

Oncogenic activity of insulin in the development of non-small cell lung carcinoma

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Abstract. Insulin is associated with the progression of numerous different types of cancer. However, the association between insulin and non-small cell lung carcinoma (NSCLC) remains unknown. The aim of the present study was to evaluate the role of insulin in the proliferation, migration and drug resistance of NSCLC cells, and to determine whether the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway was involved. NSCLC cells were treated with insulin in the absence or presence of LY294002, an inhibitor of the PI3K/Akt pathway. Following co-incubation with insulin, cell proliferation and drug resistance were measured by MTT; cell migration was examined by wound healing and Transwell assays; and the expression of cyclin A, proliferating cell nuclear antigen (PCNA), p27, matrix metalloproteinase 3 (MMP3), P-gp and proteins involved in the PI3K/Akt pathway were assessed via western blotting. The results of the current study demonstrated that insulin enhanced the proliferation, migration and drug resistance of NSCLC cells. Correspondingly, insulin upregulated the expression of cyclin A, PCNA, MMP3, P-gp and downregulated p27 expression in NSCLC cells. Following treatment with insulin, it was demonstrated that phospho-Akt expression increased in a dose-dependent manner. However, the effects of insulin on NSCLC cells was inhibited by the

PI3K/Akt pathway inhibitor LY294002. Therefore, the results of the current study indicate that insulin is associated with the development of NSCLC by activating the PI3K/Akt pathway. This may improve understanding of the mechanism of action of insulin in NSCLC in the future.

Introduction

Lung cancer is one of the most common types of cancer and remains the leading cause of cancer-associated mortality in the world (1). Non-small cell lung carcinoma (NSCLC) accounts for ~85% of all lung cancer cases (2) and the majority of patients with NSCLC are diagnosed at an advanced stage (3). Despite improvements in the diagnosis and treatment of NSCLC, the 5-year survival rate for NSCLC remains low, at between 10 and 20% (4,5). Therefore, it is important to identify key risk factors and to design novel therapeutic strategies to prevent or treat NSCLC.

Previous studies have suggested that ~20% of all types of cancer are due to obesity (6,7). Obesity is strongly associated with the development of type II diabetes mellitus, which is accompanied by elevated insulin levels (8). Epidemiological studies have demonstrated that increased insulin levels are associated with an increased risk of developing cancer, including breast, pancreatic, colon and bladder cancer (9-12). Insulin acts as a powerful mitogen and has been implicated in the onset and progression of tumors (13-15). High levels of insulin accelerated the proliferation of pancreatic ductal cells and increased migration in breast cancer and colon cancer cells (13-15). However, the effect of insulin on NSCLC has not yet been evaluated.

Activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway indicates poor patient prognosis and is associated with different types of cancer, including NSCLC, prostate and breast cancer (16-18). Furthermore, activation of the PI3K/Akt signaling pathway may promote tumor cell proliferation, migration and drug resistance (19). It has been demonstrated that insulin stimulates the PI3K/Akt signaling pathway to increase carcinogenesis in breast cancer and colon cancer cells (14). However, it remains unknown whether insulin is able to regulate the development of NSCLC by activating the PI3K/Akt signaling pathway.

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The results of the present study indicated that insulin enhanced the proliferation, migration and drug resistance of NSCLC cells. In addition, LY294002, a specific inhibitor of the PI3K/Akt signaling pathway, reversed the oncogenic effects of insulin on protein expression. The results of the present study may therefore improve understanding of the effect of insulin on NSCLC.

Materials and methods

Reagents. Insulin, LY294002, diamminedichloroplatinum (DDP), RNase A, propidium iodide (PI) and RIPA buffer were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). BCA Protein Assay kit and enhanced chemiluminescence (ECL) reagent were purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA. RPMI-1640, high-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. Transwell chambers and Matrigel Invasion Chambers were purchased from BD Biosciences, Inc., Rockville, MD, USA. Antibodies against β -actin (cat. no. 3700), phospho-Akt (p-Akt, cat. no. D25E6) and Akt (cat. no. 11E7) were purchased from Cell Signaling Technologies, Inc., Danvers, MA, USA. Antibodies against insulin receptor substrate 1 (IRS1, cat. no. ab52167) and phospho-IRS1 (p-IRS1, cat. no. ab1194) were purchased from Abcam, Cambridge, UK. Antibodies against proliferating cell nuclear antigen (PCNA, cat. no. sc-25280), cyclin A (cat. no. sc-751), p27 (cat. no. sc-528), P-glycoprotein (P-gp, cat. no. sc-55510) and matrix metalloproteinase 3 (MMP3, cat. no. sc-21732) were all purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Goat anti-mouse IgG (cat. no. 31430) and goat anti-rabbit IgG (cat. no. 31460) secondary antibodies conjugated to horse-radish peroxidase (HRP) were purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Cell culture. The human lung cancer cell lines A549, PC-9 and NCI-H1975 were provided by Dr Yan-Jun Mi (The First Affiliated Hospital of Xiamen University, Xiamen, China). Cells were maintained in RPMI-1640 medium (NCI-H1975 cells) or DMEM (A549 and PC-9 cells) supplemented with 10% FBS and 100 U/ml penicillin at 37°C in a humidified atmosphere of 5% CO₂.

Cell proliferation assay. Cell proliferation was determined using MTT, which was dissolved in dimethyl sulfoxide. Cells were suspended at a concentration of 5×10^3 /well, seeded into a 96-well plate and incubated overnight at 37°C. Following treatment with different concentration of insulin (0, 400, 800, 1600 nM), 20 μ l MTT (5 mg/ml) was added to each well and the plate was incubated under the same conditions for 4 h. Absorbance was measured at 490 nm using an auto microplate reader. All experiments were performed in triplicate.

Drug resistance assay. Cells were seeded into a 96-well plate at a concentration of 5×10^3 /well. Following treatment with 1,600 nM insulin in the presence of various concentrations of DDP (0, 5, 10, 20 and 40 μ mol) at 37°C for 48 h, 20 μ l MTT (5 mg/ml) was added to each well and the subsequent steps

were performed according to the aforementioned cell proliferation assay protocol. The group treated with PBS acted as a control.

Flow cytometry. A549 cells were synchronized through treatment with serum-free high-glucose DMEM for 24 h. Following the addition of serum-free high-glucose DMEM with or without insulin (1,600 nM) for 3 days, A549 cells were harvested, washed twice with ice cold PBS and fixed with ice cold 70% ethanol at 4°C overnight. Cells were washed with cold PBS and incubated with RNase A (100 μ g/ml) at 37°C for 30 min. Cells were subsequently stained with PI (50 μ g/ml) at 4°C for 30 min and analyzed using a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed with the ModFit 3.3 (Verity Software House, Topsham, ME, USA) software. All experiments were performed in triplicate.

Cell migration and invasion assay. Cell migration was detected by wound healing and transwell assays, which were performed following a previously described protocol (20). A549 cells were used to investigate cell migration and invasion assay due to the characteristic of easy culture ability. For the wound healing assay, scratches were made with 10- μ l micropipette tip. Then, serum-free high-glucose DMEM was added with or without insulin (1,600 nM). For the transwell assay, A549 cells were suspended at a concentration of 1×10^5 /well, seeded into the upper chamber of a 24-well plate in serum-free high-glucose DMEM with or without insulin (1,600 nM) for 24 h. A total of 500 μ l DMEM was added to the lower chamber. The cells that migrated to the lower chamber were stained with 0.05% crystal violet at room temperature for 10 min and counted using an inverted microscope. Cell invasion assays were performed using transwell invasion chambers with Matrigel. A549 cells were seeded at a density of 1×10^5 /well into the upper chamber of a 24-well plate in serum-free high-glucose DMEM with or without insulin (1,600 nM), and incubated for 24 h. The subsequent steps were performed according to the aforementioned transwell assay protocol. All experiments were performed in triplicate.

Western blotting. Western blotting was performed as previously described (20). Total protein was extracted using RIPA buffer and its concentration was evaluated using the BCA Protein Assay kit. Proteins were separated using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Following blocking with 5% milk in PBST at room temperature for 1 h, the membrane was incubated with primary antibodies (β -actin, 1:1,000; p-Akt, 1:1,000; Akt, 1:1,000; IRS1, 1:1,000; p-IRS1, 1:1,000; PCNA, 1:500; cyclin A, 1:500; p27, 1:500; P-gp, 1:500; MMP3, 1:500) at 4°C overnight followed by incubation with the appropriate HRP-conjugated secondary antibody (1:10,000) at room temperature for 1 h. The signal was subsequently visualized using an ECL reporter system (Thermo Fisher Scientific, Inc.) followed by exposure to X-ray film. All experiments were performed in triplicate.

Statistical analysis. Data are presented as the mean \pm standard error of the mean. Unpaired Student's t test or one-way

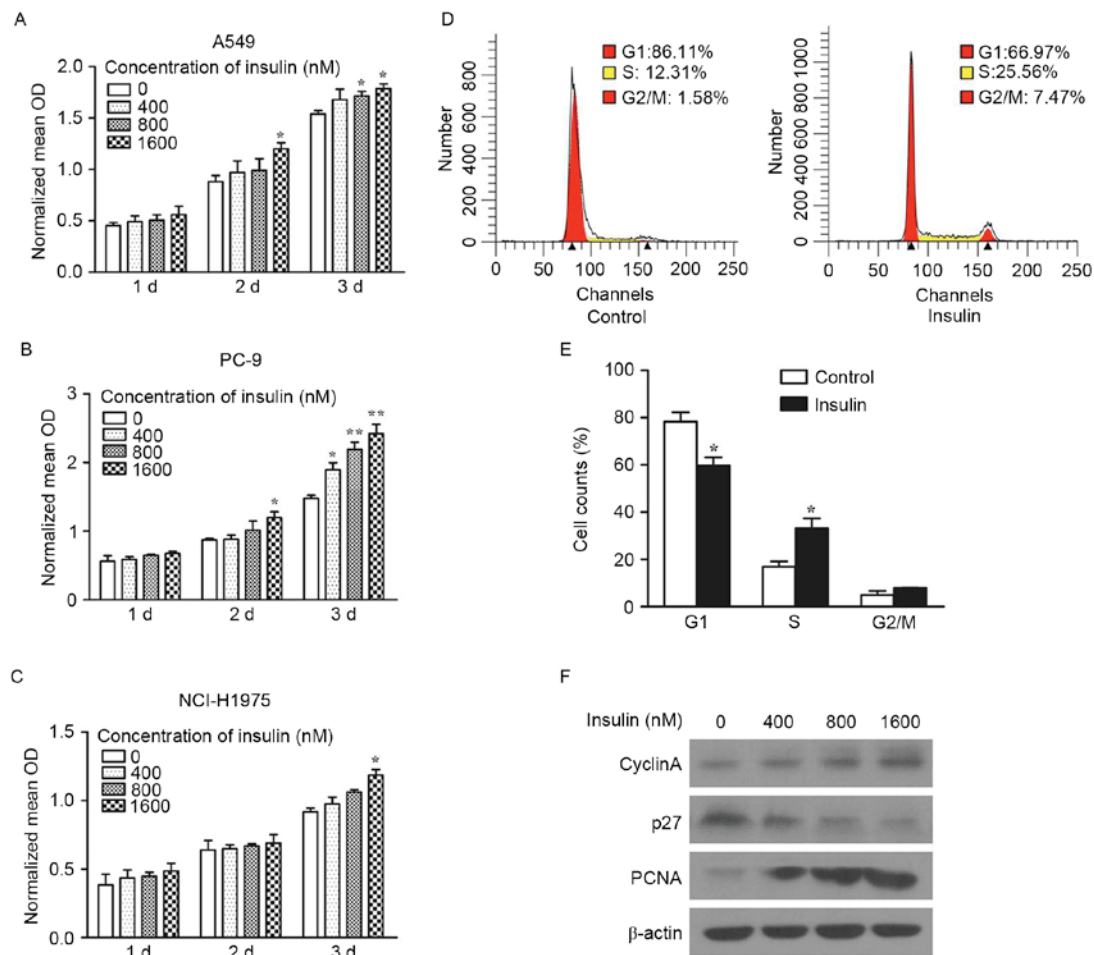


Figure 1. The proliferation of NSCLC cells following treatment with insulin. The proliferation of (A) A549, (B) PC-9 and (C) NCI-H1975 cells was measured using an MTT assay. * $P < 0.05$ and ** $P < 0.01$ vs. 0 nM insulin. (D and E) A549 cells were treated with 1,600 nM insulin for 3 days and subsequently analyzed by flow cytometry. * $P < 0.05$ vs. the control. (F) Cyclin A, p27 and PCNA expression were assessed in A549 cells by western blotting following treatment with 400, 800 and 1,600 nM insulin for 3 days. Data are presented as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$. PCNA, proliferating cell nuclear antigen; NSCLC, non-small cell lung cancer; d, days; OD, optical density.

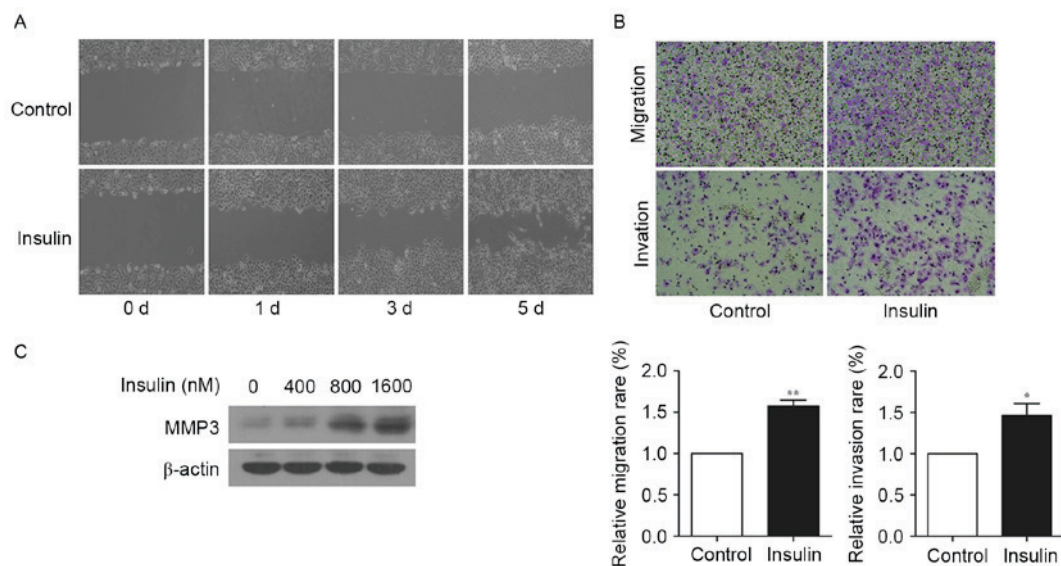


Figure 2. The migration of NSCLC cells following treatment with insulin. (A) The migration of A549 cells at 0, 1, 3 and 5 days was measured by a wound-healing assay following treatment with insulin. Magnification, $\times 400$. (B) The migration of A549 cells was measured by a Transwell assay following treatment with 1,600 nM insulin for 3 days. The migration rate was calculated and quantified. Data are presented as the mean \pm standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ vs. control. Magnification, $\times 400$. (C) The expression of MMP3 was assessed by western blotting following treatment with 1,600 nM insulin for 3 days. MMP3, matrix metalloproteinase 3.

analysis of variance were used for comparison of two or more datasets, respectively. Multiple comparison between the groups was performed using Student-Newman-Keuls method. $P < 0.05$ was considered to indicate a statistically significant difference. The data were analyzed with SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). All groups contained three replicates and all experiments were repeated three times.

Results

Insulin promotes the proliferation of NSCLC cells. An MTT assay was performed to determine the role of insulin in the proliferation of NSCLC cells. The proliferation of A549, PC-9 and NCI-H1975 cells were enhanced by insulin in a dose- and time-dependent manner (Fig. 1A-C; 0 nM insulin acted as a control; $*P < 0.05$ vs. 0 nM insulin at 2 or 3 d in A549, PC-9 or NCI-H1975 cells; $**P < 0.01$ vs. 0 nM insulin at 3 d in PC-9 cells). The enhancing effect of insulin on the proliferation of the aforementioned cells, were more obvious by day 3. In addition, the results of flow cytometry demonstrated that insulin significantly decreased the proportion of cells in the G1 phase ($P < 0.05$) and significantly increased the proportion of cells in the S-phase compared with control ($P < 0.05$; Fig. 1D and E). Western blotting was performed to measure the expression of cell cycle and growth-related proteins in A549 cells. The expression of cyclin A and PCNA were increased and of p27 protein expression was decreased in a dose-dependent manner following treatment with insulin (Fig. 1F). These results indicate that insulin promotes the proliferation of the NSCLC cells.

Insulin promotes the migration and invasion of NSCLC cells. Subsequently, the effect of insulin on NSCLC migration and invasion was detected by wound healing and Transwell assays. The ability of migration and invasion were markedly elevated by insulin in A549 cells (Fig. 2A and B). Furthermore, the number of migrating and invading A549 cells were significantly increased following treatment with insulin compared with untreated control cells (Fig. 2B; $P < 0.05$). In addition, insulin enhanced the expression of MMP3 in a dose-dependent manner in A549 cells (Fig. 2C). Taken together, these results suggest that insulin may promote the migration and invasion of NSCLC cells by regulating MMP3 expression.

Insulin increases the resistance of NSCLC cells to DDP. To investigate the effect of insulin on drug resistance, A549 and PC-9 cells were treated with different concentrations of DDP (0, 5, 10, 20 and 40 μmol) in the absence or presence of insulin. The sensitivity of A549 and PC-9 cells to DDP was significantly reduced by insulin (Fig. 3A and B; $P < 0.05$). Furthermore, the expression of P-gp in A549 cells was increased by insulin in a dose-dependent manner (Fig. 3C), indicating that insulin may reduce the sensitivity of NSCLC cells to DDP by upregulating P-gp expression.

Insulin exhibits oncogenic activity via the PI3K/Akt signaling pathway. To further explore the mechanism of insulin on cell proliferation, migration and drug sensitivity, the effect of insulin on the IRS and PI3K/Akt signaling

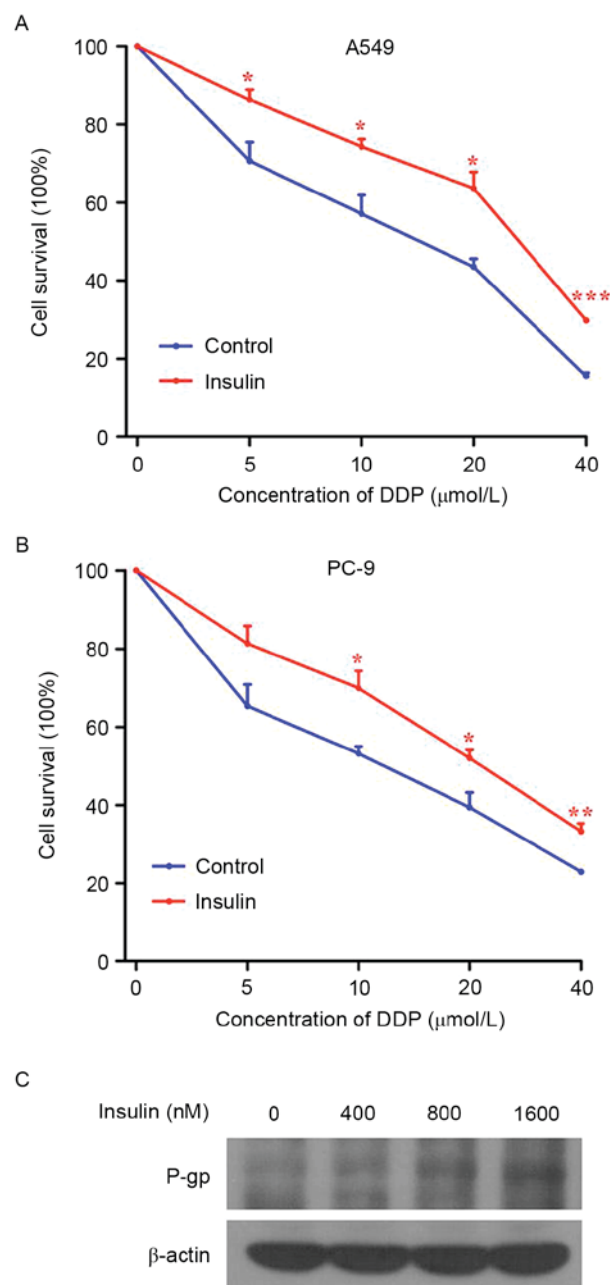


Figure 3. The susceptibility of NSCLC cells to drug treatment following treatment with insulin. Following treatment with different concentrations of DDP (0, 5, 10, 20 and 40 μmol) and/or 1,600 nM insulin for 48 h, the susceptibility of (A) A549 and (B) PC-9 cells to DDP was measured by MTT. Results are presented as the mean \pm standard error of the mean. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. 0 μmol DDP. (C) The expression of P-gp was assessed by western blotting in A549 cells following treatment with insulin for 3 days. DDP, diamminedichloroplatinum; P-gp, P-glycoprotein.

pathway in NSCLC, which has been investigated in other types of cancer (14,21), was determined by western blot analysis. The results demonstrated that insulin increased p-Akt and p-IRS1 expression in a dose-dependent manner (Fig. 4A). Furthermore, following the addition of LY294002, a PI3K/Akt inhibitor, the expression of p-Akt and p-IRS1 in A549 cells was reduced compared with cells treated with insulin alone and was similar to that of untreated control cells (Fig. 4B). Correspondingly, LY294002 also reversed the effects of insulin on the expression of cyclin A, p27, PCNA,

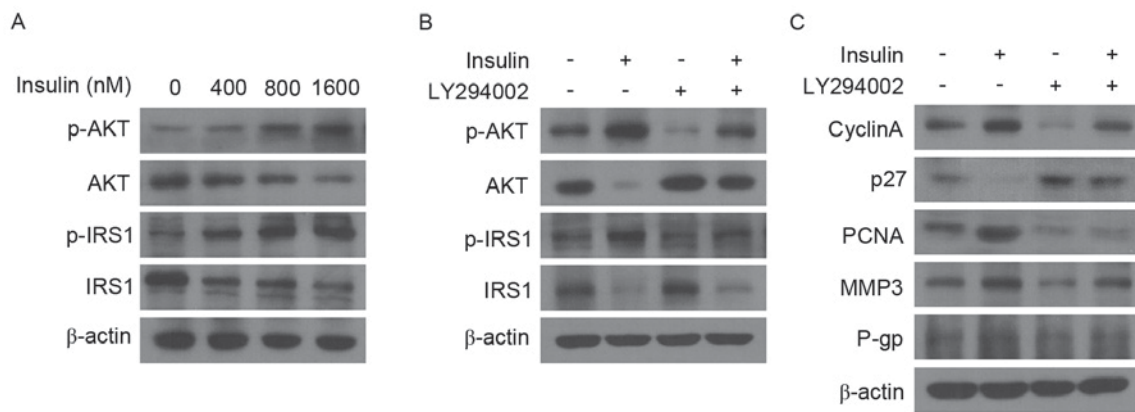


Figure 4. Insulin regulates the PI3K/Akt signaling pathway. (A) Expression of p-Akt, Akt, p-IRS1 and IRS1 were assessed by western blotting in A549 cells following treatment with 0, 400, 800 and 1,600 nM insulin for 3 days. (B and C) Western blot analysis of A549 cells exposed to 1,600 nM insulin and/or 1 μ M LY294002 for 3 days. PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; Akt, protein kinase B; p-, phosphorylated; IRS1, insulin receptor substrate 1; PCNA, proliferating cell nuclear antigen; MMP-3, matrix metalloproteinase 3; P-gp, P-glycoprotein.

MMP3 and P-gp in A549 cells (Fig. 4C). These results indicate that the PI3K/Akt signaling pathway may be involved in the oncogenic activity of insulin in NSCLC.

Discussion

The role of insulin in the pathogenesis of cancer was first reported in the 1970's (22). Insulin acts as a growth factor and may stimulate neoplastic development (23). In lung cancer, intracellular insulin activity is an important factor affecting the progression of low-grade human lung adenocarcinomas (24). Furthermore, inhaled insulin is associated with an increased incidence of lung cancer among former smokers (25).

The results of the present study revealed that insulin exhibits oncogenic activity during the development of NSCLC. Insulin markedly increased the proliferation and migration of NSCLC cells and significantly decreased the sensitivity of NSCLC cells to DDP. Cell cycle analysis demonstrated that insulin decreased the proportion of cells in the G1 phase and increased the proportion of cells in the S-phase. In addition, it was demonstrated that insulin upregulated cyclin A and PCNA expression and downregulated p27 expression in a dose-dependent manner. Furthermore, the expression of MMP3 was markedly increased following incubation with insulin in A549 cells. Lastly, insulin enhanced the expression of P-gp in a dose-dependent manner in A549 cells.

The oncogenic activity of insulin has been reported in other types of cancer tissues. Previous studies have demonstrated that insulin may contribute to the proliferation and survival of different types of cancer, including gastric, colon, pancreatic, breast and bladder cancer (14,15,21,26). It has also been demonstrated that insulin potentiates the ability of lysophosphatidic acid to stimulate cell cycle progression and DNA synthesis in MCF-7 breast cancer cells. The potentiating effect of insulin appears to increase the expression level of cyclin E and decrease the expression level of the cyclin-dependent kinase inhibitor p27^{Kip1} (27). Insulin enhances the sensitivity of gastric cancer cells to 5-fluorouracil and stimulates the migration of breast cancer cells (14,15). Insulin is also able to accelerate the metabolic capacities of cancer cells by

performing dual regulation of the glycolytic enzyme pyruvate kinase M2 (28).

Insulin binds to the insulin receptor and causes phosphorylation of insulin receptor (InsR) substrate proteins, followed by activation of downstream molecules including PI3K/Akt and MAPK. The activated PI3K/Akt signaling pathway is involved in various oncogenic functions, including the induction of cell proliferation, migration and drug resistance (19). Previous studies have demonstrated that insulin promotes the proliferation of pancreatic, bladder, breast and colon cancer cells via the PI3K/Akt signaling pathway (13,14,21). In NSCLC, overexpression of InsR predicts poor patient survival (29). IRS1 is an adaptor protein for insulin signaling and it has been suggested that IRS1 loss occurs early on in the pathogenesis of NSCLC (30). In the present study, it was demonstrated that insulin enhanced p-Akt and p-IRS1 expression in a dose-dependent manner. Furthermore, inhibition of PI3K/Akt activation by LY294002 significantly reversed the insulin-induced increases in p-Akt, p-IRS1, cyclin A, PCNA, MMP3 and P-gp expression. At the same time, levels of Akt, IRS1 and p27 expression, which were decreased by insulin, were increased following treatment with LY294002. These results suggest that the PI3K/Akt signaling pathway is crucial for the effect of insulin on NSCLC.

In conclusion, the current study demonstrated that insulin is an oncogenic factor in the development of NSCLC that may work by activating the PI3K/Akt signaling pathway. Future studies should investigate how to counteract the oncogenic activity of insulin on NSCLC.

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