Insulin Promotes the Expression of the Gluconeogenic Rate-Limiting Enzymes Phosphoenolpyruvate Carboxykinase (Pepck) and Glucose 6-Phosphatase (G6pase) through PI3k/Akt/mTOR Signaling Pathway in Goose Hepatocytes

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 of 50, 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 with 1000nM 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 glycogen synthesis 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
Insulin Promotes the Expression of the Gluconeogenic Rate-Limiting Enzymes Phosphoenolpyruvate Carboxykinase (Pepck) and Glucose 6-Phosphatase (G6pase) through PI3k/Akt/mTOR Signaling Pathway in Goose Hepatocytes

Materials and Methods

Isolation and culture of primary hepatocytes

Hepatocytes were isolated from three 10-day-old Tianfu meat-type geese 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 1×10⁶ 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 μg/mL fetal bovine serum (Clark, Australia). The hepatocytes were then plated in 60-mm culture dishes at 3×10⁶ cells per dish for total RNA and intracellular proteins isolation. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. 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 (Sellock, 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 (Sellock, USA) for 24 h or first treated with serum-free medium supplemented with1000 nM NVP-BEZ235 for 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^ΔΔCt method as described (Livak & Schmittgen, 2001). The following primer sequences were used:

- **β-actin**
  - Upstream: 5'-CAACGACGCCTCAGGTTGTTG-3', Downstream: 5'-TGGAGTTGAAGCTGGTCTCG-3';
  - 18S Upstream: 5'-TTGGTGAAGCGATTTGTCCTG-3', Downstream: 5'-ATCTCGGTGGCTGTTGGACG-3';
  - PEPCK Upstream: 5'-CTCAGGGATCGGAGATGGTGTTG-3', Downstream: 5'-GGGCAATCAATCCAGAAAATG-3';
  - G6Pase Upstream: 5'-CTCTTGCCCGACACGATGGA-3', Downstream: 5'-CTCTTGCCCGACACGATGGA-3';

β-actin Upstream 5’-CAACGACGCGCTCAGGTTGTTG-3’, Downstream 5’-TGGAGTTGAAGCTGGTCTCG-3’; 18S Upstream 5’-TTGGTGAAGCGATTTGTCCTG-3’, Downstream 5’-ATCTCGGTGGCTGTTGGACG-3’; PEPCK Upstream 5’-CTCAGGGATCGGAGATGGTGTTG-3’, Downstream 5’-GGGCAATCAATCCAGAAAATG-3’; G6Pase Upstream 5’-CTCCTGAGCGACAGATTGGA-3’, Downstream 5’-TGCAACGGAGCGACTACAGC-3’.
Insulin Promotes the Expression of the Gluconeogenic Rate-Limiting Enzymes Phosphoenolpyruvate Carboxykinase (Pepck) and Glucose 6-Phosphatase (G6pase) through PI3k/Akt/mTOR Signaling Pathway in Goose Hepatocytes

Table 1 – Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Upstream (5′-3′)</th>
<th>Downstream (5′-3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPCK</td>
<td>CAGCTACGTGTTGTAAGATGGTT</td>
<td>GGGCAATCAATCCAGAAAAATG</td>
<td>114</td>
</tr>
<tr>
<td>G6Pase</td>
<td>CTCTTGGCCGACACGGAGGA</td>
<td>TGCACGAGACGGACTACAGC</td>
<td>269</td>
</tr>
<tr>
<td>β-actin</td>
<td>CAACCGAGCGGTACAGGTGT</td>
<td>TGGAGTTGAAAGTGTCTCG</td>
<td>92</td>
</tr>
<tr>
<td>18S</td>
<td>TGTTGGAGCGATTTGTC</td>
<td>ATCTCGGTTGCTGAACG</td>
<td>129</td>
</tr>
</tbody>
</table>

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 G6Pase protein 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
level of PEPCK and on the protein activity of G6Pase. However, mRNA levels of G6Pase were different. Compared with the control group, both 1000 nM NVP-BEZ235 and 150 nM insulin significantly upregulated G6Pase mRNA level (p<0.05), and the treatment of 1000 nM NVP-BEZ235 and 150 nM insulin together upregulated 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 downregulated glucose concentrations (p<0.05). As expected, compared with 150 nM insulin alone, the treatment with both 1000 nM NVP-BEZ235 and 150 nM insulin reduced glucose concentration, despite not significantly (p>0.05).

**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 (Ziv et 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 downregulated by 10 nM insulin (Kong et al., 2013). The study on heap 1-6 cells indicated that the treatment with 100 nM insulin significantly downregulated 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 PECK were up regulated in all the insulin-treated groups. The highest up regulation of mRNA levels of G6Pase and PECK were observed at the insulin concentration of 50nM, whereas the 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 PECK 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 in HL1C 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 PECK 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 PECK are reduced when the PI3k/Akt/mTOR pathway is suppressed by NVP-BEZ2235.

Taken together, G6Pase and PECK, 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.

ACKNOWLEDGEMENTS

The work was supported by the National Natural Science Funds of China (No. 31101712), and the Research Fund for the Doctoral Program of Higher Education of China (No. 20115103120006).

REFERENCES


Insulin Promotes the Expression of the Gluconeogenic Rate-Limiting Enzymes Phosphoenolpyruvate Carboxykinase (Pepck) and Glucose 6-Phosphatase (G6pase) through PI3k/Akt/mTOR Signaling Pathway in Goose Hepatocytes


O’Brien RM, Granner DK. Regulation of gene expression by insulin. Physiological Reviews 1996;76(4):1109 –1161


Pilo B, George JC. Diurnal and seasonal variations in liver glycogen and fat in relation to metabolic status of liver and m. pectoralis in the migratory starling, Sturnus roseus, wintering in India. Comparative Biochemistry and Physiology. A Comparative Physiology 1983;74(3):601-604


