High glucose and high insulin conditions promote MCF-7 cell proliferation and invasion by upregulating IRS1 and activating the Ras/Raf/ERK pathway

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Abstract. Diabetes mellitus is associated with an increased risk of breast cancer, but the molecular mechanism underlying this association remains unclear. The aim of the present study was to investigate the effect of high glucose and high insulin conditions on MCF-7 breast cancer cells and to elucidate the molecular mechanisms underlying these effects. High glucose and high insulin conditions resulted in increased viability, proliferation, and invasion in MCF-7 cells compared with normal glucose and low insulin conditions. Reverse transcription-quantitative polymerase chain reaction and western blot analyses revealed that insulin receptor substrate 1 (IRS1) was significantly upregulated following high glucose and high insulin treatment compared with normal glucose and low insulin conditions. Furthermore, high glucose and high insulin treatment increased the Ras family of proto-oncogenes (Ras) and RAF1 proto-oncogene (Raf-1) protein expression, and activated the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. These findings suggest that high glucose and high insulin conditions promoted the proliferation and invasion of MCF-7 cells by upregulating IRS1 and activating the Ras/Raf/ERK pathway.

Introduction

Type 2 diabetes mellitus (T2DM) is a major public health problem worldwide. According to the International Diabetes Federation Diabetes Atlas 2015, there are 415 million people living with diabetes worldwide, and this number is expected to rise to 642 million by 2040 (1). T2DM constitutes >90% of the cases of diabetes, and its prevalence has been dramatically increasing in developing countries, especially in China. T2DM is characterized by hyperglycemia, hyperinsulinemia, and subclinical chronic inflammation (2,3). Diabetic patients have increased risk of several types of malignancies, including breast, pancreas, liver, urinary tract, female reproductive organ, and colorectal cancer (4). Breast cancer is one of the most common cancers worldwide and has a high mortality rate in women (5). A recently reported meta-analysis revealed that women with diabetes had a significantly higher risk (~20%) of breast cancer than those without diabetes (6). Hyperinsulinemia and T2DM were demonstrated to be independent risk factors for postmenopausal breast cancer (7). In addition, accumulating data suggest that diabetes and its complications can adversely affect cancer therapy (8) and increase mortality (9), thus affecting the outcome of breast cancer patients (10,11).

The insulin-like growth factor (IGF) and insulin receptors (IR) are important in breast cancer development and progression in T2DM patients (12-14). Insulin, not only elicits direct mitogenic effects through its actions on tumor cell growth, invasion and tumor-related angiogenesis (15), but also indirectly promotes estrogen and IGF response in both normal and malignant breast tissues (16). Insulin receptor substrate 1 (IRS1) is a regulator of insulin, IGF, and cytokine signaling, and therefore serves an important role in the proliferation, survival, and transformation of cells, by conveying signals to the phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase 1 (Akt) and extracellular signal-regulated kinase (ERK) 1/2 pathways. In general, the metabolic effects of insulin, such as glucose transport, are mediated by the PI3K pathway, whereas the mitogenic effects of insulin involve the mitogen-activated protein kinase (MAPK) pathway (17).

To date, the molecular mechanisms responsible for the association between diabetes and breast cancer are not well understood. The purpose of the present study was therefore to investigate the effect of high glucose and high insulin conditions on the proliferation and invasion of MCF-7 cells and to understand the molecular mechanisms underlying this effect.

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Materials and methods

Cell culture and treatment. MCF-7 breast cancer cells, purchased from the Institute of Cell Research of the Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) in 5% CO_2 at 37˚C. The cells were cultured in normal glucose + low insulin (5.6 mM glucose + 5 nM insulin) or high glucose + high insulin (25 mM glucose + 25 nM insulin) conditions.

Cell viability and cell proliferation assays. The MTT cell viability assay is based on the conversion of MTT to violet-colored formazan crystals by mitochondrial dehydrogenases (18). For the MTT assay, the cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. The cells were allowed to attach and proliferate for 24 h, and then subjected to the normal glucose + low insulin (5.6 mM glucose + 5 nM insulin) or high glucose + high insulin (25 mM glucose + 25 nM insulin) conditions for 24 h. Then, the cells were incubated with 0.1 mg/ml MTT at 37˚C for 4 h and lysed in dimethyl sulfoxide at room temperature for 10 min to dissolve the formazan crystals. The absorbance in each well was measured at 570 nm using a SpectraMax i3 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA) and the results were expressed as % of cell viability relative to the control cells.

For the proliferation assay, 5-Ethynyl-2'-deoxyuridine (EdU) was used. EdU is a nucleoside analog of thymidine that is readily incorporated into cellular DNA during DNA replication. Cell proliferation was evaluated using a Cell-Light EdU Apollo 567 *In Vitro* Imaging kit (Ribobio Co., Ltd., Guangzhou, China), according to the manufacturer's protocol. Briefly, the cells were incubated with 50 μM EdU for 2 h at 37˚C, fixed with 4% formaldehyde, stained with the Apollo reaction cocktail and Hoechst 33342 as a nuclear counterstain, and protected from light. Images were acquired under a fluorescent microscope and the EdU positive cells (red cells) were counted in five random fields per sample. The EdU incorporation rate was expressed as the ratio of EdU positive cells to total Hoechst 33342 positive cells (blue cells). All experiments were repeated independently at least three times. ImageJ software v1.48 (National Institutes of Health, Bethesda, MD, USA) was used to generate overlapping images (19).

Total RNA extraction and reverse transcription (RT). Total RNA was extracted from cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The total RNA was then converted to cDNA using the PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol.

Quantitative polymerase chain reaction (qPCR). qPCR was performed in a LightCycler 480 system (Roche Applied Science, Penzberg, Germany) using the SYBR Green Master Mix (Takara Bio, Inc.) (20,21). The following primers were used: IRS1, forward 5'-TTTGTGGTCCTTCCGTAGTT-3' and reverse 5'-CCTGCCCCTAATGTGATGCT-3'; β-actin, forward 5'-AAGGTGACAGCAGTCGGTT-3' and reverse 5'-GTGTGGACTTGGGAGAGG-3'. The PCR conditions were: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. IRS1 gene expression was normalized to β-actin expression for each sample. The relative mRNA expression was calculated using the -ΔΔCq method (22).

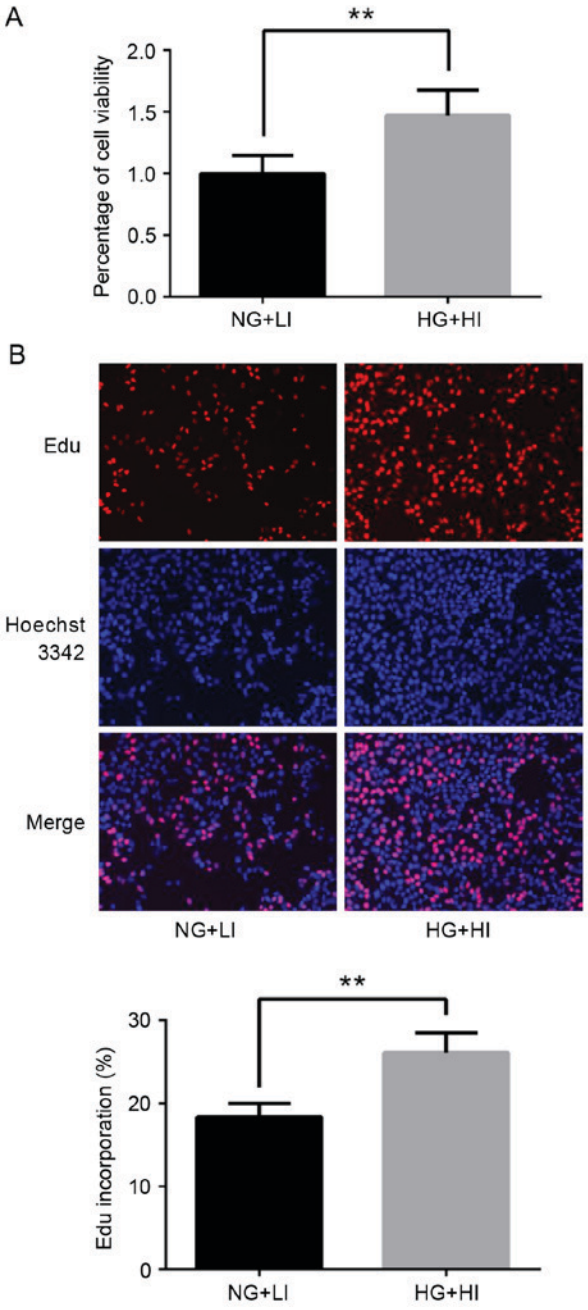

Figure 1. Effect of high glucose and high insulin on MCF-7 cell proliferation. (A) Cell viability was measured by MTT assay and presented as % relative to the control NG+LI conditions. (B) Cell proliferation was evaluated by EdU-incorporation assay at 24 h following exposure to HG+HI or NG+LI conditions. Representative images of proliferating cells labeled with EdU (red) and with a Hoechst 3342 counterstain (blue). Magnification, x100. **P<0.01. NG+LI: Normal glucose + low insulin; HG+HI: High glucose + high insulin; EdU, 5-Ethynyl-2'-deoxyuridine.

Western blot analysis. Western blot analysis was conducted as described previously (23,24). Briefly, the cells were cultured

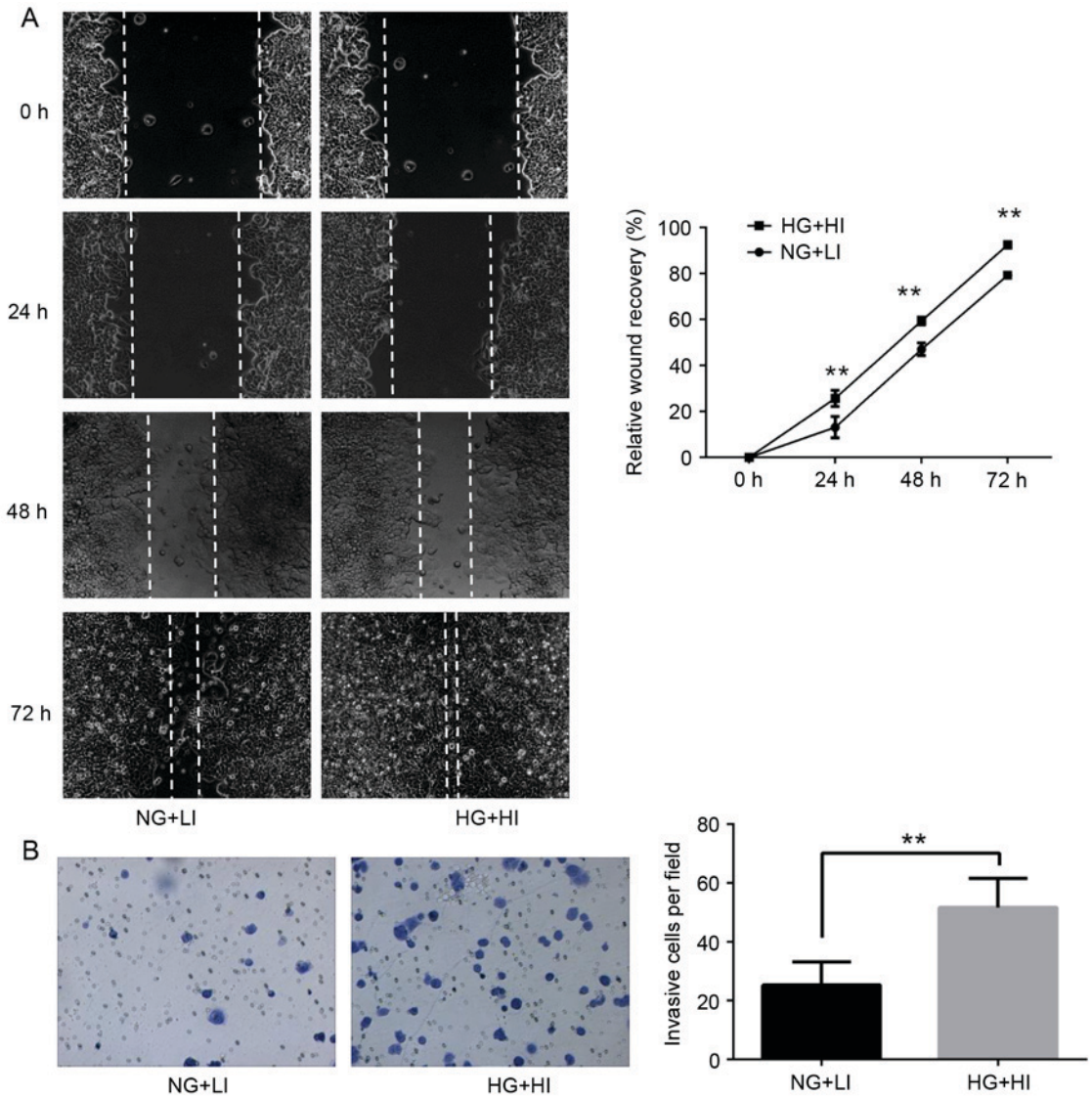

Figure 2. Effect of high glucose and high insulin on MCF-7 cell migration and invasion. (A) Migration ability was measured by wound healing assay and relative wound recovery was presented as % recovery of the wound distance at 24, 48 and 72 h relative to 0 h (magnification, x100). (B) Invasion ability was assessed using transwell invasion assays (magnification, x400). **P<0.01. NG+LI: Normal glucose + low insulin; HG+HI: High glucose + high insulin.

under normal (5.6 nM) or high-glucose conditions (25 nM) for 2 weeks. Following overnight serum starvation, the cells were then incubated with low insulin (5 nM) or high insulin (25 nM) for 2 h. Cells were washed with ice-cold PBS and lysed in Radioimmunoprecipitation Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing a 1% protease inhibitor cocktail (Beyotime Institute of Biotechnology). The cell lysates were spun at 10,000 x g for 10 min at 4˚C, and the resulting supernatant was stored at -80˚C. Protein concentration was determined using the bicinchoninic acid protein assay (Biyuntian Biotechnology Co.). The proteins were separated by 8-12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were initially blocked with 5% nonfat dry milk in TBS/0.1% Tween 20 for 1 h and then incubated with primary antibodies specific to IRS1 (cat. no. ab52167; 1:1,000; Abcam, Cambridge, MA, USA), tubulin (cat. no. MB0009; 1:5,000; Bioworld Technology, Inc., St Louis Park, MN, USA), phosphorylated (p)-ERK1/2 (cat. no. #4377; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), total ERK1/2 (cat. no. #4695; 1:1,000; Cell Signaling Technology, Inc.), RAS proto-oncogene (Ras; cat. no. ab108602; 1:1,000; Abcam) and RAF1 proto-oncogene (Raf-1; cat. no. ab137435; 1:1,000; Abcam) at 4˚C overnight. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG; cat. no. #7074; 1:1,000; Cell Signaling Technology, Inc.) at room temperature for 2 h. The immunoreactions were visualized using the Amersham Enhanced Chemiluminescence Plus western blotting detection reagents (GE Healthcare, Chicago, IL, USA), and the resulting band intensity was quantified using ImageJ software v1.48 (National Institutes of Health) (19).

Wound healing assay. To determine cell migration, MCF-7 cells were seeded in 6-well plates, incubated in normal glucose + low insulin (5.6 mM glucose + 5 nM insulin) or high glucose + high insulin (25 mM glucose + 25 nM insulin) conditions and grown to confluence overnight. Wounds were made by scraping with a sterilized 10 μl pipette tip, and cells were photographed under a phase contrast microscope (Carl

Zeiss AG, Oberkochen, Germany) at 0, 24 and 72 h. The wound width was evaluated by measuring the distance between the two edges of the scratch in 5 random fields per plate; 3 plates/group were analyzed in total. Relative wound recovery was determined using the following formula: [(Wound width $_{\text{End time point}}$-Wound width $_{\text{Starting time point}}$)/Wound width$_{\text{Starting time point}}$] x 100%. All experiments were repeated independently at least three times.

Invasion assay. The cell invasion assay was performed using 24-well transwell chambers (8.0 μm; Corning Incorporated, Corning, NY, USA). Cell suspensions were prepared in serum-free media containing 2x10^4 cells/ml, and 200 μl of cell suspension was seeded into the upper chamber of transwells that were pre-coated with Matrigel (cat. no. 354234; Corning Incorporated). Then, 600 μl of medium containing 10% serum was added to the lower chamber as the chemoattractant. After 24 h of incubation, the cells on the upper side of the transwell were removed using a cotton swab, and the filters were fixed with 100% methanol for 30 min, followed by staining with Crystal Violet Staining Solution (Beyotime Institute of Biotechnology). For each chamber, four random fields were observed by phase contrast microscopy, and the average number of invading cells in each group was counted.

Statistical analysis. All experiments were repeated independently at least three times. Results were expressed as mean ± standard deviation. Differences between two groups were assessed using the Student's t-test (two-tailed). Statistical analysis was performed using GraphPad PRISM 6 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of high glucose and high insulin on the proliferation of MCF-7 cells. Results from the MTT assay revealed that high glucose and high insulin culturing conditions resulted in increased viability in the MCF-7 cells compared with the control normal glucose and low insulin conditions (Fig. 1A). Additionally, results from the EDU cell proliferation assay demonstrated that MCF-7 cells exhibited increased proliferation under high glucose and high insulin culturing conditions, comparing with control conditions (Fig. 1B).

High glucose and high insulin conditions promote the migration and invasion of MCF-7 cells. To determine whether high glucose and high insulin conditions are associated with the progression of breast cancer, the effect of these conditions on the invasive behavior of MCF-7 cells was examined. Using a wound healing assay, the results demonstrated that high glucose and high insulin culturing conditions promoted MCF-7 cell migration, compared with control culturing conditions (Fig. 2A). In addition, the invasive ability of MCF-7 cells was significantly increased following exposure to high glucose and high insulin conditions, as determined using a transwell invasion assay (Fig. 2B). Taken together, these results indicated that exposure of MCF-7 cells to high glucose and high insulin conditions increased their migration and invasion ability.

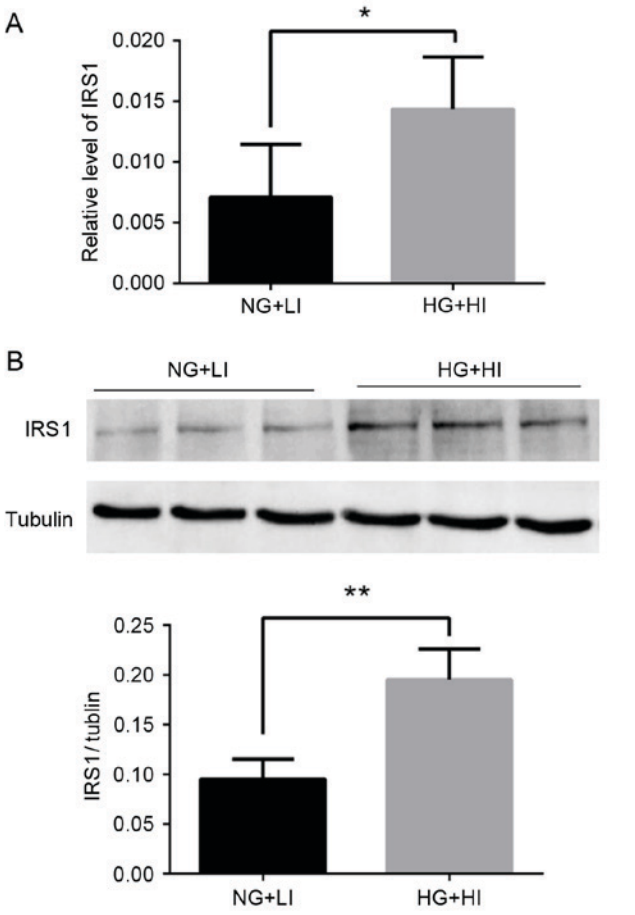

Figure 3. Effect of high glucose and high insulin on IRS1 expression. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis for the mRNA and protein expression levels of IRS1, respectively, in MCF-7 cells cultured in NG+LI and HG+HI conditions. *P<0.05 and **P<0.01. IRS1, insulin receptor substrate 1; NG+LI: Normal glucose + low insulin; HG+HI: High glucose + high insulin.

Exposure to high glucose and high insulin conditions upregulates IRS1 in MCF-7 cells. In order to explore the potential mechanisms underlying the behavior of the MCF-7 cells exposed to high glucose and high insulin, the expression of IRS1 was examined by RT-qPCR and western blotting. IRS1 mRNA and protein expression was significantly upregulated following exposure to high glucose and high insulin culturing conditions compared with normal glucose and low insulin conditions (Fig. 3).

IRS1 promotes Ras-ERK pathway activation in MCF-7 cells. IRS1 promotes activation of the downstream Ras/Raf/ERK signaling pathway, which is important for the regulation of cell proliferation, apoptosis, and differentiation (25). This pathway was thus examined in the MCF-7 cells by western blotting. The results demonstrated that ERK1/2 phosphorylation increased significantly under the high glucose and high insulin culturing conditions compared with the normal glucose and low insulin conditions (Fig. 4). In addition, under the high glucose and high insulin culturing conditions, protein expression levels of Ras and Raf-1 were significantly upregulated (Fig. 4). The results suggested that, in MCF-7 breast cancer cells, ERK may be phosphorylated by the sequential activation of Ras and Raf-1, thereby inducing cell survival, proliferation, and invasion.

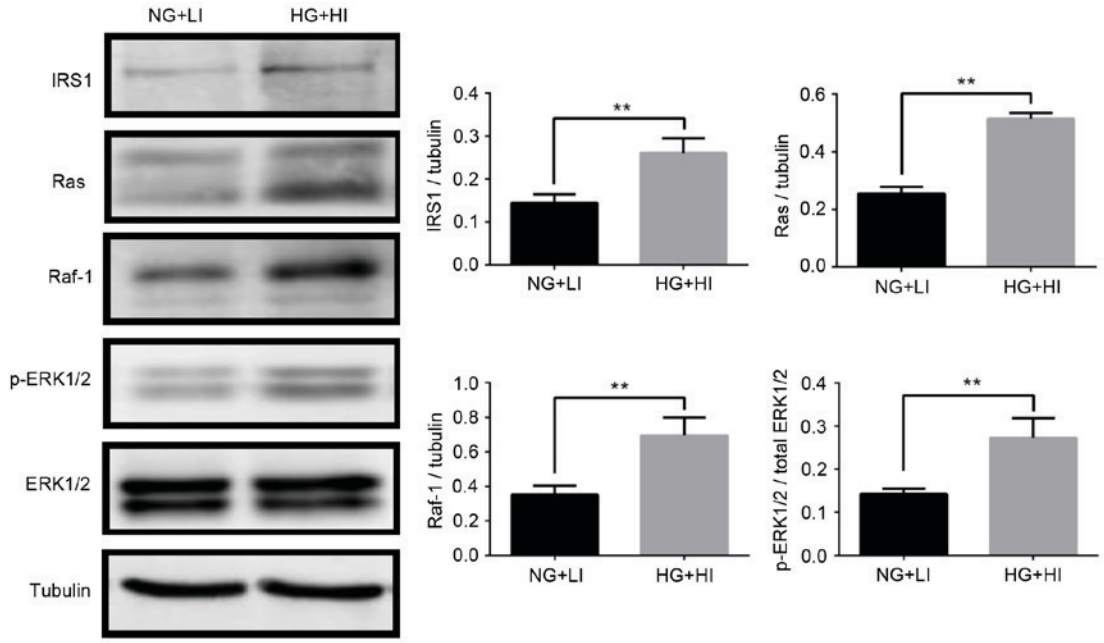

Figure 4. Effect of high glucose and high insulin on the ERK1/2 pathway. Protein expression levels of IRS1, Ras, Raf-1, p-ERK1/2 and total ERK1/2 in MCF-7 cells cultured in NG+LI and HG+HI conditions were examined by western blotting. Tubulin was used as a loading control. **P<0.01. ERK, extracellular signal-regulated kinase; IRS1, insulin receptor substrate 1; Ras, the Ras family of proto-oncogenes; Raf-1, RAF1 proto-oncogene; p, phosphorylated; NG+LI: Normal glucose + low insulin; HG+HI: High glucose + high insulin.

Discussion

With the changing global lifestyle, the number of people suffering from T2DM is constantly increasing. Female diabetic patients have an increased risk of breast cancer and its related mortality. However, the relationship between diabetes mellitus and breast cancer remains unclear. The results of the present study demonstrated that high glucose and high insulin conditions promoted the proliferation and invasion of MCF-7 breast cancer cells by upregulating IRS1 and activating the Ras/Raf/ERK pathway.

Insulin resistance, which is characterized by hyperglycemia and hyperinsulinemia, is a major cause in the pathogenesis of T2DM. Insulin is a peptide hormone produced by the pancreatic b-cells, and it is well known for its involvement in cell survival and proliferation, as well as for its effect on mitogenic signals (26). Additionally, insulin receptors are frequently overexpressed in breast cancer cells (27,28). Among the metabolic changes exhibited by cancer cells, an increase in glucose metabolism and glucose dependence is common (29). Elevated glucose levels directly promote the proliferation of tumor cells by functioning as a source of energy. As an adaptor of insulin, IRS1 has been demonstrated to act as an oncogene, and serve major roles in the growth, proliferation, migration, invasion, and differentiation of cells (30). Constitutive IRS1 activation is implicated in a variety of solid tumors, including breast cancer (31). The expression of IRS1 is increased in breast cancer tissues, with higher levels in the well-differentiated tumors compared with the poorly differentiated tumors (32). Because of the cross-talk between IGF signaling and estrogen receptor (ER) signaling pathways, IRS1 expression is regulated by estrogen in MCF-7 cells (33). MCF-7 is a hormone-dependent breast cancer cell line due to its expression of ER. Expression and activation of the ER enhances insulin mitogenicity by upregulating IRS1 and increasing PI3 K/Akt and MAPK signaling (34). High IRS1 expression is an indicator of early disease recurrence in ER-positive human primary breast tumors (35), and it indicates increased sensitivity to IGF-1 stimulated cell migration (36). Downregulation of IRS1 can suppress the growth of MCF-7 cells (36) and enhance the cytotoxic effects of tamoxifen (37). *In vivo*, transgenic mice overexpressing IRS1 have been reported to develop breast cancer and subsequent metastasis (38). Consistent with these previous studies, the present results demonstrated that IRS1 expression in MCF-7 breast cancer cells was increased following high glucose and high insulin culturing conditions compared with normal glucose and low insulin conditions.

MAPKs are known to be involved in transmitting extracellular signals that regulate cell growth, differentiation, and apoptosis (39), and to serve an essential role in MCF-7 cell cycle progression (40). Four dominating MAPK signaling cascades, the ERK1/2, ERK5, c-Jun N-terminal kinase (JNK) and p38 pathways, are implicated in normal function of mammary epithelial cells as well as the pathogenesis of breast cancer (41). Of these, the ERK-1/2 pathway is the most relevant to breast cancer (42). The Ras family of proto-oncogenes (comprising of H-Ras, N-Ras and K-Ras) encodes small GTP-binding proteins that transmit growth-promoting signals from the plasma membrane to the nucleus. This cascade involves predominantly three kinases. Upon the activation of Ras (a GTPase), Raf-1 acts as an MAPK kinase kinase and phosphorylates and activates MEK1/2, which then activates ERK1/2. Raf-1, a highly-conserved serine/threonine kinase of the MAPK pathway, serves a central role in the MAPK signaling pathway and is, thus, involved in proliferation, transformation, survival, and metastasis of cells. Active

MAPK expression is significantly higher in breast tumors than in the adjacent normal breast tissue and may be a marker of breast cancer metastasis (43). The present results revealed that ERK phosphorylation and Ras/Raf-1 protein expression were significantly increased under high glucose and high insulin conditions, thereby indicating that the enhanced proliferation and invasion of MCF-7 cells may be due to activation of the Ras/Raf/ERK pathway.

Taken together, the present findings indicate that, in MCF-7 breast cancer cells, high glucose and high insulin conditions promoted cell proliferation and invasion by upregulating IRS1 and activating the Ras/Raf/ERK pathway. A better understanding of the mechanisms underlying the effects of high glucose and high insulin on breast cancer cells may aid in developing prevention strategies against disease progression.

Acknowledgements

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