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G6Pase; insulin; PEPCK; PI3k/Akt/mTOR.

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ABSTRACT

To identify what makes insulin have an activating or inhibiting role in gluconeogenesis in goose hepatocytes and whether insulin regulates PEPCK and G6Pase through the PI3k/Akt/mTOR pathway or not, goose primary hepatocytes were isolated and cultured *in vitro*. After 12h cultured in serum-free medium, hepatocytes were incubated for 24 h in the medium with no addition (control) or with the addition of50, 100, and 150 nM of insulin, 1000 nM NVP-BEZ235, or co-addition of 150nM insulin and 1000nM NVP-BEZ235. Glucose concentration and PEPCK and G6Pase expression were determined. The results showed that PEPCK and G6Pase mRNA levels and activities were up regulated in the 50, 100, and 150nM insulin treatments, while glucose concentration was not significantly altered ($p>0.05$). Compared with the activation role of 150nM insulin alone, the co-treatment with1000nM NVP-BEZ235 and 150nM insulin significantly down regulated PEPCK mRNA level and G6Pase protein activity ($p<0.05$). However, there is a different result on mRNA level of G6Pase. In conclusion, G6Pase and PEPCK are up regulated by insulin through PI3k/Akt/mTOR pathway in goose hepatocytes. However, G6Pase mRNA and protein levels may be regulated by insulin through different signaling pathways.

INTRODUCTION

Insulin, secreted from pancreatic β cells, is a known primary regulator of glucose metabolism, by counter regulatory glucagon and growth hormone (Cryer,1993; Saltiel & Kahn,2001). As the only hormone that lowers blood glucose *in vivo*, insulin plays an important role in hepatic glucose production. In diabetic individuals, insulin action is impaired, resulting in increased hepatic glucose production (Prasad *et al.*, 2005). Many studies indicated that insulin has a close relationship with gluconeogenesis. Donkin & Armentano (1995) showed that insulin reduced gluconeogenesis and increased glycogenesis from propionate and lactate in hepatocytes of pre-ruminating calves, but had no effect on the hepatocytes of ruminating calves. Edgerton *et al.* (2009) proved that the gluconeogenic flux can be rapidly inhibited by high insulin levels in dogs. A study showed that modulated gluconeogenesis by inhibiting of the coactivator TORC2 (Dentin *et al.*, 2007).

Phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) are enzymes that limit gluconeogenesis rate (Sato *et al.*, 2011). In addition, G6Pase is a critical enzyme in the last step of the glycogenolytic pathway (Podolin *et al.*, 1999). It was shown that insulin inhibits gluconeogenesis by suppressing the expression of PEPCK and G6Pase (O'Brien & Granner, 1996). And in the fed state, the expression of these enzymes are inhibited by insulin (Ropelle *et*

al., 2009). Interestingly, Ziv *et al.* (1996) showed that insulin failed to inhibit PEPCK and G6Pase activity even during the pre-diabetic (A) state. Recent data also suggest that insulin glargine (a long-acting insulin analogue) has no effect on G6Pase or PEPCK (Xu *et al.*, 2015).

The PI3k/Akt/mTOR pathway has a primary role in many crucial cellular processes, including periodic cell proliferation, differentiation, survival, protein synthesis, and glucose metabolism (Westin, 2014; Wu & Hu, 2010; Engelman, 2009). NVP-BEZ235, an inhibitor of both PI3K and mTOR, inhibits tumor cell proliferation and induces cell cycle arrest in the G1 phase (Serra *et al.*, 2008). Previous studies have shown that the glucose metabolism can be regulated by insulin through the PI3K signal transduction cascade (Saltiel & Kahn, 2001). When this pathway is activated, the expression of G6Pase and PEPCK is reduced, thereby decreasing hepatic glucose production (O'Brien *et al.*, 2001; Yabaluri & Bashyam, 2010). Akt, as a major cellular transduction element downstream PI3K, is a member of the insulin signaling pathway (Saltiel & Kahn, 2001). The activation of mTOR by insulin is relayed through the insulin receptor/insulin receptor substrate-1 (IRS-1)/PI3K/Akt pathway (Fisher & White, 2004).

Our previous study showed that the inhibition of PI3k/Akt/mTOR pathway can reduce of lipid deposition induced by insulin in goose hepatocytes, and therefore, we hypothesized that the regulation of gluconeogenesis by insulin may be mediated by PI3k/Akt/mTOR pathway in goose hepatocytes. In the present study, two key enzymes that limit gluconeogenesis rate, PEPCK and G6Pase, were used to study the gluconeogenesis regulation by insulin in goose primary hepatocytes. The results will help to investigate the regulation mechanism of gluconeogenesis by insulin in goose hepatocytes.

MATERIALS AND METHODS

Isolation and culture of primary hepatocytes

Hepatocytes were isolated from three 10-day-old Tianfu meat-typegeese from the Experimental Farm of Sichuan Agricultural University using a modification of the "two-step procedure" described by Seglen (Seglen, 1976). Differently from Seglen, the goose liver was removed before the pre-perfusion step. Cell viability was greater than 90%, as assessed by the try pan blue dye exclusion test. Freshly isolated hepatocytes were diluted to 1×10^6 cells/mL. The culture medium was composed of DMEM (containing 1.0 g/L glucose; GIBCO, USA) with 100 IU/mL penicillin (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA), 2 mM glutamine (Sigma, USA), and 100 mL/L fetal bovine serum (Clark, Australia). The hepatocytes were then plated in 60-mm culture dishes at 3×10^6 cells per dish for total RNA and intracellular proteins isolation. Cultures were incubated at 40°C in a humidified atmosphere containing 5% CO_2. The media were renewed after 3h, and after 12h, the media was replaced with serum-free media for another 12h. Next, the cells were separately treated with serum-free media supplemented with 50, 100, or 150 nM of insulin (Sellcek, USA) and incubated for 24 h, while the control cells were cultured with serum-free medium for 24 h. In addition, some cells were treated with serum-free medium supplemented with 1000 nM NVP-BEZ235 (Sellcek, USA) for 24 h or first treated with serum-free medium supplemented with1000 nM NVP-BEZ235for 1h and then 150 nM insulin were added, and incubated for 24h.

cDNA synthesis and quantitative Real-Time PCR

RNA was extracted using the TRIzol (Invitrogen, USA), and reverse-transcribed using the Primer Script TM RT system kit for real-time PCR (TaKaRa, Japan) method, according to the manufacturer's protocol. The quantitative real-time PCR reaction contained the newly-generated cDNA template, SYBR Premix Ex Taq TM (TaKaRa, Japan), sterile water, and primers of target genes. Real-time PCR was performed on the Cycler system (one cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 40 s). For each experimental sample, a normalized target gene level, corresponding to the target gene expression level relative to the expression levels of the housekeeping genes β-actin and 18S, was determined by the $2^{-\Delta\Delta Ct}$ method as described (Livak & Schmittgen, 2001). The following primer sequences were used:

β-actin Upstream5'-CAACGAGCGGTTCAGGTGT-3',
Downstream5'-TGGAGTTGAAGGTGGTCTCG-3';
18s Upstream5'-TTGGTGGAGCGATTTGTC-3',
Downstream5'-ATCTCGGGTGGCTGAACG-3';
PEPCK Upstream5'-CAGCTACGTTGGTAAAGATGGTT-3',
Downstream5'-GGGCAATCAATCCAGAAAATG-3';
G6PaseUpstream5'-CTCTTGCCCGACACGATGGA-3',
Downstream5'-TGCACGAGACGGACTACTACAGC-3'

Table 1 – Primer sequences for real-time PCR

Gene Name	Upstream (5′-3′)	Downstream (5′-3′)	Product size (bp)
PEPCK	CAGCTACGTTGGTAAAGATGGTT	GGGCAATCAATCCAGAAAATG	114
G6Pase	CTCTTGCCCGACACGATGGA	TGCACGAGACGGACTACTACAGC	269
β-actin	CAACGAGCGGTTCAGGTGT	TGGAGTTGAAGGTGGTCTCG	92
18S	TTGGTGGAGCGATTTGTC	ATCTCGGGTGGCTGAACG	129

Intracellular protein isolation and measurement of G6Pase activity

After 24h treatment of the hepatocytes, intracellular proteins were isolated according to the following steps: first, the medium was aspirated, then a pre-cooled PBS solution was used to wash the dish three times; second, a protein lysate was added, and every dish was placed on the ice 30S, then the cells repeatedly aspirated using a pipette until all cells were suspended in the buffer; last, the cell suspension was collected by centrifugation at 4 °C, 10000 r/min for 5 min, and the supernatant was collected. The enzyme activity of G6Pase was measured using an ELISA kit (GBD, San Diego, CA, USA), according to the manufacturer's instructions.

Determination of glucose concentration in the culture medium

The culture medium was collected to determine glucose concentration. The concentration of glucose in the culture medium is considered an indicator of glucose production, which may be regulated by liver through the mobilization of glycogen and hepatic gluconeogenesis (Liang *et al.*, 2013). Glucose concentration of every treatment was detected in triplicate using Blood Glucose Meter (Sinocare Inc, China), according to the manufacturer's instruction.

Statistical Analysis

The results are presented as the mean ± standard deviation (SD). Statistical analyses were conducted using a two-tailed t-test of SAS Proprietary Software Release 8.1 (SAS Institute Inc., Cary, NC). A significance level of $p<0.05$ was accepted. Every experiment was repeated with three biological samples, and each sample was run in triplicate.

RESULTS

The effects of insulin treatment on expression of PEPCK and G6Pase

As shown in the Figure 1, compared with control group, the mRNA expression of G6Pase and PEPCK were significantly up-regulated in the 50nM and 100nM insulin treatment groups ($p<0.05$); Moreover, the protein activity of G6Pase was significantly increased by all three concentrations of insulin ($p<0.05$). The concentration of 50 nM insulin showed maximum up regulation of mRNA expression of G6Pase and PEPCK, and the 150nM insulin treatment group has the strongest effect on the increase of G6Paseprotein activity.

Effects of insulin and NVP-BEZ235 co-treatment on expression of PEPCK and G6Pase

Figure 2 summarizes the effect of insulin and NVP-BEZ235 on the enzyme activity of G6Pase and on the mRNA expression of G6Pase and PEPCK. After 24h treatment, compared with the activation role of 150nM insulin alone, 1000nM NVP-BEZ235 and 150nM insulin co-treatment had an inhibitory effect on the mRNA

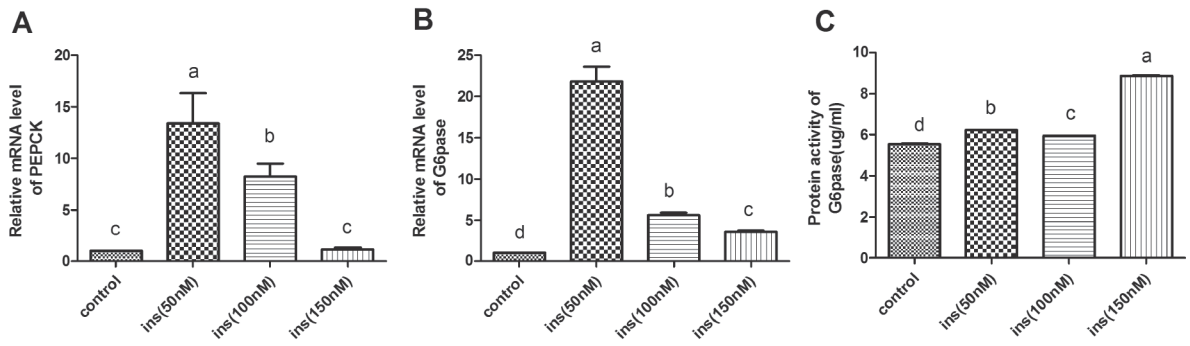

Figure 1 – Effects of different insulin concentrations on the expression of PEPCK and G6Pase. Different uppercase letters in the same set indicate difference among treatments at $p<0.05$. After 12h in serum-free medium, hepatocytes were incubated for 24 h in either with no addition (control) or with50, 100 and 150 nM of insulin. "ins" indicates insulin.

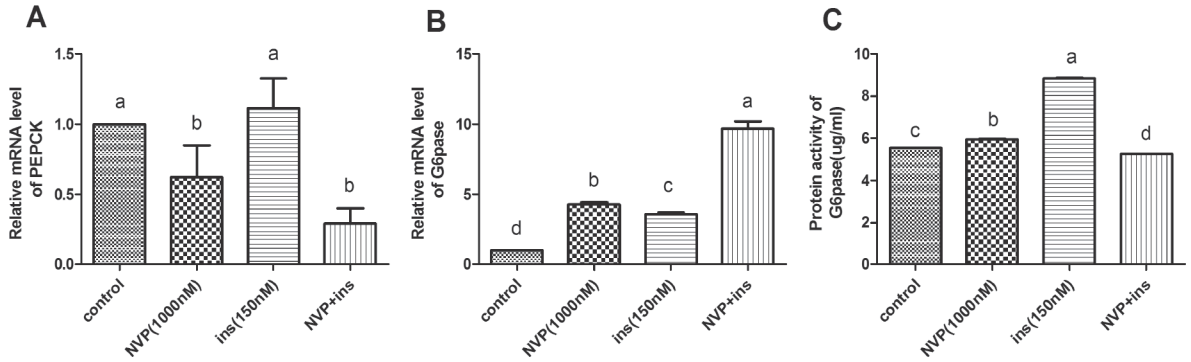

Figure 2 – Effects of insulin and NVP-BEZ235 on the expression of PEPCK and G6Pase. Different uppercase letters in the same set indicate difference among treatments at $p<0.05$. After 12h in serum-free medium, hepatocytes were incubated for 24 h with no addition (control) or with 150 nM insulin, 1000 nM NVP-BEZ235, or co-addition of 150 nM insulin and 1000 nM NVP-BEZ235. "NVP" indicates NVP-BEZ235; "NVP+ins" indicates 1000 nM NVP-BEZ235 and 150 nM insulin.

level of PEPCK and on the protein activity of G6Pase. However, mRNA levels of G6Pase were different. Compared with the control group, both1000nM NVP-BEZ235 and 150nM insulin significantly up regulated G6Pase mRNA level ($p<0.05$), and the treatment of 1000 nM NVP-BEZ235 and 150 nM insulin together up regulated G6Pase mRNA expression more significantly ($p<0.05$).

Effects of insulin and NVP-BEZ235 co-treatment or alone on glucose concentration of goose hepatocytes

Compared with the control group, Figure 3 shows that 100 and 150 nM insulin can increase hepatocyte glucose concentration, despite not significantly ($p>0.05$). Compared with the control group, both the co-treatment with 150 nM insulin and 1000 nM NVP-BEZ235 and the treatment of 1000 nM NVP-BEZ235 alone significantly down regulated glucose concentrations ($p<0.05$). As expected, compared with 150 nM insulin alone, the treatment with both 1000nM NVP-BEZ235 and 150 nM insulin reduced glucose concentration, despite not significantly ($p>0.05$).

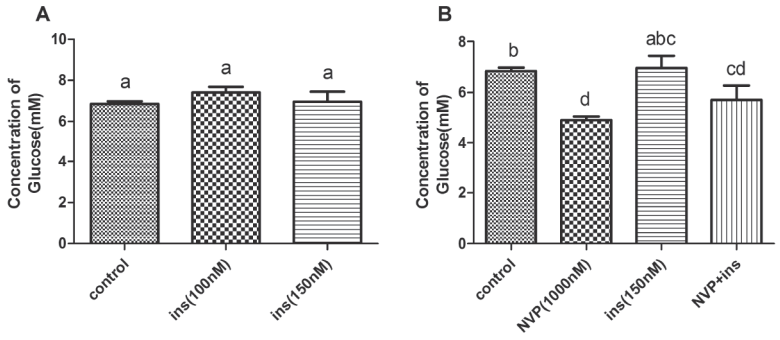

Figure 3 – Glucose concentration. Different uppercase letters in the same set indicate difference among treatments at $p<0.05$.After 12h in serum-free medium, hepatocytes were incubated for 24 h with no addition (control) or with 150 nM insulin, 1000 nM NVP-BEZ235, or co-addition of 150 nM insulin and 1000 nM NVP-BEZ235.

DISCUSSION

Since the geese are likely to show non-pathological hepatic steatosis when overfeeding, like the starling (*Sturnus roseus*), as a result of energy storage before migration (Pilo & George, 1983), goose primary hepatocytes were used to study the regulating mechanism of insulin on gluconeogenesis. Many previous studies have shown that insulin strongly inhibited the expression of G6Pase and PEPCK (O'Brien & Granner, 1996; Argaud *et al.*, 1996), however, another study showed that insulin failed to inhibit PEPCK and G6Pase activity in the pre-diabetic (A) state (Zive*t al.*, 1996). In recent years, the effects of insulin on the enzymes PEPCK and G6Pase are still not fully elucidated. Ropelle *et al.* (2009) believed that the activities of the enzymes PEPCK and G6Pase were suppressed by insulin in the fed state. In the rat H4IIE hepatoma cell line, the gene expression of G6Pase and PEPCK were also down regulated by 10 nM insulin (Kong *et al.*, 2013). The study on heap 1-6 cells indicated that the treatment with 100nM insulin significantly down regulated the expression of G6Pase and PEPCK (Yuan *et al.*, 2015). However, Xu *et al.* (2015) suggested that insulin glargine (a long-acting insulin analogue) had no effect on G6Pase or PEPCK. In extremely low birth weight infants receiving total parenteral nutrition, insulin is not able to regulate gluconeogenesis, indicating that insulin has no effect on PEPCK or G6Pase (Chacko *et al.*, 2011).

One of our aims in the present study was to identify that makes insulin have an activating or inhibiting role on the gluconeogenesis in goose hepatocytes. We found that both the mRNA expression and protein level

of G6Pase and PEPCK were up regulated in all the insulin-treated groups The highest up regulation of mRNA levels of G6Pase and PEPCK were observed at the insulin concentration of 50nM,whereasthe highest protein activity of G6Pase was detected at the insulin concentration of 150 nM (Figure 1). On the other hand, glucose concentration increases after insulin treatments were not significantly different (Figure 3A). These results indicate that insulin has an activating effect on the gluconeogenesis in goose hepatocytes at a low concentration (less than 150 nM).

The PI3k/Akt/mTOR pathway, as many studies reported, plays an essential role in multiple critical cellular activities (Westin, 2014; Yuan & Cantley, 2008). After the activation of PI3k/Akt/mTOR pathway, the expression of G6Pase and PEPCK is reduced, thereby decreasing hepatic glucose production (Yabaluri & Bashyam, 2010). Akt and mTOR (mTORC1 and mTORC2 subtypes) are the downstream genes of PI3k, but their relation is complex. The PI3K pathway was activated when mTORC1 was suppressed due to mTORC2 negative feedback, resulting in Akt phosphorylation (O'Reilly *et al.*, 2006). However, Akt activity was reduced when mTORC1 was inhibited by rapamycin for a long time (Sarbassov *et al.*, 2006). It was suggested that the inhibition of glucose production by insulin is PI3k/Akt-dependent (Kong *et al.*, 2013). When insulin was added with either PI3k inhibitor LY294002 or Akt inhibitor A6730 in H4IIE cells, the inhibition of glucose production by insulin was reversed. Eckert *et al.*(2007) demonstrated that at least two pathways are required for glucose signaling inHL1C hepatomas, one of which is dependent on a non-PI3-kinase intermediary, which is inhibited by LY294002 and LY303511. Another work suggested that treatment of L6 myotubes with 100nM insulin can increase Akt and mTOR phosphorylation (Hwang *et al.*, 2012). Saltiel & Kahn (2001) showed that glucose metabolism can be regulated by insulin through the PI3K signal transduction cascade. In our study, the results showed that the expression levels of the PEPCK gene and of the G6Pase protein were significantly lower in the co-treatment with 1000 nM NVP-BEZ235 and 150 nM insulin (Figure 2A,2C) than in the treatment with 150 nM insulin alone (Figure 2B). A similar result was obtained for glucose concentration (Figure 3B). However, to our surprise, the mRNA level of G6Pase was higher in the 150nM insulin treatment. Similarly, compared with control group, the co-treatment with NVP-BEZ235 and insulin revealed lower expression levels of the two enzymes and of glucose concentration (Figure 2A, 2C). Considering that the results of protein and mRNA levels of G6Pase were different in our study, we hypothesize that insulin regulates mRNA and protein expression of G6Pase through different pathways. The results of present study also indicate that insulin-promotion of G6Pase and PEPCK are reduced when the PI3k/Akt/mTOR pathway is suppressed by NVP-BEZ235.

Taken together, G6Pase and PEPCK, the enzymes that limit gluconeogenesis rate, are up regulated by insulin through the PI3k/Akt/mTOR pathway in goose hepatocytes. However, the regulation of mRNA and protein levels of G6Pase by insulin may be mediated by different signaling pathways.

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