Original Paper

Insulin Stimulates Goose Liver Cell Growth by Activating PI3K-AKT-mTOR Signal Pathway

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Key Words

Insulin • Goose • PI3K-Akt-mTOR signal pathway • Cell proliferation

Abstract

Background/Aims: Recent studies have suggested a crucial role for PI3K-Akt-mTOR pathway in regulating cell proliferation, so we hypothesize that insulin acts goose hepatocellular growth by PI3K-Akt-mTOR signal pathway. Because the physiological status of liver cells *in vitro* is different from that *in vivo*, a simplified cell model *in vitro* was established. **Methods:** Goose primary hepatocytes were isolated and incubated in either no addition as a control or insulin or PI3K-Akt-mTOR pathway inhibitors or co-treatment with glucose and PI3K-Akt-mTOR pathway inhibitors; Then, cell DNA synthesis and cell cycle analysis were detected by BrdU-incorporation Assay and Flow cytometric analysis; the mRNA expression and protein expression of factors involved in the cell cycle were determined by Real-Time RT-PCR, ELISA, and western blot. **Results:** Here we first showed that insulin evidently increased the cell DNA synthesis, the mRNA level and protein content of factors involved in the cell proliferation of goose primary hepatocytes. Meanwhile, insulin evidently increased the mRNA level and protein content of factors involved in PI3K-Akt-mTOR pathway. However, the up-regulation of insulin on cell proliferation was decreased significantly by the inhibitors of PI3K-Akt-mTOR pathway, LY294002, rapamycin or NVP-BEZ235. **Conclusion:** These findings suggest that PI3K-Akt-mTOR pathway plays an essential role in insulin-regulated cell proliferation of goose hepatocyte.

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Introduction

As a hormone with a number of biological effects, insulin not only displays the function of classic metabolic regulation, but also can regulate cell proliferation. Administration of insulin to newly hatched chicks improves growth performance via enhancement of cell

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proliferation in chicken myoblasts [1]. Insulin promotes cell growth by the induction of genes involved in cell proliferation [2]. PI3K-Akt-mTOR signal pathway is important for the cell growth and proliferation [3, 4]. As previously discussed, PI3K-Akt-mTOR was required for cell proliferation in different cell types [5, 6]. PI3K-Akt-mTOR pathway is known to play a major regulatory role in insulin pathway [7]. In current study, insulin could promote cell proliferation via PI3K-Akt pathway [8, 9]. However, there has no report about the role of insulin in cell proliferation of liver cells.

In our previous study, accompanied by the formation of goose hepatic steatosis induced by overfeeding, plasma insulin increased, and the PI3K-AKT-mTOR signal pathway were activated [10]. It is supposed that in the process of overfeeding to goose, insulin stimulates goose liver cell growth by activating PI3K-AKT-mTOR signal pathway. In order to clarify this hypothesis, in this study, a simplified cell model *in vitro* was established, for the physiological status of liver cells *in vitro* is different from that *in vivo*. We measured whether insulin stimulates the cell proliferation of goose liver cell and the involvement of PI3K-Akt-mTOR pathway in the process is investigated.

Materials and Methods

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of Sichuan Agricultural University at which the studies were conducted.

Primary Hepatocyte Isolation and Culture

Hepatocytes were isolated from three 30-day-old Tianfu meat geese from the Experimental Farm for Waterfowl Breeding at Sichuan Agricultural University using a modification of the "two-step procedure" described by Seglen [11]. The method differed from that of Seglen in that the liver was removed before the preperfusion step. Cultures were incubated at 40° in a humidified atmosphere containing 5% CO₂; the media was renewed after 3 h, and after 24 h, the media was replaced with serum-free media. After an additional 24 h, the cells were separately treated with serum-free media supplemented with 0, 50, 100 and 150 µmol/l of insulin and incubated for 24 h (the media was not renewed), while the control cells were cultured with serum-free media for 24 h (serum-free media was not renewed). In addition, some cells were treated with serum-free media supplemented with PI3K-Akt-mTOR pathway inhibitors (LY294002, rapamycin, NVP-BEZ235, respectively) for 24 h and then added 50 nmol/L insulin or 150 nmol/L insulin for the other 24 h together. After the incubation, the culture media and cells were cooled on ice and collected. In each case, the experiments were repeated three times.

MTT assay for cell viability

The assay for cell viability was performed according to Natali et al. [12], with some modifications. Primary cultures of goose hepatocytes were plated at a density of 0.5×10^4 cells/well in a 96-well culture dish. After the incubation with insulin and inhibitors, the cell monolayers were incubated for 4 h with 1 mg/ml of MTT. The relative cell proliferation ratio was calculated relative to the OD value at 490 nm of the control group, which was defined as 100%.

Flow cytometric/cell cycle analysis

The proportion of cells in the G0/G1, S, and G2M phases of the cell cycle was determined by flow cytometric analysis. Primary goose hepatocytes (2×10^4 cells) were plated in 24-well culture dish. After the incubation with insulin or inhibitors, cells were then harvested by trypsinization, centrifuged at 800 g for 10 min and resuspended in a final concentration of 10^6 cells/ml in hypotonic propidium iodide (PI) solution. Cells were incubated in 4°C for 30 min and analyzed on a FACScan (Becton-Dickinson, Franklin Lakes, NJ). One hundred thousand cells were collected for each sample. Excitation occurred at 488 nm and data was collected using the FL2 channel and analyzed using the software modifit. Cell proliferative index (PI)=(S+G2M)/(G0G1+S+G2M)×100.

BrdU-incorporation Assay

Primary cultures of goose hepatocytes were plated at a density of 0.5×10^4 cells/well in a 96-well culture dish. After the incubation with insulin and inhibitors, cell monolayers were then incubated for 24 h with 10 μ M bromodeoxyuridine (BrdU) in culture medium. The cells were then washed and fixed, and the incorporated BrdU was detected by a specific ELISA (Roche, Indianapolis, IN) in an ELISA reader. Meanwhile, the BrdU positive cells were detected. Briefly, after incubation with insulin and inhibitors in 35 mm culture dishes, the cells were stained with rabbit anti-BrdU antibody (1:100, Beijing Biosynthesis Biotechnology, China), then incubated with goat anti-rabbit Cy3-conjugated secondary antibody (1:300, Beijing Biosynthesis Biotechnology, China), and finally counterstained with DAPI. Cells were visualized using an upright BH2 microscope (Olympus, Japan) and quantified by counting BrdU-positive cells in 3 independent areas. BrdU-positive cells are green, and the DAPI-positive cells are blue. The experiments were repeated three times.

Measurement of protein content in culture cells

Protein content of relative protein involved in cell proliferation in culture cells were measured using respective ELISA kits according to the manufacturer's instructions. The absorbance at 450 nm was read using a plate reader. Protein content in the samples were calculated from polynomial second order or exponential standard curves obtained from the standards included in each assay.

Isolation of Total RNA and Real-Time RT-PCR

Total RNA was isolated from cultured cells using Trizol (Invitrogen, USA) and reverse-transcribed using the Primer Script™ RT system kit for real-time PCR (TaKaRa, Japan) according to the manufacturer's instructions. Specific primers were designed according to the goose gene sequences and are listed in Table 1.

Each sample was repeated in two 96-well plates and the variation of Ct between the two independent plates was 0.28 ± 0.22 , showing a fair level of inter-assay reproducibility. PCR products were then diluted 16-fold and were used to generate the calibration curve and the amplification rate (R) for each gene. For each experimental sample, a normalized target gene level (Exp), corresponding to the target gene expression level relative to β -actin, 18S and UBC (housekeeping genes) expression levels, was determined by the $2^{-\Delta\Delta Ct}$ method as previously described [13]:

$$\text{Exp}_{\text{target gene in sample}} = (1 + R_{\text{target gene}})^{Ct(\text{target gene in sample})} / (1 + R_{\beta\text{-actin or 18S or UBC}})^{Ct(\beta\text{-actin or 18S or UBC in sample})}$$

The final results were calculated by extracting the square root of the two relative mRNA levels of each gene relative to β -actin, 18S and UBC. The results for each individual were repeated three times and averaged.

Protein Analysis by Western Blotting

Total protein extracts were obtained using a reducing SDS buffer. Protein concentrations were determined on diluted samples using a Bradford procedure. Equal amounts of protein (100 μ g) were separated on a 6% SDS-PAGE and transferred on membranes. Membranes were blocked in a TBS solution

Table 1. Primer sequences for real-time PCR

Gene Name	Upstream (5'-3')	Downstream (5'-3')	Product size (bp)	Accession number
PI3K	ACCCAAGCGAGGATGAGG	TGTTGCCCGTGTGAATG	241	KF011500
Akt1	TGCTGGATAAAGATGGAC	CTGGTTGTAGAAAGGGAG	215	KF011501
mTOR	TCATTTGTTACTACCTCCCA	TTTCTAGAGCAGCTTGGCAGCCAC	93	KC424580
S6K	CTCAACTTGCTCCCTAC	AACCTCTCCAGCATCTCC	111	KC424581
4EBP1	CCACCTTCTGACCTTCCG	CATTGCTTTCTCATCGTAG	103	KF011497
Rptor	GAAAGGCAAATATCAACCG	CAGCCATCACAGACACCA	223	KF011498
P21	AGGCAACACCTGGAAGAA	ATGAGACCCACCAAGACG	207	KC424586
Cyclin D1	AGGAGCAGAAGTGCGAAGA	TGCGGTGAGAGGAATAGTTT	158	KC424583
Cyclin D2	TTCATCGCCCTTTGTGCC	ATTGCTCCCACGCTTCCA	80	KC424584
Cyclin D3	CTGGTCTCGGTGATAGCG	GACGAAAGTGATGCTGTGGC	135	KC424585
P27	CGTAAAGGCGTAGCAGAG	GCAAGTTCCAATCGGTCA	299	KF857234
Akt2	GCGATGCTCCATCACCTCC	CGCTGCGCTTCTACAACC	183	KF857233
UBC	AGGGTGGATTCTTTCTGG	ACTGAGTTTGGAGGGAGC	243	GO240773.1
β -actin	CAACGAGCGGTTTCAGGTGT	TGGAGTTGAAGGTGGTCTCG	92	M26111.1
18S	TTGGTGGAGCGATTGTGC	ATCTCGGGTGGCTGAACG	129	L21170.1

with 5% nonfat dry milk and incubated with rabbit against Cyclin D1, p21, S6K or p-S6K antibodies (1:1,000; Beijing Biosynthesis Biotechnology, China). A goat anti-rabbit horseradish peroxidase-conjugated IgG at 1:2000 (Beijing Biosynthesis Biotechnology, China) was used as the secondary antibody and the signals were detected using an ECL western blot detection kit (Beyotime Institute of Biotechnology, China). After analysis, the membranes were blotted with α -tubulin antibody at 1:1000 (Beijing Biosynthesis Biotechnology, China) to normalize for protein amount. The blot images were digitized with a luminescent image analyzer (LAS-1000, Fuji Photo Film).

Statistical Analysis

The data were subjected to ANOVA testing and the means were assessed for significance by Tukey's test. Analysis of variance and *t*-tests were performed using the SAS 9.13 package (SAS Institute Inc, Cary, NC). The results are presented as the mean $\pm$ SD. $P < 0.05$ was accepted as the level of significance. Every experiment was repeated with 3 biological samples, and each sample was run in triplicate.

Results

Effect of insulin on the cell proliferation of goose hepatocytes

The result of flow cytometric analysis indicated the treatment with insulin stimulated the DNA synthesis in a dose-dependent manner in goose hepatocytes. 100 nM insulin increased DNA synthesis by 26.3%, and 150 nM, insulin increased DNA synthesis by 55.5% (Fig. 1).

The result of BrdU stain (Fig. 2a-c) supported that insulin stimulated cell proliferation. Compared with the control group, the ration of BrdU-positive cells number after the treatment of 150 nmol/L insulin increased up to 80% (Fig. 2d). As shown in Fig. 3a, insulin at a concentration of 50, 100 or 150 nmol/L increased the cell viability, and the cell viability showed an upward trend with an increasing insulin concentration. A BrdU incorporation assay was performed to measure the changed DNA synthesis of treated cells (Fig. 3b), and

Fig. 1. Effect of insulin on cell cycle. Goose primary hepatocytes were untreated (a), cultured with 50 nmol/L insulin (b), 100 nmol/L insulin (c), or 150 nmol/L insulin (d); e, the proportion of cells in the G0/G1, S, and G2M phases of the cell cycle, cell proliferative index (PI) = $(S+G2M)/(G0G1+S+G2M) \times 100$. After 12 h in serum-free medium, hepatocytes were incubated for 24 h in either no addition as a control or 50, 100, and 150 nmol/L insulin. Cells were examined by flow cytometer.

Fig. 2. Effect of insulin on rate of BrdU-positive cells in the goose primary hepatocytes. Goose primary hepatocytes were untreated (a), cultured with 50 nmol/L insulin (b), or 150 nmol/L insulin (c). d, Rate of BrdU-positive cells, and results (mean±standard error) are expressed as the ratio of the number of BrdU-positive cells compared to the DAPI-positive cells. Different uppercase letters in the same set indicate difference among treatments at $P < 0.05$. Cells were examined by phase contrast microscopy at 200× magnification.

the data indicated the DNA synthesis rate showed an upward trend with an increasing insulin concentration, and it is consistent with the result of BrdU stain.

Fig. 3c and 3d summarized the effect of insulin on protein content of Cyclin D1 and p21. Treatment with 100 and 150 nm/L of insulin significantly increased the protein content of Cyclin D1, and significantly decreased the protein content of p21. Fig. 3e and 3f summarized the effect of insulin on mRNA expression level of genes involved in cell proliferation. Compared to the control group, insulin at all three concentrations increased the mRNA level of Cyclin D1, Cyclin D2, Cyclin D3 in a dose-dependent manner. Treatment with 100, and 150 nm/L of insulin increased the mRNA level of p21 and p27 significantly. The result of western blot (Fig. 3g) verified that insulin could stimulate the protein expression of Cyclin D1, and inhibit the protein expression of p21.

LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on cell proliferation

To verify that regulation of cell proliferation by insulin is connected to the modulation of PI3K-Akt-mTOR signaling, insulin and a PI3K inhibitor, LY294002, or a mTOR inhibitor, Rapamycin, or a Akt-mTOR dual inhibitor, NVP-BEZ235 were treated together in cell culturement. As shown in Fig. 4a-c, 20 μ mol/L LY294002, 30 nmol/L rapamycin, and 1 μ mol/L NVP-BEZ235 decreased the stimulation of insulin on the DNA synthesis rate of treated cells, and it is consistent with the result of BrdU stain (Fig. 4j) and the result of

Fig. 3. Stimulator effect of insulin on cell proliferation in the goose primary hepatocytes. (a) Cell viability detected by MTT assay; (b) DNA synthesis rate detected by bromodeoxyuridine incorporation assay; (c and d) Protein content of Cyclin D1 and p21, and the units of Cyclin D1 and p21 both are ng/ml; (e and f) Relative mRNA level of genes related with cell proliferation; (g) Western blot result of the protein expression of Cyclin D1 and p21 after insulin treatment. Different uppercase letters in the same set indicate difference among treatments at $P < 0.05$. OD = optical density.

cell cycle analysis (Fig. 5). The result of Fig. 4d-i showed, the protein content of Cyclin D1 after treatment of insulin with 20 $\mu\text{mol/L}$ LY294002, or 30 nmol/L rapamycin, or 1 $\mu\text{mol/L}$ NVP-BEZ235 together is lower significantly than that of insulin treatment. Meanwhile, the result of p21 after treatment of insulin with LY294002, rapamycin, or NVP-BEZ235 together is greater significantly than that of insulin treatment. Fig. 6a-c summarized the treatment of LY294002, rapamycin, or NVP-BEZ235 how to affect insulin-induced change on the mRNA expression level of genes involved in cell proliferation. Compared to the insulin treatment, the treatment of insulin at 50 nmol/L or 150 nmol/L with LY294002, or rapamycin, or NVP-BEZ235 together all decreased the mRNA level of Cyclin D1, Cyclin D2, Cyclin D3, significantly. The mRNA level of p21 and p27 after the treatment of insulin with three inhibitors together are all bigger than that of single insulin treatment significantly. These results indicate that insulin regulate cell proliferation mediated by PI3K-Akt-mTOR signaling pathway. The result of western blot (Fig. 6d) verified that PI3K-Akt-mTOR signaling pathways inhibitors abolished the effect of insulin on the protein expression of Cyclin D1 and p21.

Fig. 4. LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on cell proliferation in the goose primary hepatocytes. (a, b and c) DNA synthesis rate detected by bromodeoxyuridine incorporation assay; (d, e, and f) Protein content of Cyclin D1, and the units of Cyclin D1 are ng/ml; (g, h, and i) Protein content of p21, and the units of p21 are ng/ml; “50INS, 150INS, LY, RAP, NVP” under the X axis indicate the treatment: 50 nmol/L insulin, 150 nmol/L insulin, 20 μ mol/L LY294002, 30 nmol/L rapamycin, and 1 μ mol/L NVP-BEZ235; (j) rate of BrdU-positive cells, the numbers “1-12” under the X axis indicate the treatment: 1, control; 2, 20 μ mol/L LY294002; 3, 30 nmol/L rapamycin; 4, 1 μ mol/L NVP-BEZ235; 5, 50 nmol/L insulin; 6, 50 nmol/L insulin + 20 μ mol/L LY294002; 7, 50 nmol/L insulin + 30 nmol/L rapamycin; 8, 50 nmol/L insulin + 1 μ mol/L NVP-BEZ235; 9, 150 nmol/L insulin; 10, 150 nmol/L insulin + 20 μ mol/L LY294002; 11, 150 nmol/L insulin + 30 nmol/L rapamycin; 12, 150 nmol/L insulin + 1 μ mol/L NVP-BEZ235. Different up-percase letters in the same set indicate difference among treatments at $P < 0.05$. OD = optical density.

Insulin stimulate PI3K-Akt-mTOR pathway

Fig. 7a summarized the effect of insulin on mRNA expression level of genes involved in PI3K-Akt-mTOR pathway. Compared to the control group, insulin at all three concentrations increased the mRNA level of PI3K, Akt1, Akt2, mTOR, Rptor, 4EBP1, and S6K in a dose-dependent manner. Fig. 7b showed, treatment with 100 or 150 nmol/L of insulin significantly increase the protein content of PI3K, Akt, mTOR, 4EBP1, and S6K. The result of western blot in Fig. 7c showed insulin stimulated of the protein expression of S6K and p-S6K. These results indicate that insulin treatment could stimulate PI3K-Akt-mTOR signaling pathways.

Fig. 5. LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on cell cycle in the goose primary hepatocytes. Goose primary hepatocytes were untreated (a), cultured with 50 nmol/L insulin (b), 20 μ mol/L LY294002 (c), 50 nmol/L insulin + 20 μ mol/L LY294002 (d), 30 nmol/L rapamycin (e), 50 nmol/L insulin + 30 nmol/L rapamycin (f), 1 μ mol/L NVP-BEZ235 (g), 50 nmol/L insulin + 1 μ mol/L NVP-BEZ235 (h). Cells were examined by flow cytometer.

LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on PI3K-Akt-mTOR signal pathway

To further verify that activation of PI3K-Akt-mTOR signal pathway by insulin, the collective effect of insulin and PI3K-Akt-mTOR signal pathway inhibitors on the protein content and mRNA level of PI3K-Akt-mTOR signal pathway was assessed. As shown in Fig. 8a-e, after collective treatment with insulin and 20 μ mol/L LY294002, 30 nmol/L rapamycin, and 1 μ mol/L NVP-BEZ235, the protein content of PI3K, Akt, mTOR, 4EBP1, and S6K is lower than that after single insulin treatment. The result of western blot in Fig. 8f showed that, compared with the stimulation role of insulin alone, the collective treatment of insulin at 150 nmol/L with 20 μ mol/L LY294002, or 30 nmol/L rapamycin, or 1 μ mol/L NVP-BEZ235 decreased the protein expression level of S6K and p-S6K. Fig. 9 summarized the three PI3K-Akt-mTOR signal pathway inhibitors decreased the activation of insulin on the mRNA expression level of genes involved in PI3K-Akt-mTOR pathway. Compared to the result of insulin treatment, the mRNA level of PI3K, Akt1, Akt2, mTOR, Rptor, 4EBP1, and S6K all decreased, significantly after the collective treatment of insulin at 50 nmol/L or 150 nmol/L with 20 μ mol/L LY294002, or 30 nmol/L rapamycin, or 1 μ mol/L NVP-BEZ235. These results further verify that the activation role of insulin in cell proliferation is mediated by PI3K-Akt-mTOR signaling pathways.

Discussion

Previous research has already demonstrated insulin play a role in overfeeding-induced goose fatty liver and hepatic lipids deposition [14, 15]. However, whether insulin regulates

Fig. 6. LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on relative mRNA level and protein expression of genes related with cell proliferation. (a) relative mRNA level after treatment of LY294002 and insulin together or alone; (b) relative mRNA level after treatment of Rapamycin and insulin together or alone; (c) relative mRNA level after treatment of NVP-BEZ235 and insulin together or alone; Different uppercase letters in the same set indicate difference among treatments at $P < 0.05$; (d) Western blot result of the protein expression of Cyclin D1 and p21 after the treatment of insulin and LY294002, Rapamycin or NVP-BEZ235 together or alone. The numbers “1, 2, 3, 4, 5, 6, 7, 8” under the blot indicate control, 150 nmol/L insulin, 20 μ mol/L LY294002, 30 nmol/L rapamycin, 1 μ mol/L NVP-BEZ235, 150 nmol/L insulin + 20 μ mol/L LY294002, 150 nmol/L insulin + 30 nmol/L rapamycin, and 150 nmol/L insulin + 1 μ mol/L NVP-BEZ235, respectively. Each blot is a representative of three independent experiments.

the cell growth of goose liver is unclear. In this study we demonstrated that insulin promoted an increase in proliferation of goose hepatocytes at 50, 100 and 150 nmol/L, respectively. Such doses are much higher than physiological concentration of insulin (0.1-1 nmol/L). Similar comparable dose-dependent growth response to insulin has been observed in other cell lines such as MCF-7 breast cancer cells [16]. After culture with three PI3K-Akt-mTOR pathway inhibitors, LY294002, Rapamycin, or NVPBEZ235, the cell proliferation was inhibited. The inhibiting role of NVPBEZ235 was evident than that of LY294002 and rapamycin. When PI3K-Akt-mTOR pathway was blocked by LY294002, Rapamycin, or NVPBEZ235, respectively, insulin still promoted cell growth as compared to the cells treated with inhibitor alone, but its effect was lower than the insulin treatment alone. Cell proliferation driven by insulin was effectively reverted using the dual PI3K/mTOR inhibitor, NVP-BEZ235, both *in vitro* and *in vivo* [17]. We further found that when PI3K was blocked by LY294002, insulin still increased

Fig. 7. Treatment with insulin stimulate PI3K-Akt-mTOR pathway. (a) Relative mRNA level of genes related with PI3K-Akt-mTOR pathway; (b) Protein content of factors involved in PI3K-Akt-mTOR pathway, and the unit of PI3K is pmol/ml, the units of Akt1 and mTOR are pg/ml, the units of S6K and 4EBP1 are ng/ml; (c) Result of western blot for S6K and p-S6K. Each blot is a representative of three independent experiments. Different uppercase letters in the same set indicate difference among treatments at $P < 0.05$.

the gene expression and activity of PI3K downstream targets Akt and mTOR. These results suggested that alternative pathways to the activation of Akt and mTOR might be involved in insulin-induced cell proliferation.

mTOR is a downstream target of Akt. Interestingly, rapamycin inhibited the mRNA and activity of mTOR, but also inhibited the mRNA and activity of PI3K and Akt. However, in other reports, rapamycin inhibits mTOR, and it can lead to activation of upstream proteins such as Akt, due to the loss of a feedback loop mechanism [18, 19]. The reason why rapamycin inhibited PI3K and Akt in goose liver cells remains to be determined. mTOR seems to be involved only partly in the insulin action on cell proliferation, because the inhibition of mTOR by rapamycin did not completely abolish insulin-induced cell proliferation. These results suggest that at least the two following pathways are involved in insulin-induced cell proliferation regulation, a PI3K-Akt-dependent/mTOR-independent pathway and a PI3K-Akt/mTOR-dependent pathway. It has been shown that insulin could revert the inhibition of the dual PI3K/mTOR inhibitor NVPBEZ235 induced-decrease of cell proliferation in goose. Insulin increased the levels of phosphorylated mTOR and p-S6K in parental HepG2 cells and HepG2 cells overexpressing constitutively active Akt/PKB cell lines. Rapamycin treatment partially decreased the phosphorylation of mTOR but completely abolished the phosphorylation of p-S6K in the absence as well as presence of insulin in both cell lines [20]. In this study, rapamycin caused inhibition of cell proliferation both in the absence and presence of insulin, but the inhibitory effect was reverted evidently in the absence of insulin. It is possible that the presence of insulin might lead to phosphorylation of p-S6K and/or

Fig. 8. LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on the protein level of factors involved in PI3K-Akt-mTOR signal pathway. (a - e) Protein content of factors involved in PI3K-Akt-mTOR pathway, and the unit of PI3K is pmol/ml, the units of Akt1 and mTOR are pg/ml, the units of S6K and 4EBP1 are ng/ml. The numbers "1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12" under the X axis indicate control, 20 μ mol/L LY294002, 30 nmol/L rapamycin, 1 μ mol/L NVP-BEZ235, 50 nmol/L insulin, 50 nmol/L insulin +20 μ mol/L LY294002, 50 nmol/L insulin +30 nmol/L rapamycin, 50 nmol/L insulin +1 μ mol/L NVP-BEZ235, 150 nmol/L insulin, 150 nmol/L insulin +20 μ mol/L LY294002, 150 nmol/L insulin +30 nmol/L rapamycin, and 150 nmol/L insulin +1 μ mol/L NVP-BEZ235, respectively; (f) Western blot result of the protein expression of S6K and p-S6K after the treatment of insulin and LY294002,

Rapamycin or NVP-BEZ235 together or alone. "INS, LY, RAP, NVP" under the blot indicate 150 nmol/L insulin, 20 μ mol/L LY294002, 30 nmol/L rapamycin, and 1 μ mol/L NVP-BEZ235. Each blot is a representative of three independent experiments.

reversal of mTOR activity even in the presence of rapamycin and this might be the cause of cell proliferation even in the presence of rapamycin. Alternatively, there may be other kinases controlling the activated cell proliferation, although the dual PI3K/mTOR inhibitor NVPBEZ235 was applied in these cells.

Insulin activated the mRNA level or protein content of cyclin D1, cyclin D2, and cyclin D3, three critical regulators of cell proliferation, and it inhibited the mRNA level or protein content of p21 and p27, two cell cycle inhibitors. Meanwhile, insulin stimulated the mRNA level and protein content of genes involved in the PI3K-Akt-mTOR pathway. The culture with three PI3K-Akt-mTOR pathway inhibitors, LY294002, Rapamycin, or NVPBEZ235, decreased the mRNA level or protein content of Cyclin D1, Cyclin D2, and Cyclin D3, increased the mRNA level or protein content of p21 and p27. However, after the treatment of LY294002, Rapamycin, or NVPBEZ235, insulin could restore the mRNA level or protein content of relative factors, which means insulin could stimulate cell proliferation mediated-by PI3K-

Fig. 9. LY294002, Rapamycin or NVP-BEZ235 decreased the stimulation of insulin on relative mRNA level of genes related with PI3K-Akt-mTOR pathway. Different uppercase letters in the same set indicate difference among treatments at $P < 0.05$. The numbers “1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12” under the X axis indicate control, 20 $\mu\text{mol/L}$ LY294002, 30 nmol/L rapamycin, 1 $\mu\text{mol/L}$ NVP-BEZ235, 50 nmol/L insulin, 50 nmol/L insulin + 20 $\mu\text{mol/L}$ LY294002, 50 nmol/L insulin + 30 nmol/L rapamycin, 50 nmol/L insulin + 1 $\mu\text{mol/L}$ NVP-BEZ235, 150 nmol/L insulin, 150 nmol/L insulin + 20 $\mu\text{mol/L}$ LY294002, 150 nmol/L insulin + 30 nmol/L rapamycin, and 150 nmol/L insulin + 1 $\mu\text{mol/L}$ NVP-BEZ235, respectively.

Akt-mTOR pathway, and cyclin D family played an important role. Reports said the mice or MIN6 cells overexpressing a constitutively active form of Akt showed enhanced beta cell proliferation that is associated with increased protein levels of cyclin D family and p21 [21, 22]. Our result indicated p21 played an inhibiting role in insulin-stimulated cell proliferation, but the experiments in p21 deficient mice have demonstrated that p21 is not essential for maintaining beta cell function and cell cycle arrest *in vivo* [23]. In contrast, transgenic mice overexpressing p21 show decreased beta cell replication [24]. Thus, the mechanisms of p21 involved in the regulation of cell proliferation are different in different cell types.

In summary, our data demonstrated that insulin promoted cell proliferation. PI3K-independent activation of Akt might contribute to the cell proliferation of insulin on goose primary hepatocytes. And at least the two following pathways are involved in insulin-induced cell proliferation regulation, a PI3K-Akt-dependent/mTOR-independent pathway and a PI3K-Akt-mTOR-dependent pathway.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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