RESEARCH ARTICLE

Insulin Promotes Proliferation and Migration of Breast Cancer Cells through the Extracellular Regulated Kinase Pathway

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Abstract

The present study was undertaken to determine the roles of insulin in the growth of transplanted breast cancer in nude mice, and the proliferation and migration of MCF-7 human breast cancer cells and assess its influence on downstream signaling pathways. In a xenograft mouse model with injection of MCF-7 human breast cancer cells, tumor size was measured every other day. The insulin level and insulin receptor (IR) were increased in the breast cancer patient tissues. Insulin injected subcutaneously around the tumor site in mice caused increase in the size and weight of tumor masses, and promoted proliferation and migration of MCF-7 cells. The effects of insulin on the increase in the proliferation and migration of MCF-7 human breast cancer cells were abolished by pretreatment with the extracellular regulated kinase (ERK) inhibitor PD98059. Insulin increased the phosphorylation of ERK in the MCF-7 cells. These results indicate that insulin promotes the growth of breast cancer in nude mice, and increases the proliferation and migration of MCF-7 human breast cancer cells via the ERK pathway.

Keywords: Breast cancer - insulin - proliferation - migration - extracellular regulated kinase

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Introduction

Breast cancer is associated with reproductive factors (Kobayashi et al., 2012), genetic background (Cao et al., 2013; Xia et al., 2014), body composition (Irwin et al., 2009; Schmitz et al., 2005), nutrition (Hauner et al., 2011; Yaw et al., 2014) and obesity (Demark et al., 2012; Sangrajrang et al., 2013), and was the most common cancer in women (Jemal et al., 2010). Currently, three major strategies have been linked to an increase in breast cancer risk: obesity, lack of physical exercise, and high levels of saturated dietary fat (Alegre et al., 2013). Although progress has been made in reducing incidence and mortality rates and improving survival, cancer still accounts for more deaths than heart disease (Jemal et al., 2010).

Obesity has been revealed as one of the breast cancer risk factors, known to be associated with high levels of circulating insulin (Minatoya et al., 2013). The insulin and insulin like growth factor (IGF) signaling systems are implicated in breast cancer biology (Yang et al., 2012). Specific obesity-associated factors, including leptin, insulin and inflammatory mediators, seem to influence breast cancer growth and prognosis independently of estrogens and at least in part by interacting with estrogen signalling at a cellular level (Maccio et al., 2011). In breast carcinomas, increased levels of insulin-like growth factor 1 (IGF-1) can act as a mitogen to augment tumorigenesis (Martin et al., 2012). The insulin receptor (IR) plays a crucial role in mediating the metabolic and proliferative functions triggered by the peptide hormone insulin (Foti et al., 2009). However, the precise relationship between insulin/IR and breast cancer is not well understood.

It has been demonstrated that extracellular regulated kinase (ERK) played a novel role in the protection from hepatovascular metabolic remodeling and vascular diseases in obese-induced insulin resistance (Kujiraoka et al., 2013). Insulin may promote proliferation of skeletal myoblast cells through phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) and MEK/ERK pathways (Yu et al., 2013). However, insulin signaling mechanisms in the breast cancer are not well clear.

The present study was designed to determine the roles of the insulin in the growth of breast cancer in the nude mice and the proliferation and migration of MCF-7 human breast cancer cells and its downstream signaling pathway.

Materials and Methods

Animals and xenograft model

Female nude mice (4-6 weeks, 15-20 g) were purchased from the Chinese Academy of Medical Sciences Laboratory Animal Center (Beijing, China). The animals were housed in a temperature- and humidity-controlled room with a 12-hour on-off light cycle and given free access to food and water. To establish the xenograft mouse

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model, MCF-7 human breast cancer cells were dilute with phosphate-buffered saline (PBS) at a concentration of 5×10^7 cells/ml, and 100μl MCF-7 human breast cancer cells in PBS were injected subcutaneously into the left back armpit of each mouse (Yu et al., 2010). Tumor size was calculated according to the following formula: length × width × depth × 0.5236 (Savry et al., 2013).

Measurement of insulin levels

Blood samples were collected into a tube containing EDTA-K2. Immediately after centrifugation (Eppendorf AG, Hamburg, Germany), the plasma was frozen at -70°C until being assayed. Insulin concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Systems Laboratories, Webster, TX, USA) following the manufacturer's instructions.

Western blotting

Tissues or 5×10^6 cells were lysed in modified RIPA or lysed directly in 1×SDS loading buffer. Proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was probed with rabbit polyclonal antibody against IR (1:500; Cell Signaling Technology, Inc.), ERK or P-ERK (1:500: Cell Signaling Technology, Inc., OR, USA) followed by incubation with the secondary antibodies (1:5000; Bioworld Technology Inc., Louis, MN, USA). The bands were visualized by enhanced chemiluminescence using ECL (Pierce Chemical) and captured on X-ray films. The total amount of IR is expressed as the percentage of IR to GAPDH protein, and phosphorylated ERK level was normalized to the ERK protein level.

Cells and cell culture

MCF-7 human breast cancer cells were purchased from the Chinese Academy of Medical Sciences Cancer Institute (Beijing, China) and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen). Cells were subcultured every 3-5 days to maintain logarithmic growth until a sufficient number of cells (5×10^7 cells/ml) were obtained for transfer to nude mice.

Cell proliferation assay

Cell proliferation was assessed by bromodeoxyuridine (BrdUrd) incorporation using a BrdUrd ELISA colorimetric assay (Roche Diagnostics, Mannheim, Germany). To determine the proliferation of MCF-7 cells, the cells were initially plated at a density of 2×10^5 per 60 mm dish. After the cells had been incubated, they were counted using a hemocytometer and then plotted.

Cell migration assay

Cells (10^5 cells/well) were suspended in 0.5 ml of 10% FBS DMEM and placed in the top chamber of the well; 0.75ml of 10% FBS DMEM was added to the bottom compartment. Following 48h incubation, non-migrating cells were scraped from the membrane of the top compartment, and cells that had migrated through the membrane were fixed and stained using the Protocol Diff-Quik stain set (Siemens, Munich, Germany). Membranes were excised and mounted on a standard microscope slide (Curtin Matheson Scientific, Inc., Houston, TX, USA). The numbers of cells that migrated were determined from five random high-power fields (HPFs) visualized at 200× magnification.

Chemicals

Insulin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). PD98059, the inhibitor of ERK, was purchased from Cayman Chemical (Ann Arbor, MI, USA). The two chemicals were dissolved in PBS. The doses of insulin, PD98059 in this study were 100nM, 20μM, respectively.

Statistical analysis

Data were analyzed by using SPSS for Windows, release 18 (IBM, Armonk, NY, USA). Comparisons between 2 observations were assessed by Student's paired t-test. One-way or two-way ANOVA was used followed by the Bonferroni test for post hoc analysis when multiple comparisons were made. All of the data were expressed as the mean±SE. A value of $p<0.05$ was considered statistically significant.

Results

The levels of insulin and insulin receptor

Insulin level was increased in the plasma from breast cancer patients, and the expression of IR was also increased in the tissues from breast cancer patients (Figure 1).

Effects of insulin on the tumor size and weight

Injection of insulin subcutaneously around the tumor site caused an increase in the mean tumor weight after 3 weeks and the size of tumor from 9 days after treatment (Figure 2).

Effects of insulin on proliferation and migration

Insulin promoted the proliferation of MCF-7 cells after both 48h and 72h treatment, insulin also induced an increase in the migration of MCF-7 cells compared with PBS (Figure 3).

Effects of ERK inhibitor on proliferation and migration

ERK inhibitor PD98059 inhibited the proliferation of

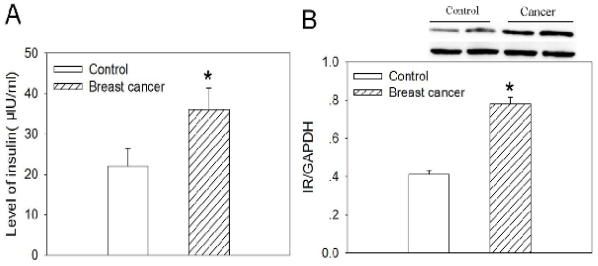

Figure 1. Insulin Level and Insulin Receptor (IR) Expression in the Breast Cancer Patient Tissues. A, The level of insulin in the breast cancer patient tissues; B, The expression of the IR in the breast cancer patient tissues. Values are mean±SE. *$p<0.05$ versus Control

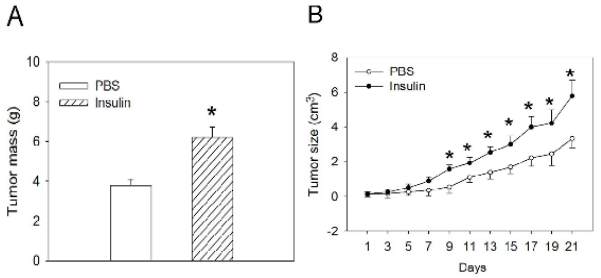

Figure 2. Effects of Insulin on the Tumor Size and Weight. A, effects of the subcutaneous around the tumor site injection of PBS, insulin on the tumor size; B, effects of the subcutaneous around the tumor site injection of PBS, insulin on the tumor weight. The tumor size was measured every other day and the tumor weight was measured 3 weeks after treatment. Values are mean±SE. *p<0.05 versus PBS

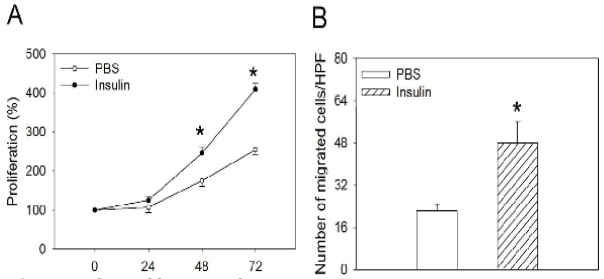

Figure 3. Effects of Insulin on Proliferation and Migration. A, effects of MCF-7 cells treated with PBS, insulin on proliferation. B, effects of MCF-7 cells treated with PBS, insulin on migration. Values are mean±SE. *p<0.05 versus PBS

MCF-7 cells compared with PBS after 48 h, and abolished the increased proliferation induced by insulin. PD98059 also inhibited migration of MCF-7 cells compared with PBS, and abolished the increase in the migration caused by insulin (Figure 4).

Effects of insulin on ERK phosphorylation

Insulin increased the expression of phosphorylated of ERK in the MCF-7 cells 2h after treatment compared with PBS (Figure 5).

Discussion

Insulin use was associated with risk of cancer at several sites and increased the risk for cancer, such as pancreas, liver, kidney, stomach and breast cancer (Karlstad et al., 2013). Elevated insulin level leads to increased secretion of estrogen by binding to the circulating sex hormone binding globulin (SHBG). The increased estrogen-mediated downstream signaling favors breast carcinogenesis (Khan et al., 2013). The role of the insulin receptor as either mediating the effects on tumors or as compensating for the insulin-like growth factor receptor has arisen (Belardi et al., 2013). Insulin, through ERK signaling, induced a proliferation in vascular smooth muscle cells (VSMCs) (Gomez et al., 2013). The present study demonstrates new findings that insulin promotes the growth of breast cancer in the nude mice and increases the proliferation and migration of MCF-7 human breast cancer cells via ERK pathway.

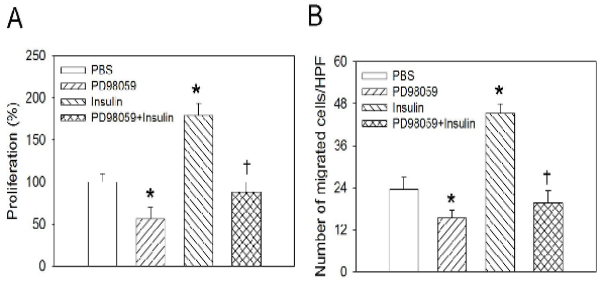

Figure 4. Effects of ERK Inhibitor PD98059 on Proliferation and Migration. A, effects of MCF-7 cells treated with PBS, ERK inhibitor PD98059, insulin or insulin after pretreatment with PD98059 on the proliferation. B, effects of MCF-7 cells treated with PBS, ERK inhibitor PD98059, insulin or insulin after pretreatment with PD98059 on the migration. Values are mean±SE. *p<0.05 versus PBS; and †p<0.05 versus insulin

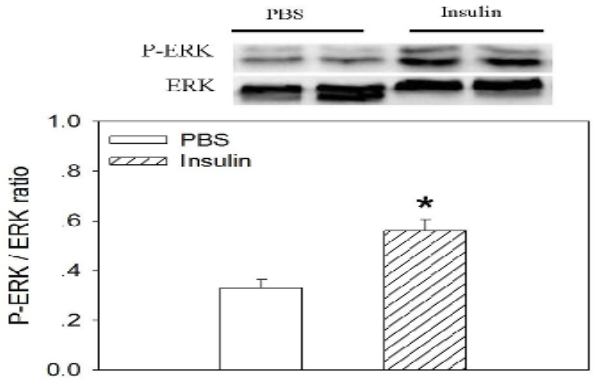

Figure 5. Effects of insulin on ERK phosphorylation. Insulin increased the phosphorylation of ERK in the MCF-7 cells 2 h after treatment. Values are mean±SE. *p<0.05 versus PBS.

It has been shown that high serum levels of insulin-like growth factor I (IGF-I) are associated with an increased risk of sporadic breast cancer. Furthermore, insulin and markers of insulin resistance, such as abdominal obesity, high blood glucose, high serum testosterone and metabolic syndrome, may affect both breast cancer incidence and prognosis (Pasanisi et al., 2013). The insulin family of proteins is ubiquitously expressed and has pleiotropic effects on metabolism and growth. Insulin, IGF-1 and particularly IGF-2 have been identified as tumor promoters (Belardi et al., 2013). In the present study, the insulin level and insulin receptor (IR) were increased in the breast cancer patient tissues. Insulin injected subcutaneously around the tumor site caused an increase in the size and weight of tumor mass. These results demonstrated that insulin play an important role in the growth of breast cancer.

Anti-human hepatocyte growth factor (HGF) antibody and an inhibitor for insulin-like growth factor-1 (IGF-1) receptor inhibited MCF-7-E10 cell growth by the breast cancer extracts (Yamaguchi et al., 2013). IGF-2 stimulate the proliferation of human estrogen receptor (ER) positive breast cancer cells (Salisbury et al., 2013). Endogenous hyperinsulinemia and insulin receptor (IR)/IGF-I receptor (IGF-IR) phosphorylation in tumors are associated with a worse prognosis in women with breast cancer. In vitro,

insulin stimulation of the IR increases proliferation of breast cancer cells (Gallagher et al., 2013). In the present study, insulin promoted the proliferation of MCF-7 cells, and insulin also promoted the migration of MCF-7 breast cancer cells.

In 3T3-L1 adipocytes, ERK played an important role in the activation of myosin light chain kinase during insulin-stimulated glucose uptake (Woody et al., 2013). Insulin, through ERK signaling, induced a proliferation in vascular smooth muscle cells (Gomez et al., 2013). However, whether insulin is through ERK signaling in the breast cancer to regulate the proliferation and migration is not well clear. In the present study, the effects of insulin on the increase in the proliferation and migration of MCF-7 human breast cancer cells was abolished by pretreatment with the ERK inhibitor PD98059. Insulin increased the phosphorylation of ERK in the MCF-7 cells. These results demonstrated that ERK signaling plays an important role in the proliferation and migration of breast cancer.

In conclusion, insulin promotes the growth of breast cancer in the nude mice and increases the proliferation and migration of MCF-7 human breast cancer cells via ERK pathway.

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