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Insulin promotes proliferation, survival, and invasion in endometrial carcinoma by activating the MEK/ERK pathway

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ABSTRACT

The involvement of insulin in endometrial carcinoma (EC) was investigated using radioimmunoassay, Western blot, immunoprecipitation, MTT, and Annexin V-FITC/PI assays in tissue samples and cultured cells. Serum levels of insulin, p-p52Shc, p-p46Shc, Shc·Grb2 complexes, p-MEK, p-ERK, and cyclin D1 were elevated in patients with EC. Expression of key proteins in the MEK/ERK pathway, including p-p52Shc, Shc·Grb2 complexes, p-MEK, p-ERK, and cyclin D1, was significantly higher in patients with advanced FIGO stage, high grade, and lymph-node metastasis and correlated positively with serum insulin concentration. Insulin promotes Ishikawa 3-H-12 cell proliferation, survival, and invasion, and these effects induced by insulin were significantly blocked by MEK inhibitor PD98059. Insulin thus promotes EC cell proliferation, survival, and invasion via the MEK/ERK pathway.

1. Introduction

Endometrial carcinoma (EC) is the most common gynaecologic cancer in developed countries [1]. Although overall cancer incidence rates in women have decreased, the incidence of EC has increased. Indeed, during 2012, an estimated 47,130 new cases will be diagnosed and 8010 deaths will be attributed to EC in the United States [2]. The incidence of EC has also been increasing in China during the past 20 years, with an increase of over 100% in the overall death rate from EC during this time [3]. Identifying the molecular alterations underlying EC will help researchers and physicians better understand EC tumourigenesis and progression and could aid in the development of novel therapeutic approaches.

The known risk factors for EC include obesity, type 2 diabetes mellitus [4], hypertension, and polycystic ovary syndrome (PCOS) [5]. The common pathophysiologic basis of these diseases is insulin resistance [6,7], and studies have further indicated that insulin resistance is a major risk factor for EC [8,9]. During insulin resistance, glucose levels increase due to the reduced sensitivity of insulin-responsive tissues to insulin. To overcome this, pancreatic β cells produce more insulin, resulting in increased levels of insulin in the circulation, a phenomenon known as compensatory hyperinsulinemia. Insulin is a metabolic hormone required for normal growth and development, lipid metabolism, and maintenance of whole-body glucose homeostasis [10]. In addition to its metabolic effects, abnormalities in insulin secretion and action are well documented in carcinogenesis. Insulin regulates the expression of more than 150 genes, including genes that regulate cell growth and proliferation, the cell cycle, apoptosis, and differentiation [11]. Furthermore, insulin has been reported to be mitogenic and anti-apoptotic in a variety of *in vitro* cancer models, including EC cell lines [12,13].

Insulin promotes tumour cell proliferation and survival through two major pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the Ras/mitogen-activated protein kinase (MAPK) pathway. Upon insulin binding, insulin receptors become autophosphorylated and thus activated. This stimulates phosphorylation of the insulin receptor substrate (IRS) proteins. The activated receptor or phosphorylated IRS proteins can activate the PI3K/Akt pathway, which is critical for the metabolic effects of insulin, or can recruit the Shc, Grb2, and son-of-sevenless (SOS) proteins, thereby triggering activation of Ras. Activated Ras then activates Raf-1 kinase, leading to phosphorylation and activation of MEK1/2 which, in turn, phosphorylates and activates ERK1/2 [14–16]. The ERK pathway drives the cellular response to extracellular stimuli in part through modulation of cyclin D1 expression [17].

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Catalytic activation of ERK appears to be required for the induction of cyclin D1 expression in embryo fibroblast cell lines, human airway smooth muscle cells, and pancreatic ductal epithelial cells [18–20]. The MEK/ERK signalling pathway has been implicated as a key regulator of cell proliferation, apoptosis, and metastasis [21–23]. Xu et al. suggested that insulin may be required for cell sensitization to growth hormone-induced ERK1/2 activation and proposed a potential cellular mechanism by which insulin may promote the growth of rat H4IIE hepatoma cells [21]. Lathi et al. demonstrated that during hyperinsulinemia, insulin can activate the MAPK pathway in a dose-dependent manner in decidualized human endometrial stromal cells [24]. Our earlier study demonstrated that insulin can promote EC cell proliferation and inhibit apoptosis, at least in part via the PI3K/Akt pathway (data not shown). However, it is unknown whether the MEK/ERK pathway also plays a role in regulating these processes in EC cells in response to insulin stimulation.

In this study, we investigated the role of insulin in EC. Specifically, we aimed to elucidate the mechanism by which insulin exerts its effects on EC cell proliferation, survival, and invasion. We hypothesised that insulin stimulates EC cell proliferation, inhibits apoptosis, and promotes EC cell invasion through the MEK/ERK pathway. To test this hypothesis, we evaluated the expression and phosphorylation levels of key proteins in the MEK/ERK pathway, including Shc, GrB2, Ras, MEK, ERK, and cyclin D1 in EC tissues. In addition, we measured the effects of insulin on proliferation, apoptosis, invasion, and ERK pathway activation in the Ishikawa 3-H-12 cell line, which is an endometrial cancer cell line. We also investigated whether the MEK inhibitor PD98059 could block the effects of insulin. Our results showed that the insulin/MEK/ERK signalling pathway was over activated *in vivo* in EC tissues. *In vitro*, insulin promoted Ishikawa 3-H-12 cell proliferation, survival, and invasion by activating the MEK/ERK pathway. These results suggested that insulin plays an essential role in EC growth and progression through the MEK/ERK pathway.

2. Materials and methods

2.1. Reagents

RIPA cell lysis buffer, protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin), human recombinant insulin, and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] and DMSO solutions were purchased from Sigma–Aldrich (Missouri, USA). The BCA Protein Assay Kit for quantitation of total protein and the Enhanced Chemiluminescence (ECL) Detection Kit were obtained from Pierce (Illinois, USA). Antibodies directed towards Shc, p-Shc (Tyr317), Grb2, Ras, cyclin D1, and β-actin, as well as peroxidase-conjugated goat anti-rabbit antibody IgG, were from Santa Cruz Biotechnology (California, USA). PD98059, a specific inhibitor of the ERK activator MEK, as well as antibodies directed towards MEK, p-MEK(Ser217/221), ERK, and p-ERK (Thr202/Tyr204) were purchased from Cell Signalling Technology (Massachusetts, USA). Monoclonal mouse anti-INSR (α-subunit) was obtained from NeoMarkers, Ins (California, USA). Histostain™-Plus Kit was from Zhongshan Golden Bridge Biotechnology (Beijing, China). TRIzol and Superscript II Reverse Transcriptase were purchased from Invitrogen (California, USA). DNA molecular weight markers and Pyrobest™ DNA Polymerase Taq were purchased from TaKaRa Bio (Dalian, China). Primers were synthesized by Aoke Biotechnology (Beijing, China). Propidium iodide (PI) and RNase A were from Dingguo Biotechnology (Beijing, China). The Annexin V-FITC/PI Apoptosis Detection Kit was purchased from BD Biosciences (California, USA). Matrigel and fibronectin (FN) were purchased from Sigma–Aldrich.

2.2. Patient samples

Fresh tissues were obtained by surgical excision from 62 ECs, 17 atypical hyperplasia endometria (AHE), and 25 normal endometria (NE) (proliferative and secretory phase) at the General Hospital of Tianjin Medical University. The study was approved by the local ethics committee and the informed consent for the use of the tissue was obtained. Tissues were dissected and snap-frozen in liquid nitrogen within 30 min of resection of the uterus and then stored at −80 °C. Fasting serum blood samples were drawn from an antecubital vein of patients with EC, AHE, and normal endometria. The blood was centrifuged to separate the serum. Serum levels of insulin were measured by radioimmunoassay (RIA) using insulin RIA kits from the Beijing Zhongjia Institute of Biological Technology (Beijing, China). The age of the EC patients ranged from 32 to 83 years (mean ± SD = 53.00 ± 9.80 years), and none had radiation or chemotherapy treatment before surgery. Tumour stage, grade, and histology were assessed according to the FIGO system.

2.3. Cell culture

The Ishikawa 3-H-12 human endometrial cancer cell line, which is a cell line of moderately differentiated adenocarcinoma cells that express estrogen receptors, was kindly provided by Professor Nishida of the Kasumigaura National Hospital (Ibaraki-ken, Japan). Ishikawa 3-H-12 cells were seeded at a density of 5×10^5 cells per mL, cultured in RPMI 1640 medium supplemented with 10% FBS, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was isolated from Ishikawa 3-H-12 cells according to the TRIzol protocol. RNA yield and purity were determined by spectrophotometry. Aliquots of 2 μg of total RNA were used for first strand cDNA synthesis in 40 μL reactions that included 200 units of M-MLV reverse transcriptase. The PCR primers were as follows. Cyclin D1: forward 5′-CTGGCCATGAACTACCTGGA-3′ and reverse 5′-GCTACACTTGATCACTCTGG-3′ (flanking a 483-bp region); β-actin: forward 5′-CCTGGGCATGGAGTCCTGTG-3′ and reverse 5′-AGGGGCCGGACTCGTCATAC-3′ (flanking a 305-bp region). After cDNA synthesis, the cyclin D1 and β-actin genes were amplified. PCR conditions were as follows: denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. The PCR products were separated in 2% agarose gels, stained with ethidium bromide, and visualised under UV transillumination.

2.5. Western blot analysis

Frozen tissues and cells were homogenised in lysis buffer containing 25 mM Tris/HCl (pH 8.0), 140 mM NaCl, 2 mM EDTA, 1 mM $NaVO_4$, 1% Nonidet-P40, 1 mM PMSF, 5 μg/mL aprotinin, and 2 μg/mL leupeptin. Supernatant fractions were collected and the protein content was determined with the BCA Protein Assay Kit according to the manufacturer's instructions. Proteins were separated by electrophoresis in 12% SDS–polyacrylamide gels (PAGEs) and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies against Shc, p-Shc (Tyr317), Grb2, Ras, MEK, p-MEK(Ser217/221), ERK, p-ERK (Thr202/Tyr204), cyclin D1, or β-actin overnight at 4 °C. After washing with phosphate-buffered saline solution (PBS), membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. After a second round of washes in PBS, protein bands were visualised with ECL. Band intensities were determined by densitometry, and the results were expressed as the ratio of target protein to β-actin or as the ratio of a specific phosphorylated protein to the total amount of that protein.

2.6. Immunoprecipitation analysis of Shc·Grb2 complex formation

For immunoprecipitation analysis, 500 μg of protein in lysis buffer was incubated with anti-Shc primary antibody for 1 h at 4 °C. Protein A/G-agarose beads were added, and incubation was continued overnight at 4 °C. The beads were washed three times with lysis buffer, and the immunoprecipitated proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, incubated overnight at 4 °C with the anti-Grb2 primary antibody, washed in PBS-T, and then incubated with horseradish peroxidase-labelled secondary antibody for 1 h at room temperature. Protein bands were visualised with ECL.

2.7. MTT assay

To evaluate the effect of insulin on cell proliferation, Ishikawa 3-H-12 cells were seeded in 96-well plates at a density of 5×10^3 cells per well in RPMI 1640 medium supplemented with 10% FBS and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, the cells were serum-starved for 24 h and then treated with the MEK inhibitor PD98059 at 0, 0.1, 1, 10, or 50 μM in triplicate wells. After 30 min, insulin was added to a final concentration of 10^{-8} M. Cell viability after 24, 48, and 72 h was determined using the MTT assay. At the appropriate time, 20 μL of MTT stock solution (5 mg/mL) was added to each well. After a 4 h incubation at 37 °C, 200 μL DMSO was added to each well. The plates were incubated for an additional 20 min at 37 °C, and the absorbance was measured at 570 nm with a microplate reader.

2.8. Annexin V-FITC/PI assay

Apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection Kit according to the manufacturer's directions. Briefly, cultured Ishikawa 3-H-12 cells were washed twice with cold PBS, resuspended in 200 μL of 1× binding buffer, and stained with 5 μL Annexin V-FITC plus 5 μL PI. The cells were then incubated for 15 min at room temperature in the dark. After the addition of 300 μL of 1× binding buffer to each tube, cells were analysed within 60 min by flow cytometry using