

The proliferative role of insulin and the mechanism underlying this action in human breast cancer cell line MCF-7

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Summary

Purpose: To explore the proliferative role of insulin and the potential mechanism in human breast cancer cell line MCF-7.

Methods: MCF-7 cells were treated with different concentrations of insulin. Morphological observation and methylthiazolyltetrazolium (MTT) assay were used to detect the proliferation of MCF-7 cells. The expression of insulin receptor mRNA was detected by RT-PCR, with or without mitogen activated kinase (MAPK) signals blocked by their inhibitors: SP600125, the inhibitor of Jun N-terminal kinase (JNK), and PD98059, the inhibitor of extracellular signal-regulated (ERK1/2).

Results: Insulin increased the proliferation of MCF-7 cells in a concentration-independent manner. Western blotting implicated that 100 nM insulin induced activation of JNK and ERK. The expression of phosphorylated JNK was detected within 5 min after insulin treatment and sustained for an hour, while ERK was activated later than JNK, at the 30th

min after insulin treatment. Then we reevaluated the proliferative effect promoted by insulin after MAPK signaling pathway was blocked by its inhibitors. The results showed that after inhibiting the activities of JNK and ERK, the proliferative effect of insulin was also attenuated. Western blot results showed that, while blocking the MAPK signaling pathway, 10 μ M (ERK pathway inhibitor) could significantly inhibit phosphorylated activation of ERK1/2 with 200 nM insulin at 30 min, while no obvious inhibition with 20 μ M SP600125 (JNK pathway inhibitor) of phosphorylated activation of ERK1/2 was noticed.

Conclusion: It seems that insulin can stimulate the proliferation of MCF-7 cells in vitro. MAPK signal transduction pathway involves the proliferative effect, which also regulates the expression of insulin receptor, mediates and amplifies the insulin signaling pathway.

Key words: breast cancer, ERK, insulin, JNK, mammary carcinoma

Introduction

Breast cancer is the most common malignancy in females and the most common cause of death in women aged 45-55 years. Obesity is usually associated with hyperinsulinemia, and apart from important metabolic effects, insulin has strong mitogenic effects, especially related to breast cancer [1]. Therefore, studies on the proliferation of breast cancer cells under the influence of insulin are important for elucidating part of the mechanisms of breast cancer development, and offer clues for therapeutic interventions; however, the specific mechanism of insulin on human breast cancer cell and related signal transduction pathways are unclear.

In our study, we investigated the growth kinetics effects of human MCF-7 breast cancer cell line and the kinase phosphorylation of MAPK pathway under the influence of insulin.

Methods

MCF-7 cell culture

MCF-7 cells were generously provided by General Surgery Laboratory, First Affiliated Hospital of Nanjing Medical University. The cells were cultured in incubator in DMEM/F12 medium with 100 U/ml penicillin, 100 μ g/ml streptomycin and 4% fetal bovine serum (FBS), in 5% CO₂ at 37° C. Cells were digested for 8 min using

0.25% trypsin and were inoculated in 96-well plate (0.4×10^4 cells/well) and in 6-well plate (8×10^5 cells/well). After 24 h the medium was replaced with DMEM/F12 containing 0.1% FBS, then 16 h later, the intervention experiment was performed: cell proliferation and insulin receptor protein levels were detected after incubating with 0, 25, 50, 100, 200 nM insulin (Humulin R, 400 U: 10 ml) for 24 h; phosphorylated JNK and ERK1/2 expression was detected after incubation of 100 and 200 nM insulin for 0, 5, 15, 30 and 60 min. Then 20 $\mu\text{mol/L}$ JNK inhibitor SP600125 (Calbiochem, La Jolla, CA), and 10 $\mu\text{mol/L}$ ERK1/2 inhibitor PD98059 (Calbiochem, La Jolla, CA) were added into MCF-7 cells. One hour later cells were incubated with 0, 25, 50, 100, 200 nM insulin for 24 h, then cell proliferation was assessed. On the other hand, phosphorylated ERK1/2 level was measured after incubation of 200 nM insulin with the cells for 30 min.

MCF-7 cell proliferation

Cell inoculation and intervention

100 μl 0.4×10^4 MCF-7 cells were inoculated to 96-well plate. Culture and intervention were performed according to the experiment's need; 5 multiple pores were set up per group and no cells as blank control group.

MTT assay

After intervention, 10 μl MTT solution (5 mg/ml, AMRESCO, USA) were added into each well, incubation continued for 4 h, the supernatant was removed and 100 μl DMSO (AMRESCO, USA) were added into each well. When the colored materials were dissolved completely, the 96-well plate was read by enzyme-linked immunoassay instrument (the absorbance wavelength was 490 nm; A value).

Cell proliferation rate

The cell proliferation rate was calculated as follows: cell proliferation rate = (A value of the experimental group - A value of control group) / A value of the control group $\times 100$.

Morphology

The dynamic morphology of MCF-7 cells was observed under CKX41 inverted phase contrast microscope (Olympus, Japan).

Western blot

Briefly, the total protein of MCF-7 cells was extracted, protein concentration was detected using the bicinchoninic acid (BCA) method, then the protein was run on SDS-PAGE gel; anti-mouse ERK1/2 (p44/p42) antibody, anti-rabbit phosphorylated ERK1/2 (Thy202/Tyr204) antibody, anti-rabbit phosphorylated JNK (p54/p46) antibody, and anti-rabbit insulin receptor- β were all purchased from Cell

Signaling Technology Corporation, and used according to manufacturer's instructions last developed with ECL kit (Pierce, USA).

Statistical analysis

Data were expressed as means \pm standard deviation and were analyzed using the paired ANOVA method. The SPSS version 15.0 package was used for statistical considerations and a p-value < 0.05 was considered statistically significant.

Results

Growth effects of insulin on breast cancer cell morphology

MCF-7 cells were dispersedly distributed, sparse, and their shape was more structured with 0.1% FBS for 24 h. Cell density increased, and cell accumulation and gross morphology was disorderly layer-arranged, the cell size and shape were uneven, and multinuclear and macronuclear cells increased with 25 nM insulin for 24 h.

MTT assay of MCF-7 proliferation kinetics

MTT colorimetric results showed that all concentrations of insulin (25 nM, 50 nM, 100 nM, 200 nM) could promote MCF-7 cell proliferation compared with the control group. Significant difference ($p < 0.01$) was detected after 24 h between 25 nM and 50 nM insulin; the maximum rate of cell proliferation was seen in incubation with 25 nM insulin for 24 h (44.5%; Table 1).

Phosphorylated ERK1/2 and JNK expression

β -actin was used for internal reference, and semi-quantitative analysis showed that phosphorylated JNK protein expression increased significantly ($p < 0.05$) after 5 min in the groups with 100 nM and 200 nM compared with 0 min, and the increase continued to 60 min. Phosphorylated ERK1/2 expression significantly increased after 30 min in the group with 100 nM insulin with the increase of insulin concentration; 200 nM insulin could cause rapid activation of phosphorylated ERK1/2 and phosphorylated ERK1/2 protein expression increased after 5 min, suggesting that insulin could

Table 1. Comparison of absorbance in MCF-7 cells with different concentration of insulin

	Insulin (nM) 24 h				
	0	25	50	100	200
A	0.0623 \pm 0.07	0.090 \pm 0.008*	0.081 \pm 0.007*	0.071 \pm 0.007	0.066 \pm 0.003

* $p < 0.01$ compared to control group, A: absorbance

promote cell proliferation through activation of JNK and ERK1/2 signal pathway; moreover, the higher the insulin concentration, the faster activation of ERK1/2 and the longer continuation of phosphorylation (Figure 1, A and B). Ten μM PD98059 (ERK pathway inhibitor) could significantly inhibit phosphorylated activation of ERK1/2 with 200 nM insulin at 30 min, while the inhibition of 20 μM SP600125 (JNK pathway inhibitor) to phosphorylated activation of ERK1/2 was not obvious (Figure 1C).

MCF-1 cell proliferation by MAPK pathway inhibitors

MTT colorimetric results showed that MCF-7 cell proliferation was not inhibited significantly by MAPK inhibitors without insulin ($p>0.05$); MCF-7 cell proliferation which was induced by insulin (25 nM, 50 nM, 100 nM and 200 nM) could be inhibited with 10 μM PD98059 for 1 h, and inhibition was statistically significant in the group with 25 nM insulin ($p<0.05$; insulin+PD98059 group vs. the same concentration insulin group); the proliferation which was induced by insulin (25, 50, 100 and 200 nM) was clearly inhibited with 20 μM SP600125 for 1 h ($p<0.01$); the inhibition of SP600125 was stronger than that of PD98059 ($p<0.01$). Compared with blocking ERK1/2 pathway, inhibition of JNK activation could be more effective in blocking MCF-7 cell proliferation with insulin (Table 2).

Discussion

The number of obese population is rapidly expanding in many parts of the world, constituting a threat to public health and economy. A current study proved that obesity is related with increasing incidence and mortality of some diseases, such as diabetes mellitus, cardiovascular diseases, breast cancer and other tumors [2]. Breast cancer is the most common malignancy in women and epidemiological studies [3,4] have suggested that its incidence in postmenopausal women is associated with obesity; however, but the exact mechanism by which obesity contributes to increasing can-

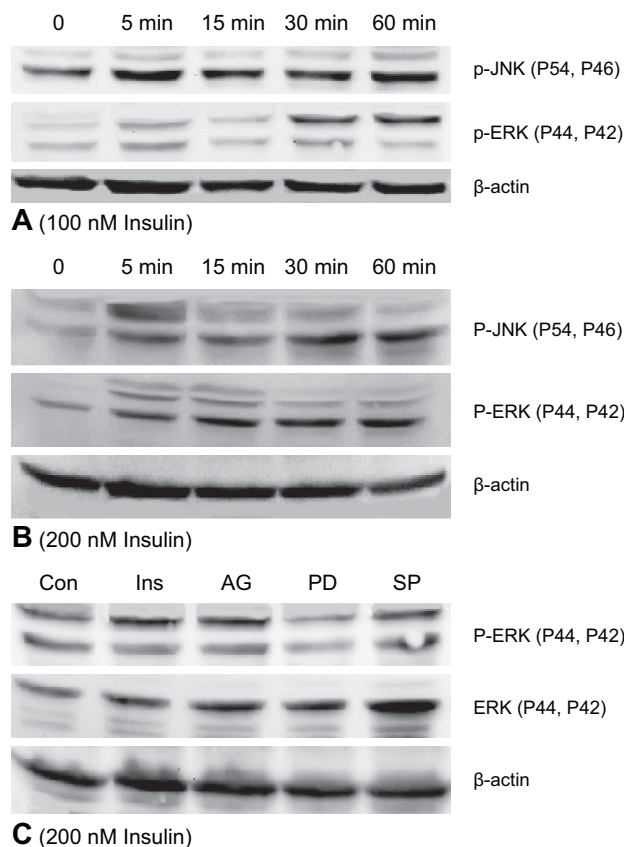


Figure 1. Insulin influenced phosphorylated JNK and ERK1/2 protein expression; MAPK signal pathway inhibitors inhibited the phosphorylation caused by insulin. **A:** group with 100 nM insulin intervention; **B:** group with 200 nM insulin intervention; **C:** MAPK signal pathway inhibitor inhibited phosphorylation caused by 200 nM insulin for 30 min (Ins: 200 nM insulin, PD: 10 μM PD98059, SP: 20 μM SP600125).

cer risk is still unclear. The most likely mechanism of carcinogenesis is a change of hormone levels including insulin, which destroys the balance between cell proliferation and apoptosis [5]. Epidemiology, case-control studies, and animal experiments all have confirmed that the incidence of breast cancer increases in people with hyperinsulinemia, and fasting plasma insulin levels and incidence of breast cancer have a positive correlation [6]. Further clinical studies [7,8] confirmed that patients with breast cancer who had higher fasting insulin and

Table 2. Influence of proliferation with different concentration of insulin by signal pathway inhibitors (A value, mean \pm standard deviation)

	Insulin (nM) 24 h				
	0	25	50	100	200
Ins	0.0623 \pm 0.007	0.090 \pm 0.008*	0.081 \pm 0.007*	0.071 \pm 0.007	0.066 \pm 0.003
Ins+PD	0.060 \pm 0.009	0.076 \pm 0.013 Δ	0.069 \pm 0.012	0.061 \pm 0.006	0.056 \pm 0.001
Ins+SP	0.056 \pm 0.004	0.054 \pm 0.006 Δ *	0.050 \pm 0.003 Δ *	0.045 \pm 0.003 Δ *	0.046 \pm 0.005 Δ

Compared to control group (0 nM), * $p<0.01$. Group with inhibitors vs. the same concentration insulin group, Δ $p<0.05$ and Δ $p<0.01$. Insulin+SP group vs. insulin+PD group with the same insulin concentration, * $p<0.01$. Among groups, * $p<0.01$. Ins: 200 nM Insulin, PD: 10 μM PD98059, SP: 20 μM SP600125

C peptide levels had poor prognosis. The intervention measures such as exercise and reducing caloric intake could reduce the risk of developing breast cancer, suggesting that insulin was associated with the pathogenesis of breast cancer [9,10].

We used recombinant human insulin to stimulate the human breast cancer cell line MCF-7 *in vitro*. The results showed that 25-200 nM insulin could promote MCF-7 cell proliferation, while a significant difference emerged between the 25 nM and 50 nM groups, the 25 nM insulin group showing the highest rate of proliferation. Promoting MCF-7 cell proliferation effect of insulin was related to its promoting mitotic effect, but the exact molecular mechanism is still unclear, suggesting it might be related to abnormal activation of intracellular proliferation-associated signal pathways. MAPK signal transduction pathway is an important cell survival and anti-apoptotic signaling pathway mediating signal transduction of many growth factors (e.g. insulin) and cytokines. JNK, p38 and ERK1/2 are three key members of the MAPK pathway. JNK and p38 were activated by cell stress and related to apoptosis [11], but it was also proved that JNK was very important for IL-3-mediated cell survival [12]. The activation of the ERK1/2 pathway, induced by mitogens or growth factors, is usually considered as a cell survival signal with antiapoptotic effect [13]. It has been confirmed that MAPK signal pathway was expressed abnormally or activated abnormally in a variety of human tumors and tumor cell lines, which suggested that abnormal regulation of this pathway was closely related to carcinogenesis. Compared with normal breast tissue and benign tumors, the increased activity and expression of MAPK pathway was related to the malignant phenotype of breast cancer [14]. Therefore, it is necessary to study the breast cancer cell proliferation mechanism in relation with insulin and the relationship with the MAPK signal transduction pathway. Western blot results showed that 100 nM insulin could induce JNK protein phosphorylated activation after 5 min and this could continue to 60 min, while the activation of ERK1/2 protein was relatively delayed, with activation starting after 30 min; however, with increased insulin concentration (200 nM), the ERK1/2 phosphorylated activation was rapid and strong. Therefore, the incubation time and concentration of insulin inducing the degree and speed of ERK1/2 and JNK phosphorylated activation, and influencing breast cancer cell proliferation should be a subject of thorough studies. Elucidation of its mechanism may render it into a new target for interventional therapy of breast cancer. To further confirm whether these effects were related to cell proliferation, specific or non specific, we used additionally the specific inhibitors (PD98059 and SP600125) of ERK1/2 and JNK

pathways to study the relationship between the activation of MAPK pathway and insulin-promoting MCF-7 cell proliferation effect. The results showed that incubation of 10 μ M PD98059 and 20 μ M SP600125 with MCF-7 cells could significantly inhibit the proliferation-promoting effects of insulin, and the inhibition of SP600125 was stronger. These observations revealed that the pro-proliferation effect of insulin on MCF-7 cells was related to the phosphorylation activation of JNK and ERK1/2. Inhibition of cell proliferation by blocking the JNK pathway was more effective than blocking ERK1/2, showing that the contribution to insulin-induced cell proliferation of these two pathways was different.

In this study we investigated whether the recombinant human insulin influenced the proliferation of human breast cancer cell line MCF-7, and to this purpose we used related signal pathway inhibitors to intervene with the above-mentioned effect. The results showed that recombinant human insulin could stimulate MCF-7 cell proliferation, its mechanism was associated with intracellular MAPK pathway activation, and the ERK1/2 and JNK proliferation-promoting pathways were also involved in MCF-7 cell proliferation. All these suggested that insulin affected the MCF-7 cell activation signal pathways, however, the different components of signal pathways had different regulatory intensity of the ERK1/2 and JNK pathways. A previous study [15] has shown that ERK1/2 signal pathway was the major pro-proliferation pathway in cancers of the pancreas, colon, lung and others. However, the JNK pathway played the main role in insulin-induced cell proliferation of breast cancer cells. Different results may be due to the different cell types and stimuli.

In conclusion, the active change of MAPK signaling pathway associated with phosphorylated JNK may play an important positive role in the occurrence and development of breast cancer in obese patients. Blocking the insulin signal transduction is expected to become a new target of breast cancer molecular therapy, and the study of insulin-MAPK signal pathway can provide some experimental and theoretical basis for early diagnosis, prognosis and guidance of treatment of breast cancer.

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