INTRODUCTION

The incidence of obesity and breast cancer has been continuously increasing worldwide. Obesity is an important and modifiable risk factor for the development and progression of postmenopausal breast cancer [1]. An increase in breast cancer risk associated with higher body weight among postmenopausal women has been attributed to elevations in circulating estrogen levels [2]. Although it is clear that estrogen plays an important role in various aspects of breast cancer development, other factors which have been interested in proteins synthesized in adipose tissue, which may influence breast cancer development, can be involved [3,4]. Leptin is one of these proteins and a 16-kDa protein hormone product of the obese (ob) gene as well as a potent adipocytokine [5]. Leptin binds to the leptin receptor (Ob-R), belonging to the class I cytokine receptor superfamily, which activates the janus kinase signal transducer, an activator of transcription (JAK/STAT) and the Ras/extracellular signal-regulated kinase (ERK1/2) signal transduction pathways [6]. A number of isoforms of Ob-R have been identified, but the long isoform (Ob-Rb) has been noted to contain an active intracellular signaling domain and to have the ability to activate the intracellular JAK/STAT, Ras/ERK1/2, and PI3K-Akt-GSK3 pathways [7]. Short isoforms (Ob-Ra) lacks major domains and mainly activate MAPK and have little effect on STAT activation [8]. In the breast, leptin is required for normal mammary gland development and lactation, however, leptin may also contribute to tumorigenesis in mammary tissue [9]. Several actions of leptin, including stimulation of normal and tumor cell growth, cellular migration and invasion, and enhancement of angiogenesis, suggest that this hormone could promote an aggressive estrogen-indepen-dent breast cancer phenotype [10]. Recent studies have suggested that the leptin and estrogen systems are involved in functional cross-talk. For instance, leptin has been shown to...
modulate aromatase activity either in a positive or negative fashion [11-14]. Furthermore, 17β-estradiol (E2) has been shown to up-regulate the synthesis of leptin mRNA and protein in adipocytes [15]. E2 can also modulate Ob-R expression [16], possibly through a putative estrogen responsive element in the Ob-R gene promoter [17]. In MCF-7 cells, leptin has been shown to induce aromatase gene expression, elevating aromatase activity and increasing estrogen synthesis [11]. Leptin has also been shown to enhance estrogen receptor α (ERα)-dependent transcription by decreasing ERα ubiquitination and degradation, especially in the presence of the antiestrogen ICI 182,780 [18]. Furthermore, leptin has been shown to transactivate ERα via the ERK1/2 pathway [19]. Therefore, we hypothesized that circulating leptin plays an important role in tumor development especially in ER-positive breast cancer cells, which are known to have significant resistance to hormone therapy in ER-positive breast cancer. We studied the effect of leptin on the response to tamoxifen in ER-positive breast cancer cells and investigated changes in the activation of multiple leptin stimulated Ob-R signal transduction pathways.

**METHODS**

**Cell culture and therapeutic agents**

MCF-10A, MCF-7, MDA-MB231, MDA-MB453, and MDA-MB468 cell lines were obtained from the American Type Culture Collection (Manassas, USA); T-47D, HCC-70, SK-BR3, HCC-1954, Hs578T, and HCC-38 cell lines were obtained from Korea Cell Bank (Seoul, Korea). MCF-10A normal breast cell line was grown in DMEM/F12 (Gibco-BRL, Gaithersburg, USA) media with 5% horse serum (Invitrogen, Cergy Pontoise, France), 1% penicillin/streptomycin (Gibco-BRL), 0.5 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, USA), 100 ng/mL cholera toxin (Sigma-Aldrich), 10 µg/mL insulin (Sigma-Aldrich), and 20 ng/mL recombinant human EGF (Invitrogen). MDA-MB231, MDA-MB453, MDA-MB468, and Hs578T cell lines were cultured in DMEM (Gibco-BRL) with 10% fetal bovine serum (FBS; Hyclone Labs Inc., Logan, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). All other cell lines were grown in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in a humidified atmosphere, 5% CO2 at 37°C. The culture medium was changed every 3 to 4 days. Recombinant human Leptin was purchased from PeproTech Inc. (Rocky Hill, USA), Tamoxifen and 17β-E2 were purchased from Sigma-Aldrich and dissolved according to the manufacturer’s instructions.

**Charcoal-stripped FBS and cell stimulation**

To remove endogenous steroid hormones from the serum in cell culture medium, we used dextran-coated charcoal stripping FBS. Briefly, dextran-coated charcoal was prepared by stirring 2.5% (w/v) Norit-A charcoal and dextran T-70 (0.025% w/v) in PBS and then storing for 18 hours at 4°C. The solution was centrifuged to pellet the charcoal at 1,000 g for 5 minutes, and the supernatant was decanted and replaced with the same volume of FBS. The tube was vortexed to thoroughly mix the charcoal with the serum and then incubated for 12 hours at 4°C. The stripped serum was passed through a prefiler and a 0.45 micron filter before sterilizing through a 0.2 micron filter. MCF-7 and T-47D (hormone receptor-positive) cell lines were used in this study. Cell cultures were changed to phenol-red free media containing 10% charcoal-stripped FBS for 5 days prior to treatment. Cells were treated with 50 and 100 ng/mL leptin, 10 and 20 nM E2, and 10 and 20 µM tamoxifen. All experiments were repeated 3 to 4 times.

**Western blot analysis and antibodies**

Cells were washed twice with PBS and then suspended in a total protein extraction kit (Intron Biotechnology, Seongnam, Korea) on ice for 15 minutes. Lysates were cleared by centrifugation at 13,000 rpm for 20 minutes. Protein concentrations were measured with the Bradford assay using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer’s instructions. Equal amounts of cell lysates were separated by SDS-PAGE gels, and then transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, USA). Blots were blocked with blocking buffer (5% non-fat dry milk in TBS-T) for 1 hour, followed by incubation overnight at 4°C with Leptin and Ob-R rabbit polyclonal and β-actin mouse monoclonal IgG antibodies. Antibody against the Ob-R (SC-1834) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Leptin (LS-C25172) and β-actin (AC-15) antibodies were purchased from LSBio (Seattle, USA) and Sigma (St. Louis, USA), respectively. The following antibodies were used to study leptin receptor signal transduction pathways: anti-Hsc70 mAb (Assay Designs, Ann Arbor, USA), anti-ERK1/2 Thr180/Tyr182 mAb (Cell Signaling, Beverly, USA), anti-phospho-ERK1/2 Thr180/Tyr182 mAb (Cell Signaling), anti-STAT3 pAb (Cell Signaling), anti-phospho-STAT3 Tyr205 pAb (Cell Signaling), anti-p38 MAPK pAb (Cell Signaling), anti-phospho-p38 MAPK Thr180/Tyr182 pAb (Cell Signaling), and anti-ERα mAb (Cell Signaling). Primary antibodies were detected using horseradish peroxidase-linked antimouse or anti-rabbit conjugates as appropriate (Dako, Glostrup, Denmark), and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, USA).
Protein expression levels were quantified using ImageJ software (National Institute of Health, Bethesda, USA) for the detection of the intensity of protein bands. Blots were washed 3 times in TBS-T, and incubated with peroxidase-conjugated affinipure rabbit anti-mouse IgG (1:5,000 dilution; Jackson ImmunoResearch, West Grove, USA) or peroxidase-conjugated affinipure mouse anti-rabbit IgG for 1 hour at room temperature. After washing with TBS-T 4 times for 5 minutes each, immunocomplexes were visualized by enhanced chemiluminescence (Amersham Biosciences).

Cell proliferation assay
For cell proliferation assays, cells were harvested, counted and plated at 2500 MCF-7 cells and 2000 T-47D cells per well in 96-well plates and placed in an incubator overnight. Afterwards, the cells were grown in serum-free medium for 18 hours, and then leptin and E2 were added according to the above mentioned concentrations followed by incubation for 24 hours. The cells were then treated with tamoxifen, and after 48 hours, cell viability was determined with the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Madison, USA) following the manufacturer’s protocol. Cell proliferation assays were performed at least 3 times for both cell lines.

Induction of cell signaling
MCF-7 and T-47D cells were synchronized in serum-free medium and then stimulated with estradiol 20 nM, leptin 50 ng/mL, and tamoxifen 10 µM. Stimulation of Ob-Rb was assessed at different time points from 5 minutes to 6 hours. Hsc70 was used as a loading control at each time point for both cell lines. Phosphorylated levels of ERK1/2, STAT3, p38 MAPK, and ERα were assessed by Western blotting using 25 µg of protein and specific antibodies.

Statistical analysis
Graphs were generated and quantitative results were compared using paired Student’s t-test with SigmaPlot® (Statistical Solutions Ltd., Cork, Ireland). p-values were two-sided, and a p < 0.05 level of probability was accepted as being statistically significant.

RESULTS
Leptin and Ob-Rb expression in breast cancer cell lines
To study the effect of leptin on tamoxifen action, we first determined the expression level of leptin and Ob-R in various breast cancer cell lines. Leptin expression was found in MCF-7, T-47D, MDA-MB453, HCC-70, and MDA-MB468 cells. These results were confirmed by quantitative analysis using ImageJ densitometry (Figure 1A). Notably, leptin expression was increased in ER-positive breast cancer cell lines compared to other types of breast cancer cells. Ob-Rb was detected in the MCF-7, SK-BR3, MDA-MB453, and MDA-MB231 cell lines.
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Leptin enhances the proliferation of MCF-7 cells

For cellular proliferation experiments, we chose to use MCF-7 and T-47D cells because they had differential expression patterns. MCF-7 cells have an ER-positive, leptin-positive, and Ob-Rb-positive expression profile, whereas T-47D cells have an ER-positive, leptin-positive, and Ob-Rb-negative expression profile. In the cell viability assay, E2 at 10 and 20 nM had a significant proliferative effect on MCF-7 cells compared to the respective E2 10 and 20 nM control treatments ($p<0.001$). Leptin at 50 ng/mL had no proliferative effect on MCF-7 cells ($p=0.211$), however, there was a significant proliferative effect of leptin at 100 ng/mL ($p<0.001$) (Figure 2A).

In the T-47D cell line (Figure 2B), E2 at 10 nM increased cell proliferation but was not statistically significant ($p=0.123$). However, E2 at 20 nM had a significant proliferative effect compared to the control ($p=0.048$). Leptin at 50 and 100 ng/mL had no proliferative effect on T-47D cells ($p=0.565$ and $p=0.641$, respectively). Tamoxifen at 10 and 20 µM had a significant inhibitory effect on proliferation of both MCF-7 and T-47D cells in a dose dependent manner. However, leptin at 50 and 100 ng/mL had no additional proliferative effect on MCF-7 cells compared to E2-only treated control MCF-7 cells under both E2 10 and 20 nM treatments. Leptin did not affect the activity of tamoxifen treatment compared to tamoxifen-only treated control MCF-7 cells. Similar results were obtained in T-47D cells (data not shown). Thus, the results conclusively establish that leptin does not interfere with the effect of E2 or tamoxifen in either MCF-7 or T-47D cells.

Leptin inhibited the antiestrogenic effect of tamoxifen under E2 treated conditions

In MCF-7 cells, leptin at 50 and 100 ng/mL had no significant effect on tamoxifen response in E2 10 nM treated cells (Figure 3A). Interestingly, in E2 20 nM treated MCF-7 cells (Figure 3B), leptin at 50 ng/mL had a significant inhibitory effect on the response to both 10 and 20 µM tamoxifen ($p<0.001$ and $p<0.001$, respectively). In T-47D cells, when leptin 50 ng/mL was added to the combination of E2 10 nM and tamoxifen 10 µM, leptin significantly inhibited the antiestrogenic effect of tamoxifen ($p=0.037$) (Figure 3C). Leptin at 50 ng/mL also had a significant inhibitory effect on tamoxifen response with E2 at 20 nM ($p=0.021$) (Figure 3D). In cell signaling, when leptin was added to the combination treatment of E2 and tamoxifen, ERK1/2 and STAT3 were activated compared to the E2 and tamoxifen only combination at 1 hour in MCF-7 cells (Figure 4A). In contrast, in T-47D cells, when leptin was added to the combination of E2 and tamoxifen, leptin did not have any effect on ERK1/2 and STAT3 signaling (Figure 4B). The p38 MAPK and ERα signal transduction pathways were not activated or altered by treatment with leptin in either MCF-7 or T-47D cells.
DISCUSSION

The goal of hormone therapy is to block estrogen-induced proliferation of breast cancer cells. The ER is expressed in approximately 70% of patients with breast cancer. In spite of high levels of ER expression, many ER-positive patients demonstrate resistance to hormone therapy. The Ob-R and ERα have been found to be coexpressed in malignant mammary tissue and in breast cancer cell lines [20]. Therefore, it is possible that the Ob-R and ERα signal transduction pathways are involved in functional cross-talk that contributes to tumor development and progression. Leptin is also known to cross-talk with and transactivate several signal transduction pathways, including estrogen, the EGF receptor and HER2 signal transduction pathways, that are targets of breast cancer therapy [21]. Therefore, we anticipated that leptin would play some role in resistance to antiestrogenic treatment, and our results showed that leptin inhibited the antiestrogenic effect of tamoxifen in MCF-7 and T-47D ER-positive breast cancer cells under E2 stimulation. This finding was supported by a recent study by Chen et al. [22].

Leptin is known to activate the STAT3 signal transduction pathway in ERα-positive breast cancer cells which are already resistant to antihormones. Thus, high leptin levels in obese breast cancer patients may contribute to the development of antiestrogen resistance [23]. These results were confirmed by...
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Figure 4. Leptin activates multiple signal transduction pathways in MCF-7 cells compared to T-47D cells. MCF-7 and T-47D cells were synchronized in serum-free medium and then stimulated with estrogen (20 nM), leptin (50 ng/mL), and tamoxifen (10 µM). Stimulation of Ob-Rb was assessed at different time points from 5 minutes to 6 hours. Hsc70 was used as a loading control at each time point for both cell lines. Activation (phospho) and levels of ERK1/2, STAT3, p38 MAPK, and estrogen receptor α (ERα) were assessed by Western blotting in 25 µg of protein using specific antibodies. Figure 4 shows multiple signal transduction pathways being activated at 1 hour. Tamoxifen 10 µM effectively inhibited the activity of total- and phospho-ERK1/2 and STAT3 in both cell lines. (A) In MCF-7 cells, when leptin was added to the combination of estrogen and tamoxifen, leptin induced activation of total- and phospho-ERK1/2, total- and phospho-STAT3 signaling (grey box). There were no significant differences in total- and phospho-ERK1/2 and STAT3 in both cell lines. (B) In contrast to the results obtained in MCF-7 cells, in T-47D cells, there was no activation of the phospho-ERK1/2 and phospho-STAT3 signal transduction pathways. Also, total- and phospho-p38 MAPK, ERα signaling were not activated by addition of leptin (grey box).

leptin-induced activation of multiple signal transduction pathways in the current study. When leptin was added to E2 and tamoxifen combination treatment, remarkably, leptin activated total- and phospho-ERK1/2, total- and phospho-STAT3 compared to E2 and tamoxifen combination alone in MCF-7 cells (Figure 4A). These results suggest that leptin inhibits the antiestrogenic effect of tamoxifen under E2 stimulation through activation of the ERK1/2 and STAT3 signal transduction pathways. In contrast to the results obtained in MCF-7 cells, in Ob-Rb negative T-47D cells in which leptin also had an inhibitory effect on tamoxifen activity (Figure 3C and 3D), the phospho-ERK1/2 and phospho-STAT3 signal transduction pathways were not activated but total ERK1/2 and total STAT3 were found to be activated (Figure 4B). These results suggest that leptin exerts its activity not only through the Ob-Rb, but also through cross-talk with other signal transduction pathways implicated in tumorigenesis. A recent discovery revealed that ERα turnover is differentially regulated depending on whether the receptor is free (not bound by ligand), agonist bound, or antagonist bound and whether signal transduction pathways (e.g., MAP kinases) are induced by other cell surface receptors [24]. Thus, it is possible that leptin can exert its action only on antiestrogen ICI 182,780-dependent estrogen receptor processing. Indeed, in different assays, no effects of leptin were observed in the basal or E2-induced activity of ERα [18]. This phenomenon was also shown in our study; leptin had no additional proliferative effect on E2-only treatment in both MCF-7 and T-47D cells. However, in E2 and tamoxifen combination treatment, leptin showed an inhibitory effect on tamoxifen in both MCF-7 (Ob-Rb positive) and T-47D (Ob-Rb negative) cells. That is, leptin can exert its action only on tamoxifen-dependent ER processing, displaying its maximal activity at a concentration of 50 ng/mL.

Previous studies have demonstrated in vitro that leptin stimulates cancer cell proliferation [25] as well as inhibits apoptosis [26] and induces angiogenesis [27]. Leptin was recently reported to stimulate the proliferation of various tumor cell types (prostate, colorectal, lung) [28]. Similar results have been described in breast cancer in which leptin was shown to stimulate the proliferation of various breast cancer cell lines [20]. Chen et al. [29] found no significant effect of surgical tumor removal on high serum leptin concentrations in their breast cancer patients. This finding is consistent with the hypothesis that production of leptin by tumor cells is a minor
source of the adipokines, while unexcised adipose tissue remains the major contributor to circulating levels of leptin [3]. Therefore, serum leptin levels are considered an important factor which affects the tumor environment in breast cancer [30]. In the current study, leptin significantly inhibited the anti-estrogenic effect of tamoxifen in the presence of E2 in ER-positive breast cancer cells. That is, increased serum leptin levels in breast cancer patients could be a cause of endocrine-resistance, especially for those with high serum E2 levels. Therefore, inhibition of leptin, including lowering of circulating leptin levels, could be an effective measure to overcome hormone resistance as well as antitumor activity. Although large-scale clinical studies are required to draw clinically meaningful conclusions, we found that leptin may be a promising target for the treatment of ER-positive breast cancer.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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