Decreased fasting blood glucose is associated with impaired hepatic glucose production in thyroid-stimulating hormone receptor knockout mice

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Abstract. Our previous study reported that thyroid-stimulating hormone (TSH) promotes cholesterol synthesis via the cyclic adenosine monophosphate/protein kinase A/cAMP regulatory element-binding protein (cAMP/PKA/CREB) pathway after binding to TSH receptors (TSHR) in the liver. The hepatic cAMP/PKA/CREB pathway also plays an important role in maintaining fasting glucose homeostasis. These findings implied a possible role for TSH in hepatic glucose metabolism. In this study, we used TSH receptor knockout mice (Tshr-ko mice) to clarify the effect of Tshr deletion on hepatic glucose metabolism, and investigated whether the effects of TSH directly regulate hepatic gluconeogenesis in HepG2 cells. Tshr-ko mice exhibited decreased fasting blood glucose levels, increased insulin sensitivity but normal level of fasting plasma insulin. Tshr deletion impaired hepatic glucose production by down-regulating the expression of glucose-6-phosphatase (G6P) and phosphoenolpyruvate pyruvate carboxylase (PEPCK) mRNA, two rate-limiting enzymes in hepatic gluconeogenesis, and enhancing the abundance of hepatic glucokinase (GK), the first enzyme regulating glycogen synthesis. Moreover, Tshr deletion inhibited the protein expression of hepatic phospho-CREB and increased the protein expression of hepatic phospho-AMP-activated protein kinase (p-AMPK), two up-stream regulators of PEPCK and G6P mRNA. In HepG2 cells, TSH increased the expression of G6P and PEPCK at mRNA level. These results indicated the simulative effects of TSH on hepatic glucose production in vivo and in vitro, suggesting a novel role for TSH in hepatic glucose metabolism.

Key words: Thyroid-stimulating hormone receptors (TSHR), Gluconeogenesis, cAMP regulatory element binding protein (CREB), AMP-activated protein kinase (AMPK)

SUBCLINICAL HYPOTHYROIDISM (SCH) is characterized by an increased level of plasma TSH and normal levels of free thyroxin (FT4) and free triiodothyronine (FT3). With increasing evidence from clinical studies, the association between SCH and other diseases, such as hypercholesterolemia [1] and coronary heart disease [2], is receiving increasing attention. Some studies have also found associations between SCH and insulin resistance [3-5] or fasting hyperinsulinemia [6]. Amati [7] reported that SCH patients had significantly lower improvements in insulin sensitivity relative to euthyroid group under similar regular exercise and body weight loss. And some studies [8, 9] observed an improvement of insulin sensitivity when the level of serum TSH was reduced within the normal range in SCH patients. Moreover, in the population of nondiabetic elderly men, it was reported that the TSHR-Asp727Glu polymorphism was associated with insulin resistance, suggesting that TSHR plays a role in glucose metabolism [10]. Taken together, these clinical studies strongly suggest a possible role for TSH in glucose metabolism.

TSH is synthesized and secreted by the pituitary gland and directly regulates thyroid function via binding to TSH receptor located in the membrane of thyrocytes. Recently, multiple studies have found that extrathyroidic tissues express TSHR [11]. Our previous studies [12, 13] demonstrated that functional TSHR was expressed...
samples were collected. The liver, heart and kidney were weighed, and the liver was divided into three parts. One part was frozen in liquid nitrogen until analysis, and the other two parts were separately fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining or Carnoy’s fluid for Periodic Acid Schiff (PAS) staining. All animal experiments were performed according to the Shandong University Institutional Animal Care and Use Committee (Jinan, China).

**Blood parameters**

The serum TT4, insulin, glucagon and insulin-like growth factors 1 (IGF-1) concentrations were measured using radioimmunoassay (Jiuding, Tianjing Biomedical Engineering limited company and HTA CO., LTD., China). The serum TSH level was assayed using an ELISA kit (Uscn Life Science Inc.). The blood glucose level was detected with an OneTouch Ultra glucometer (Johnson & Johnson, USA). Insulin sensitivity was assessed with the insulin sensitivity index (ISI) as follows: \( \text{ISI} = \frac{1}{(\text{FBG} \text{ (mmol/L)} \times \text{FINS} \text{ (mIU/L)})} \) [19] (data were calculated after natural logarithmic transformation because this index was not normally distributed).

**Metabolic studies**

To evaluate the glucose metabolism in Tshr-ko mice, oral glucose tolerance tests (OGTT), insulin tolerance tests (ITT) and pyruvate tolerance tests (PTT) were performed as described previously [20, 21]. In brief, for OGTT, the mice were administered glucose (2 g/kg body weight) after an 18 h fast, and the levels of blood glucose were measured at 0, 30, 60 and 120 minutes using a glucometer. For ITT, mice fasted for 5 h were injected intraperitoneally with insulin (1.0 U/kg body weight, human insulin, Lily), and blood glucose levels were detected at 0, 15, 30, 60 and 90 minutes after insulin injection. For PTT, mice fasted for 18 h were injected intraperitoneally with pyruvate (2 g/kg body weight) dissolved in saline, and blood glucose were measured at 0, 30, 60 and 90 minutes after pyruvate injection. The area under the curve (AUC) was calculated.

**Cell Culture**

The HepG2 human hepatoma cell line was purchased from Chinese Academy of Science (Shanghai). HepG2 cells were cultured in Eagle’s minimum essential medium (EMEM) (GIBCO) containing 10% fetal bovine serum and 100 U/mL penicillin-streptomycin in a humidified incubator of 95% air and 5% CO2 at
37°C. When the cells reached 80-90% confluence, the cells were exposed to 0.2 µm TSH for 20h under serum-free medium.

**RNA extraction and quantitative real-time PCR analysis**

Total RNA was extracted from HepG2 cells and mouse liver tissue using Trizol (TAKARA), and single-strand cDNA was synthesized from 5 µg of total RNA with random hexamer primers (TAKARA) in a 10-µL final volume. Real-time PCR was carried out with SYBR Premix Ex Taq II (TAKARA) in a final volume of 20 µL containing 250 ng of reverse-transcribed total RNA, 400 nM of primers, and 10 µL of 2×SYBR Premix Ex Taq II. PCR was performed for 40 cycles at 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 30 sec in a LightCycler instrument (Roche Diagnostics). We determined the relative quantification of gene expression by the 2^−ΔΔCT method [22]. The sense and antisense PCR primers used are listed in Table 1.

**Western blot analysis**

Liver tissue samples were homogenized in total protein extract solution and separated by centrifugation after sufficient lysis. The supernatant was collected, and the protein concentration was determined using the BCA method. Proteins (100 µg) were separated using SDS-PAGE with 12% polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked (0.5% non-fat milk powder in TBST) for 1 h before incubation with primary antibodies at 4°C overnight. The primary antibodies: phospho-CREB (Ser-133) (1:1000), CREB (1:1000), phospho-GSK3β (Ser-9) (1:1000), GSK3β (1:1000), phospho-AMPK (Thr-172) (1:1000), AMPK (1:1000) and β-actin (1:10000) were from Cell Signaling Technology (Boston, USA). After incubation with either anti-rabbit or anti-mouse secondary antibody, the immune complexes were detected. The relative target protein levels were normalized to β-actin in the same membrane.

**Histology and PAS staining**

Liver paraffin-embedded tissues were sectioned at 5 µm and stained with H&E. For hepatic glycogen staining, samples were stained with Periodic Acid Schiff according to the manufacturer’s instructions (Yili Company, Beijing, China).

**Hepatic glycogen assay**

The hepatic glycogen content was assayed as described previously [23]. Briefly, 100 mg liver was homogenized in 1.5 mL of 30% KOH and dissolved at 100°C for 15 min. The glycogen content was determined by adding 2.5 mL anthrone reagent (2 mg anthrone/mL sulfuric acid) and measuring the absorbance at 620 nm. Glucose solution was used as a standard.

**Statistics**

The data are presented as the mean ± SD. Statistical significance was tested with Student’s t test and analysis of variance of repeated data with SPSS 17.0. Differences were considered statistically significant at p < 0.05.

**Results**

**Tshr-ko mice exhibit decreased fasting blood glucose but normal plasma insulin level**

When fed the non-supplemented diet, Tshr-ko mice exhibit decreased fasting blood glucose but normal plasma insulin level.
exhibited decreased serum T4/T3 levels and elevated TSH level [18]. Therefore, in our study, all Tshr-ko mice were fed a diet containing 100 ppm thyroid powder to eliminate the effect of abnormal thyroid hormone levels on glucose metabolism. As shown in Table 2, 6-week-old and 8-week-old Tshr-ko mice fed the supplemented diet exhibited equal levels of serum TSH and TT4 compared with the same age of wild-type mice (all \( p > 0.05 \)), however, the ratio of organ weight to body weight (including the liver, heart and kidney) in Tshr-ko mice did not show significant differences compared with wild-type mice (all \( p > 0.05 \)). To estimate the potential effect of Tshr deletion on liver function, liver histology were observed. The results showed that liver function was normal in Tshr-ko mice relative to wild-type mice based on the findings that liver H&E staining did not show obvious changes (Fig. 1A). Moreover, no significant differences were observed in plasma insulin (Fig. 1B), glucagon (Fig. 1C) and IGF-1 concentrations (Fig. 1D) between Tshr-ko mice and wild-type mice (all \( p > 0.05 \)), but the level of fasting glucose was reduced to 69% in Tshr-ko mice (\( p < 0.01 \), Fig. 1E). In line with decreased fasting glucose, Tshr-ko mice had increased insulin sensitivity index relative to wild-type mice (\( p < 0.01 \), Fig. 1F).

**Table 2** General characterization of Tshr-ko mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Wild-type</th>
<th>Tshr-Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>23.77±1.70</td>
<td>18.76±1.99*</td>
</tr>
<tr>
<td>Liver weight (%)</td>
<td>3.93±0.50</td>
<td>3.82±0.23</td>
</tr>
<tr>
<td>Heart weight (%)</td>
<td>0.51±0.08</td>
<td>0.57±0.08</td>
</tr>
<tr>
<td>Kidney weight (%)</td>
<td>1.26±0.08</td>
<td>1.27±0.19</td>
</tr>
<tr>
<td>TT4 (μg/dL, 6wk)</td>
<td>5.03±0.24</td>
<td>4.69±0.78</td>
</tr>
<tr>
<td>TT4 (μg/dL, 8wk)</td>
<td>3.96±1.57</td>
<td>4.36±1.17</td>
</tr>
<tr>
<td>TSH (pg/mL)</td>
<td>464.20±103.37</td>
<td>492.49±198.04</td>
</tr>
</tbody>
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Values are given as means ± SD. The data were analyzed by Student’s \( t \) test. \( n=6~8 \) animals /group. BW, body weight; TT4, total T4 \( *p < 0.05 \)

Fig. 1 Histology of liver and glucose metabolic parameters in Tshr-ko mice

Wild-type (WT) and Tshr-ko (KO) aged 8-week-old littermate male mice were used. (A) The sections of liver tissues were stained with H&E, and representative sections are shown. Magnification, ×200. (B, C and D) Fasting plasma insulin, glucagon and IGF-1 concentrations were detected using RIA. (E) Fasting blood glucose was measured with glucometer. (F) Insulin sensitivity was assessed with the insulin sensitivity index. Results are presented as mean ± SD. \( n=8~10 \) animals/group. \( *p < 0.01 \)


**Fig. 2** *Tshr* deletion improves glucose tolerance and reduces gluconeogenesis

Time curve of glucose levels and area under the curves (AUC) following oral glucose tolerance test (OGTT, A), intraperitoneal insulin tolerance test (ITT, B) and pyruvate tolerance test (PTT, C) in wild-type (WT) and *Tshr*-ko (KO) mice, respectively. Data are presented as means ± SD, n=3~7 animals/group. *p < 0.05, #p < 0.01

*Tshr* deletion improves glucose tolerance and reduces hepatic gluconeogenesis

To evaluate the effect of *Tshr* deletion on whole body glucose metabolism, we performed OGTT to observe glucose clearance, ITT to observe insulin-stimulated glucose disposal and PTT to observe gluconeogenesis. In OGTT (Fig. 2A), *Tshr*-ko mice displayed lower levels of blood glucose throughout the test compared with wild-type mice (all *p < 0.05). At 30 min after glucose loading, the blood glucose level in *Tshr*-ko mice was 62% that of wild-type mice (*p < 0.05). In ITT (Fig. 2B), both groups showed the lowest level of blood glucose at 30 min after the i.p. insulin injection, and the glucose level at 30 min was reduced to approximately 40% of glucose at 0 min, which revealed that the amount of insulin injected was appropriate. However, neither time point showed a significant difference in blood glucose throughout the test when compared the two types of mice (all *p > 0.05*). PTT has been identified as an indicator to reflect changes in hepatic glucose production because pyruvate is a substrate for glucose production and the liver is a major organ for gluconeogenesis [24, 25]. In PTT, both groups achieved peak blood glucose levels at 30 min after the i.p. pyruvate injection. And *Tshr*-ko mice showed significantly lower glucose levels than wild-type mice at all time points after the i.p. pyruvate injection (all *p < 0.01*, Fig. 2C). The AUC results also supported that *Tshr* deletion improved pyruvate tol-
analyzed to assess whether TSH influence gluconeogenic gene expression by using real-time PCR. Compared to wild-type mice, the expression levels of PEPCK and G6P mRNA were reduced by 36% and 47%, respectively, in Tshr-ko mice (Fig. 3C & 3D, all \( p < 0.05 \)).

Moreover, we also determined the abundance of hepatic glucokinase (GK). The results showed that the expression level of GK mRNA was 1.38-fold increase in Tshr-ko mice (Fig. 3E, \( p < 0.05 \)). In HepG2 cells, the abundance of PEPCK and G6P mRNA were significantly increased to 268% and 159% after 20-hours exposure of TSH, respectively, suggesting a direct action of TSH on gluconeogenic gene expression (Fig. 4, all \( p < 0.05 \)).

**Effects of Tshr deletion on proteins involved in regulating hepatic glucose metabolism**

Previous studies indicated that CREB is a downstream molecule of TSH and that it plays a vital role in promoting PEPCK and G6P mRNA expression [13, ...
Therefore, we evaluated the protein expression of p-CREB and CREB in Tshr-ko mice. In parallel with the changes in PEPCK and G6P mRNA, the expression of phospho-CREB was diminished in Tshr-ko mice \( (p < 0.05) \), while the expression of total CREB was unaltered \( (p > 0.05) \) (Fig. 5A).

AMPK is a key regulatory protein in glucose signaling transduction. The expression of total AMPK showed no significant difference between Tshr-ko mice and wild-type mice (Fig. 5B, \( p > 0.05 \)). However, the expression level of phospho-AMPK was significantly increased in the liver of Tshr-ko mice (Fig. 5B, \( p < 0.05 \)). Moreover, as one of the downstream target proteins of AMPK, the expression level of phospho-GSK3β was also enhanced in Tshr-ko mice (Fig. 5C, \( p < 0.05 \)).

**Discussion**

In the present study, we confirmed the association between TSH and hepatic glucose metabolism for the
first time. We found that Tshr deletion in mice elicited fasting hypoglycemia and enhanced insulin sensitivity but did not change plasma insulin levels. The decreased fasting glucose level was due to abnormal hepatic glucose production, including a decrease in hepatic PEPCK and G6P mRNA and an increase in hepatic GK mRNA, and we also found that TSH directly up-regulates the mRNA abundance of gluconeogenic genes in vitro. Moreover, our data indicated that the underlying mechanism of fasting hypoglycemia in Tshr-ko mice may be related to the inhibition of hepatic phospho-CREB and the activation of hepatic AMPK.

In the fasting state, glucagon promotes hepatic glucose production and has a hyperglycemic effect via activating enzymes required for gluconeogenesis. Our findings showed a normal level of fasting serum glucagon in Tshr-ko mice, making it unlikely that the decreased fasting glucose was due to altered glucagon level. And we also eliminate the effect of fasting hypoglycemia of IGF-1 level could reduce blood glucose level based on the finding that there was no significant difference of IGF-1 level between 8-week-old Tshr-ko mice and wild-type mice. However, Xing et al. [27] has reported lower IGF-1 concentrations in their Tshr-ko mice. The difference maybe due to the different age of mice measured the serum IGF-1 level. Xing measured the level of serum IGF-1 when the mice were 21 days old. However, we measured at the age of 8 weeks. Our data implied that Tshr deletion did not affect serum IGF-1 level at the age of 8 weeks. Moreover, we eliminated the negative effects of liver injury on hepatic gluconeogenesis because of the generally normal liver histology in Tshr-ko mice compared with wild-type mice.

Hepatic glucose output is vital for maintaining fasting glucose homeostasis. In the fasted state, liver produces glucose by either gluconeogenesis or glycogenolysis to provide energy for maintaining normal functions. PEPCK and G6P are rate-limiting enzymes that control hepatic gluconeogenesis [28], and G6P also regulates the last step of glycogenolysis. The transcription of PEPCK and G6P mRNA determines the rate of hepatic gluconeogenesis [29]. In Tshr-ko mice, the decreased gene expression of liver PEPCK and G6P mRNA indicated a reduced capacity of hepatic gluconeogenesis and glycogenolysis. Consistent with these results, a lower AUC of the pyruvate tolerance test and increased storage of liver glycogen were observed in Tshr-ko mice. To estimate the effect of TSH on gluconeogenesis in vitro, the expression of PEPCK and G6P mRNA in HepG2 cells were determined after stimulation by TSH. The results confirmed a direct action of TSH on gluconeogenic genes in HepG2 cells. Hepatic gluconeogenic gene expression is mediated by multiple transcriptional factors, such as CREB, which has binding sites in the promoter of PEPCK and G6P genes [26]. In our study, we observed Tshr deletion leading to the down-regulation of phospho-CREB expression, but the expression of total CREB was unchanged, suggesting that Tshr deletion may impair hepatic glucose production partly by inhibiting CREB phosphorylation.

Recently, several studies reported that long-term AMPK activation could directly repress the gene expression of PEPCK and G6P and inhibit hepatic glucose output [30]. And TSH has been reported to inhibit the phosphorylation of AMPK in the thyroid gland [17]. To determine whether Tshr deletion influenced AMPK activity in the liver, we measured the protein expression of hepatic phospho-AMPK. We found that phospho-AMPK expression was significantly increased in Tshr-ko mice. As AMPK downstream target protein, in parallel with phospho-AMPK, the expression of phospho-GSK3β was also enhanced in Tshr-ko mice. Increased GSK3β phosphorylation plays a critical role in AMPK-induced suppression of hepatic gluconeogenesis [16]. Moreover, GSK3β is also an upstream inhibitor of glycogen synthesis. As the first enzyme in glycogen synthesis, GK was markedly increased determined by the level of GK mRNA in the liver of Tshr-ko mice. These results indicated that the increased glycogen storage in the liver of Tshr-ko mice could be explained not only by reduced glycogenolysis, but also by enhanced glycogen synthesis. Taken together, the changes of gluconeogenesis, glycogenolysis, and glycogen synthesis in the liver of Tshr-ko mice were partly due to the increase of AMPK activation. Furthermore, it was well known that activation of AMPK could enhance insulin sensitivity. Hepatic AMPK activation explains the improvement in insulin sensitivity observed in Tshr-ko mice. However, we did not observe obvious glucose changes between the two groups in the insulin tolerance test. One possible explanation was that the insulin tolerance test is a crude technique to estimate insulin-stimulated glucose disposal because rapid declines in glucose usually lead to changes in many hormones regulating glucose metabolism during the insulin tolerance test [31]. As a result, the glucose levels of the insulin tolerance test may be due to a complex interaction of hormones, not only the action of insulin. In our
study, a more accurate technique, such as a hyperinsulinenic-euglycemic clamp, is needed to quantitatively assess insulin sensitivity. This was a limitation of our study. However, a previous report has shown that ISI is significantly correlated with the glucose turnover rate measured by a hyperinsulinenic-euglycemic clamp \( r > 0.7, p=0.0001 \) \[19\], which suggests that ISI can be a simple method for estimating insulin sensitivity to some extent.

Thyroxine (T4) stimulates hepatic gluconeogenesis, and even a small change in T4 levels can affect glucose metabolism \[32\]. In our study, we fed Tshr-ko mice supplemented diet to eliminate the effect of T4 on hepatic gluconeogenesis. We found that both 6-week-old and 8-week-old Tshr-ko mice have equivalent serum TT4 levels with that of wild-type mice at the same age (Table 2), indicating that the supplement diet was appropriate for the Tshr-ko mice at the age of 6-8 weeks. Although there was a slightly higher level of serum TT4 in 8-week-old Tshr-ko mice, the differences in serum TT4 and TSH levels were not statistically significant between the two types of mice. In addition, a higher serum TT4 level in Tshr-ko mice was hypothesized to elevate fasting glucose, which was in contrast to the fasting hypoglycemia observed in Tshr-ko mice. Taken together, these data indicated that we could ignore the extra effect of T4 on hepatic gluconeogenesis when comparing Tshr-ko mice with wild-type mice.

Our study confirmed a novel role for TSH in hepatic glucose metabolism. The inhibition of hepatic PEPCK and G6P and enhanced expression of GK contributed to the development of fasting hypoglycemia in Tshr-ko mice. And TSH had direct impact on gluconeogenesis \[in vitro\]. Tshr deletion affected the expression of proteins involved in regulating hepatic glucose production. Further studies are needed to elucidate how TSH affects AMPK activation and whether other insulin target organs are involved in reducing fasting glucose. Our study provides possible pathological implications for the pathogenesis of abnormal glucose metabolism in SCH patients.

Acknowledgements

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