBNEC growth. Primary cultured HBCECs and HBNECs were treated with 0, 1.5, 3, 6 and 12 nM leptin. The average sensitive dose of leptin to HBCECs and HBNECs was calculated and compared. ** $P < 0.01$, significant difference between the HBNECs group compared to HBCEC group. (B) Effect of leptin on primary cultured HBCEC growth. Primary cultured HBCECs were treated with 0, 3, 6 and 12 nM leptin. * $P < 0.05$, significant difference in HBCEC growth compared to the control group. (C) Effect of zeranol on primary cultured HBCEC growth. Primary cultured HBCECs were treated with 0, 5, 10 and 20 nM zeranol. * $P < 0.05$, significant difference in HBCEC growth compared to the control group.

Fig. 1A, the average sensitivity concentration of leptin differs significantly between matched HBNECs and HBCECs: the average sensitivity concentration of leptin for HBNECs and HBCECs is 5.5 and 2.0 nM, respectively. Fig. 1B and C show that both leptin and Z significantly increased HBCEC growth in a dose-dependent manner ($P < 0.05$). Leptin at 3 nM and Z at 5 nM significantly increased the growth of HBCECs isolated from human breast cancer patients after 24-h treatment, and treatment with 5 nM Z stimulated cell growth more than that of 3 nM leptin. The pre-lp group grew faster than the post-lp group (Fig. 2A), and the cells in the pre-lp group grew faster than those treated with leptin alone (Fig. 2B). As shown in Fig. 2A, compared to the combination of 3 nM leptin with 1.25

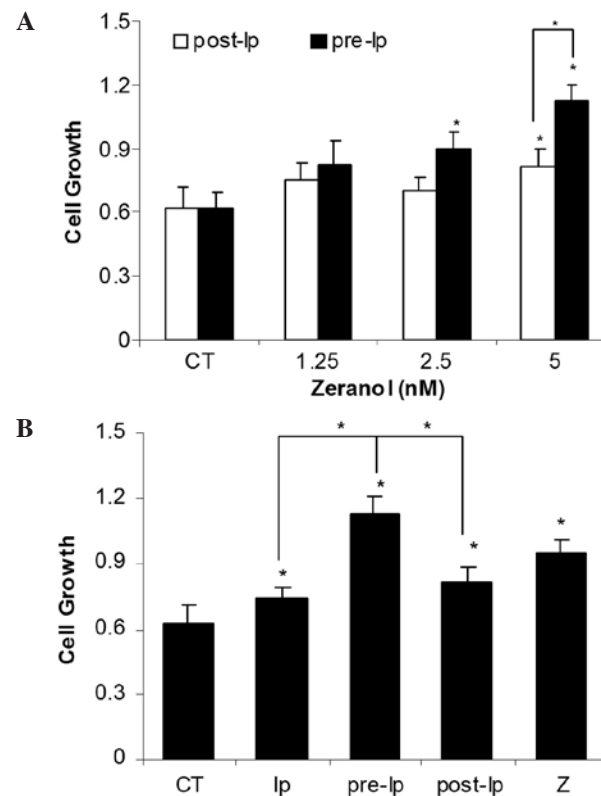


Figure 2. (A) Effects of leptin and zeranol on the proliferation of primary cultured HBCECs. HBCECs were treated with 1.25, 2.5 and 5 nM zeranol or 3 nM leptin in the 1st and/or 2nd 24 h. * $P < 0.05$, significant differences in HBCEC growth compared to the control group and between the pre-lp and post-lp groups. (B) Effects of 3 nM leptin and 5 nM zeranol on the proliferation of primary cultured HBCECs. HBCECs were treated with 3 nM leptin or 5 nM zeranol in the 1st or 2nd 24 h. HBCEC growth was most stimulated in the pre-lp group. * $P < 0.05$, significant differences in HBCEC growth compared to the control group.

and 2.5 nM Z, the combination of 3 nM leptin with 5 nM Z had the greatest effect on increasing the sensitivity of primary cultured HBCECs to Z for proliferation.

Pre-treatment with leptin significantly increased cyclin D1 expression in ERα-positive HBCECs. HBCECs used were ERα-positive (figure not shown). Fig. 3A shows that *cyclin D1* mRNA expression levels of the HBCECs were significantly increased in the lp and the pre-lp groups compared to the CT group ($P < 0.05$).

Zeranol (5 nM) significantly increases ObR expression in HBCECs. As shown in Fig. 3B, ObR gene expression was significantly increased in the Z and post-lp groups compared to the control ($P < 0.05$).

Discussion

Cyclin D1 is a cell cycle regulator that plays an important role in cell growth. The cyclin-dependent kinases (CDKs) cannot regulate cell growth without the cyclin subunit. By binding to cyclin D, cyclin D-CDK 4/6 constitutes the engine of the cell cycle machinery and affects the G_1 phase of cell growth. The cyclin D1 level is modulated by changes in growth factors in the medium used to culture cells. Leptin stimulates breast

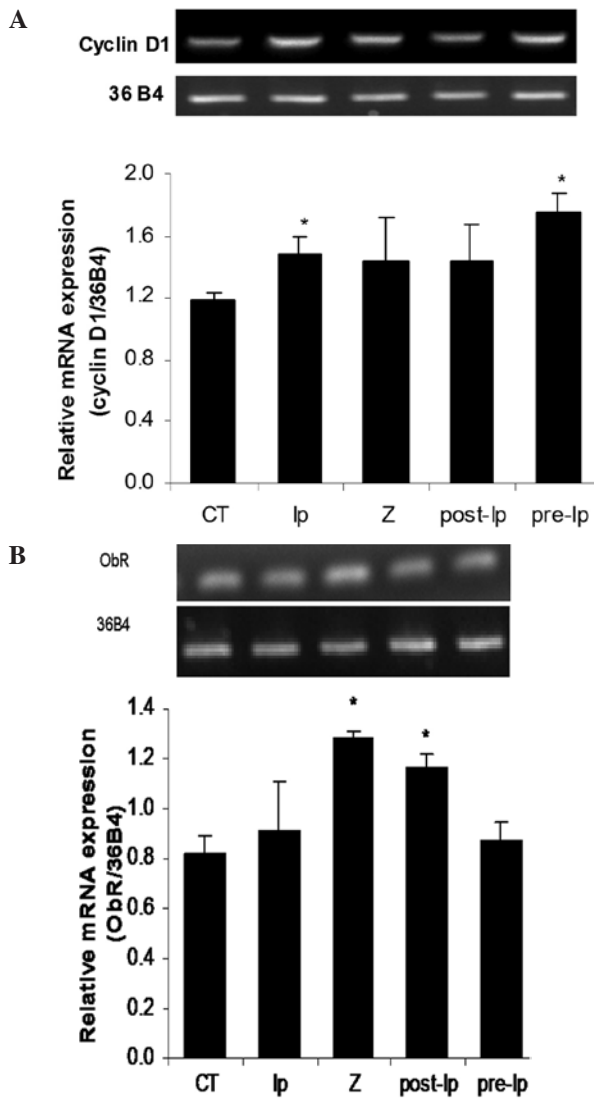


Figure 3. (A) Effects of leptin and zanol on cyclin D1 expression in HBCECs. HBCECs were treated with 3 nM leptin or 5 nM zanol in the 1st or 2nd 24 h. * $P < 0.05$, significant differences in cyclin D1 expression compared to the control group. (B) Effects of leptin and zanol on ObR expression in HBCECs. HBCECs were treated with 3 nM leptin and 5 nM zanol in the 1st or 2nd 24 h. * $P < 0.05$, significant differences in ObR gene expression compared to the control group.

cancer cell growth by up-regulating *cyclin D1* expression (35). Moreover, Garofalo *et al* found that leptin modulates estrogen synthesis and ER α activity (7,36). Besides controlling the cell cycle, cyclin D1 was found to be associated with ER (37). Cyclin D1 binds to the ER and stimulates its transcriptional activities. The cyclin D1/ER complex may play a role in stimulating tumor cell proliferation. Since leptin stimulates estrogen biosynthesis through the induction of aromatase activity and the modulation of ER α activity, it has been characterized as a growth factor for breast cancer. High levels of leptin in obese breast cancer patients may play a significant role in breast cancer cell proliferation, invasion and metastasis (7,38). The molecular action of leptin was initiated by leptin binding to the leptin receptor ObR. It is necessary to understand how leptin and Z affect cyclin D1 and ObR mRNA expression in breast cancer epithelial cells isolated from the cancerous tissues of human breast cancer patients.

Our data indicate that 5 nM Z alone does not affect cyclin D1 expression in primary cultured human breast cancer epithelial cells, while HBCECs exposed to 3 nM leptin for 24 h and then to Z in the following 24 h do significantly exhibit an increase in the expression of *cyclin D1* as compared to the control group. Our results, determined using a cell proliferation assay, showed that ER α -positive HBCEC proliferation was significantly increased by treatment with 5 nM Z for 24 h, and that treatment with 5 nM Z stimulated cell growth more than 3 nM leptin. However, when these cells were pre-treated with 3 nM leptin for 24 h, 2.5 nM Z for 24 h significantly increased HBCEC growth, compared to their respective control groups. A significant difference in cell growth was found between the pre-lp and post-lp groups (Fig. 2A). The pre-lp group demonstrated the highest stimulation of HBCEC growth compared to any other group. This result supports our hypothesis that in the pre-lp group, the sensitivity of primary cultured HBCECs to Z was increased. This is partially explained by the fact that the pre-lp group exhibits higher expression of cyclin D1. Thus, the combination of exposure of leptin and Z is more harmful to humans than leptin and Z alone. Leptin expression is up-regulated in obesity, primary breast cancer and lymph node metastasis (7,21,35). The serum level of leptin in cancer patients is higher than that in a healthy control group (26). The prognosis for ObR-positive breast cancer patients is worse than that for those without leptin or ObR expression (2). Since leptin increased HBCEC sensitivity to Z, it is possible that Z affected obese breast cancer patients more than cancer patients of average weight. Thus, the prognosis for obese breast cancer patients is worse, since more breast cancer patients exhibit leptin expression (9). Moreover, our MTT assay demonstrated that primary cultured HBCECs are more sensitive to leptin and Z than MCF-7 Adr cells (39).

A previous study found higher serum levels of leptin in a breast cancer patient group than in a control group. Our results also show that the average dose of 2 nM leptin significantly stimulates HBCEC growth. This suggests a possible relationship between obesity and breast cancer. Moreover, the potential risk for breast cancer in obese individuals may be attributed to the consumption of Z-containing beef products. Other researchers found relationships between the consumption of red meat in adult women and an increase in breast cancer risk (40). An association between higher intake of red meat in adolescence and an increase in breast cancer risk in pre-menopausal women has also been demonstrated (41). We also found that Z residues were detectable in meat harvested from Z-implanted beef heifers, though at levels much lower than the FDA legal limit of 150 ppb (data not shown).

On the other hand, researchers have found that leptin receptors are expressed in a wide range of tumor cells, and that leptin and estrogen play roles in sustaining the growth of estrogen-dependent breast cancer cells (38). Similar to how estrogen modulates ObR expression in some estrogen-responsive tissues (25,27), our previous data also indicate that 30 nM Z increases ObR expression in MCF-7 Adr cells, thus affecting leptin action (39). With Z treatment, the expression of ObR was increased in the HBCEC cells, and the action of leptin was amplified. This result also suggests that Z may be more harmful to obese breast cancer patients than to normal weight breast cancer patients in terms of breast cancer development (32).

In conclusion, Leptin and Z enhance HBCEC growth via increasing *cyclin D1* mRNA expression, and Z strengthens the effect of leptin. Our laboratory is the first to reveal that leptin increases the sensitivity of ER α -positive HBCECs to Z. Furthermore, Z strengthens the potency of leptin by increasing *ObR* expression in ER α -positive HBCECs. Further investigation of interactions involving leptin and Z in human breast cancer epithelial cells, and the measurement of biologically active Z metabolites contained in sera and meat harvested from Z implanted beef heifers, is in progress at our laboratory.

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