Effects of different glucose concentrations on the leptin signaling pathway in MCF-7 and T47D breast cancer cells

Pinar Buket Demirel1, Umit Ozorhan2, Bilge Guvenc Tuna3, Margot Cleary4, Soner Dogan2

1 Maltepe University, Faculty of Medicine, Department of Medical Biology and Genetics, Istanbul, Turkey
2 Yeditepe University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey
3 Yeditepe University, Faculty of Medicine, Department of Biophysics, Istanbul, Turkey
4 University of Minnesota, Hormel Institute Medical Research Center, MN, USA

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Abstract
Aim: Leptin activates multiple intracellular signaling pathways, including JAK/STAT, by binding to its receptor. Leptin is also an important regulator of glucose homeostasis. Although both glucose and leptin increase breast cancer cell proliferation in vitro, whether the enhancing effect of glucose on the proliferation of breast cancer cells is mediated by the leptin signaling pathway is not known. The aim of this study was to investigate the effect of different glucose concentrations on the leptin signaling pathway in MCF-7 and T47D breast cancer cells.

Material and Methods: MCF-7 and T47D cell proliferation in different glucose concentrations (2.5 mM, 5 mM, 25 mM, or 50 mM) were assayed using CCK-8 assay. Leptin, leptin receptors (ObR, ObRb) as well as STAT3 mRNA and protein levels in both cell lines in different glucose concentrations were examined by RT-PCR and western blot, respectively.

Results: Incubation in 2.5 mM, 5 mM, 25 mM, or 50 mM glucose for 72h significantly increased the proliferation of both MCF-7 and T47D cells compared to 0 mM glucose incubated cells (P<0.001). mRNA levels of leptin, ObR, ObRb or STAT3 in 2.5 mM, 5 mM, 25 mM, or 50 mM glucose incubated cells were not significantly different in both cell lines compared to 0 mM (p>0.05). However, ObR protein levels in MCF-7 cells incubated in 25 mM glucose was significantly lower compared to 0 mM glucose by western blot (p<0.05).

Conclusion: Our data suggest that the enhancing effect of glucose on breast cancer cell proliferation is not mediated by the JAK/STAT pathway.

Keywords: Leptin; glucose; breast cancer; JAK/STAT; MCF-7; T47D

INTRODUCTION
Recent studies have revealed that diabetes, a group of chronic metabolic diseases characterized by hyperglycemia, is linked to an increased risk of breast cancer. About 90% of all diabetes cases are type 2 diabetes, associated with reduced insulin secretion and insulin resistance (1). A number of meta-analyses reported that type 2 diabetes is associated with a statistically significant risk of breast cancer development especially in post-menopausal women (2, 3). Diabetes is also associated with an increased mortality in breast cancer patients as 5-year mortality rates are significantly higher in breast cancer patients diagnosed with type 2 diabetes compared to the breast cancer patients without type 2 diabetes (4).

Obesity, an established risk factor for type 2 diabetes also increases the risk of breast cancer especially in post-menopausal women (5). Since both obesity and type 2 diabetes are related to insulin resistance, the association between obesity, diabetes and breast cancer is attributed mostly to the insulin resistance, which is involved in the worse prognosis of breast cancer in diabetic (4) and obese (6, 7) patients.

Regulation of glucose homeostasis is mediated by not only insulin, but also leptin. Several studies suggested that glucose is a regulator of leptin expression and secretion: Infusion of glucose in humans to prevent hypoglycemia also prevents decrease of serum leptin levels (8). Besides, changes in serum leptin levels during caloric restriction...
are correlated with the changes in serum glucose levels in humans (9). Leptin mRNA levels were also shown to be associated with serum glucose levels in mice and removing the effects of glucose diminishes the effects of leptin (10). Additionally, gestational diabetes mellitus patients with impaired fasting glucose have higher serum leptin levels compared to individuals with normal glucose tolerance (11).

Leptin is a 167-amino acid peptide hormone that is expressed as a product of the obese (Ob) gene mainly in the adipose tissue, and is critical in the regulation of appetite, energy balance and insulin resistance (12,13). Serum leptin levels are positively correlated with the total adipose-tissue mass and increase in obesity (14, 15). Levels of serum leptin are also significantly higher in breast cancer patients compared to healthy individuals (16). Consistently, leptin enhances proliferation, survival and anchorage independent growth of breast cancer cells (17) and is also suggested as an inducer of angiogenesis (18), which is an established biomarker of a poor prognosis in invasive breast cancer (19).

Leptin exerts its biological functions through binding to its receptor (ObR), which is a member of the class I cytokine receptor family. ObR has six isoforms (ObRa-ObRf) generated as a result of alternative splicing. All ObR isoforms share an identical extracellular domain but differ in the length of their intracellular domains. Among these isoforms, only ObRb contains a long intracellular domain and has a full signaling potential. Leptin binding to ObRb triggers activation of a broad array of signaling pathways, including the JAK2/STAT3 pathway, which mediates the effects of leptin on cell survival and proliferation (20, 21). Consistently, leptin receptor is also overexpressed in breast cancer especially in higher grade tumors (22).

Although both glucose and leptin have been reported to increase proliferation of breast cancer cells in vitro, the interaction between glucose and leptin signaling on cell proliferation remains largely unknown (23, 24). In this study we aim to investigate the effect of different glucose concentrations on leptin signaling pathway in MCF-7 and T47D breast cancer cells, and determine whether the positive effect of glucose on breast cancer cell proliferation is mediated by the leptin signaling pathway.

**MATERIAL and METHODS**

**Cell Culture**

Two ER (estrogen receptor) positive breast cancer cell lines (T47-D, MCF-7) were obtained from the American Type Culture Collection (ATCC, VA, USA). These two cell lines differ by their TP53 characteristics. MCF-7 cells have wild type TP53 while T47-D cells have mutant TP53 (25). Both cell lines were grown in complete DMEM medium (Gibco, NY, USA), 10% heat inactivated FCS (Atlanta Biologics, GA, USA), 1% Pen/Strep (Gibco, NY, USA) then incubated in 5% CO2 at 37°C until it reaches 70% confluency prior to the experiments.

**Experimental Design**

After reaching confluency, T47-D and MCF-7 cells were washed with PBS and then trypsinized at 37°C to collect cells in normal cells media. Cells were centrifuged for 7-10 min at 1000 rpm, resuspended in medium and equal numbers of cells (1 x 106 cells/plate) were placed in petri dishes. Cultures were incubated overnight to allow the cells to attach. Following the overnight incubation, cell medium was removed, replaced with fresh DMEM containing 0 mM, 2.5 mM, 5 mM, 25 mM or 50 mM glucose and incubated at 37°C for 72 hours. Following the 48-hour incubation in 0 mM, 2.5 mM, 5 mM, 25 mM or 50 mM glucose, the cultures were treated with 100 ng/ml leptin (Cell Sciences, MA, USA) for 24 more hours to evaluate the effects of leptin on the expression of leptin receptor (ObR and ObRb) protein levels in different glucose concentrations. Average of two to three different petri dishes for each glucose concentration was taken as one sample (duplicate or triplicate).

**CCK-8 Growth Assay**

T47-D and MCF-7 cells were trypsinized (Gibco, NY, USA) and harvested. Harvested cells were plated in 96 well plate at a density of 5x103 cells/well and allowed to attach for about 24 h. On the following day, cell medium was removed and replaced with fresh DMEM containing different concentrations of glucose (0 mM, 2.5 mM, 5 mM, 25 mM, and 50 mM) and incubated at 37°C. After 48 h, 72 h, and 96 h later cell proliferations were measured using Cell Counting Kit-8 according to manufacturer’s instructions (Dojindo Laboratories, Japan). 10 μl of CCK-8 reagent was added into each well and incubated at 37°C in a 5% CO2 incubator for 3 h. Optical density (OD) of formazan dye were measured by Multiskan EX ELISA reader (Thermofisher, MA, USA) at 450 nm (with 620 nm reference). Cell proliferation assay were performed at least in triplicate.

**RT-PCR**

mRNA expression levels of leptin and long form (ObRb) and total form (ObR) of leptin receptors and STAT3 were measured in T47D and MCF-7 samples using reverse transcription polymerase chain reaction (RT-PCR) analysis. Briefly, total RNA was extracted from harvested cells using a RNeasy mini kit in accordance with the manufacturer’s protocol (Qiagen, CA, USA). Total RNA that isolated from cells were quantified using a spectrophotometer, and 200 ng of total RNA were used for reverse transcription. In reverse transcription, reverse transcriptase (Superscript II), random hexamers, oligo(dT) primers and RNase inhibitor to cDNA synthesis from mRNA (Invitrogen, CA, USA). After cDNA synthesis, RT-PCR were performed using primers for leptin, forward 5'-CCTGTGCGCTTTGTCCTATCTG-3' and reverse 5’-AGGCAAGCTTGAGACCTG-3'; ObR, forward 5’-CAGATTGGATATGGCTTAAAGT-3' and reverse 5’-GTTAAAATTCCAAGGGAAGC-3'; ObRb, forward 5’-ACACTGTATAATTCCACACCCAGAG-3' and reverse 5’-TGGATAAACCCCTTGGCTTCTCA-3'; Stat3 forward 5’-CAGGTTGTCAGATCACATGGG-3' and reverse 5’-TTATTCCAAACTGCAACTGATG-3'. PCR were performed with 94°C for 4 minutes for denaturing, 35 cycles of 94°C
for 30 seconds, 62°C for 30 seconds, 72°C for 1 minute, and final extension at 72°C for 10 minutes. RNA/DNA free water was used as negative control and β-actin primers were used as housekeeping. Products of PCR were separated on ethidium bromide stained 1% agarose gel electrophoresis. Product size was determined using DNA ladder. Agarose gel image were obtained and band intensities were by densitometry using the program UN-SCAN-IT gel (Silk Scientific, UT, USA). Results were normalized using β-actin as housekeeping. DNA samples were isolated from agarose gel and sent to the Advanced Genetic Analysis Center of the University of Minnesota (MN, USA) for sequencing to confirm the products (leptin, ObR, ObRb, STAT3 and β-actin).

**Western Blotting**

Total soluble protein from the cultured T47D and MCF-7 cells were isolated using the Mammalian Cell - PE LB™ kit (Genotech, MO, USA) containing EDTA, DTT and protease inhibitor was used. Cell lysates were sonicated and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected and protein amounts were quantified and standardized. 20 mg of protein were run for each sample in 10% SDS-PAGE. Proteins were transferred into Immobilon-P PVDF membranes (Millipore, MA, USA). Membranes were blocked with PBST with 5% milk. Ob-R (Abcam, MA, USA), Ob-Rb (Linco Res, MO, USA) primary antibodies were incubated in PBST with 5% milk overnight. Membranes were then washed and incubated with alkaline phosphatase conjugated IgG antibodies that used as secondary antibodies on the membrane. Protein expressions were detected by enhanced chemiluminescence (ECF substrate, Amersham, NJ, USA) and measured in a Storm 840 system (Amersham, NJ, USA). Bands were quantified with Un-Scan-It gel version 6.1 digitizing software (Silk Scientific, UT, USA) and normalized to β-actin.

**Statistical Analysis**

One-way Anova test and Tukey’s multiple comparison tests were performed to determine statistical differences among the groups. All analyses were conducted in Graphpad Prism 7.0. Data represent mean ± standard deviation (SEM). Differences were accepted statistically significant when p<0.05. “n value” for each comparison is given in the figure legends.

**RESULTS**

MCF-7 and T47D cells were cultured in medium containing different concentrations of glucose for 2, 3 or 4 days and cell proliferation was analyzed by the CCK-8 assay. In MCF-7 cells, incubation in 2.5 mM, 5 mM, 25 mM, or 50 mM glucose significantly increased proliferation compared to 0 mM glucose at all times tested (P<0.05) (Figure 1A). Similarly, incubation in 2.5 mM, 5 mM, 25 mM, or 50 mM glucose significantly increased the proliferation of T47D cells relative to 0 mM glucose at all times tested (P<0.001) (Figure 1B).

In order to investigate whether the positive effect of glucose on the proliferation of MCF-7 and T47D cells is mediated by the leptin pathway, we examined the mRNA expression of leptin, leptin receptors (ObR, ObRb) and STAT3 by RT-PCR in both cell lines incubated in different glucose concentrations (Figure 2). 72- hour incubation in 2.5 mM, 5 mM, 25 mM, or 50 mM glucose did not significantly affect the mRNA levels of leptin, ObR, ObRb or STAT3 compared to 0 mM glucose in both MCF-7 (Figure 2A) and T47D (Figure 2B) cells (p>0.05). However, when we examined the protein levels of leptin, ObR, ObRb and STAT3 by western blot (Figure 3), we observed that the ObR protein levels in MCF-7 cells incubated in 25 mM glucose was significantly lower compared to 0 mM glucose incubated cells (p<0.05). Besides, although not statistically significant, ObR and ObRb protein levels
in all other tested glucose concentrations were also lower compared to 0 mM (Figure 3A). On the other hand, although not statistically significant, incubation with 2.5 mM, 5 mM, 25 mM, or 50 mM glucose led to an increase in the protein levels of ObR and ObRb relative to 0 mM in T47D cells (Figure 3B).

Both MCF-7 and T47D cells are known to express leptin, which may bind to leptin receptors on these cells and affect their proliferation (26). Therefore, we also examined the levels of leptin receptors by western blot in the presence of leptin addition in the concentration of 100 ng/ml for 24 hours (Figure 4). Although not statistically significant, ObR and ObRb protein levels in MCF-7 cells were decreased in the presence of leptin addition at all tested glucose concentrations relative to 0 mM (Figure 4A), similar to the effect observed with no leptin addition (Figure 3A). However, in 0 mM glucose incubated T47D cells leptin addition increased the ObR and ObRb protein levels (Figure 4B) compared to 0 mM glucose incubated T47D cells in the absence of leptin addition (Figure 3B). These results indicate that leptin addition enhances the
expression of ObR and ObRb proteins in the absence of glucose in T47D cells.

DISCUSSION

In this study we hypothesized that glucose increases proliferation through the leptin pathway in breast cancer. Leptin has been shown not to significantly affect the proliferation of estrogen receptor-negative breast cancer cells (27). Therefore, the leptin signaling pathway under different glucose concentrations was investigated in estrogen receptor positive MCF-7 and T47D cell lines. First, the effect of different glucose concentrations on the proliferation of MCF-7 and T47D cells were tested. Consistent with the previous literature (23,28,29) incubation in 2.5, 5, 25, and 50 mM glucose increased the proliferation of both breast cancer cell lines (Figure 1). Next, we investigated whether glucose incubation increases proliferation of breast cancer cells through the leptin pathway. Leptin pathway activation has been shown to trigger cell proliferation through JAK/STAT, MAPK and PI3K-Akt signaling cascades (30). Leptin binding to its receptor, ObRb, leads to phosphorylation of JAKs. Activated JAKs phosphorylate the ObRb tail, creating binding sites for STATs. Thus, leptin enhances proliferation by STAT-regulated gene activation (31). Leptin has also been reported to enhance breast cancer cell proliferation via the MAPK pathway through ERK phosphorylation (32). However, to the best of our knowledge, there are no previous studies related to the effect of glucose on leptin pathway in breast cancer cells. Therefore, we examined the activation of the JAK/STAT pathway in the presence of varying glucose concentrations. We did not observe any significant differences in the mRNA (Figure 2) and protein levels (Figure 3) of leptin, ObR/ObRb, and STAT3 in different glucose concentrations. Finally, we investigated whether leptin itself has an effect on ObR and ObRb protein expression levels in MCF-7 and T47D cells. Our data reveal that leptin addition does not dramatically affect the ObR and ObRb protein levels in MCF7 cells in the presence of tested compared to no leptin addition (Figure 4A). Whereas, in T47D cells leptin addition leads to increased ObR and ObRb protein expression levels in the absence of glucose (Figure 4B), suggesting that leptin alone has an effect on the expression of ObR and ObRb proteins in T47D cells.

CONCLUSION

Taken together our data revealed that the positive effect of glucose on breast cancer cell proliferation is not through the JAK/STAT pathway. However, leptin may still be involved in glucose-enhanced proliferation of breast cancer cells through proliferation pathways other than JAK/STAT.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study is conducted with commercially available breast cancer cell lines. Therefore, it does not require ethical consent.

Pinar Buket Demirel ORCID: 0000-0001-7627-0291
Umit Ozorhan ORCID: 0000-0002-7661-2818
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