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Hyperhomocysteinemia stimulates hepatic glucose output and *PEPCK* expression

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Homocysteine is an intermediate in the sulfur amino acid metabolism. Recent studies suggested that there might be links between hyperhomocysteinemia and insulin resistance. In the present study, we investigated the effect of homocysteine on glucose metabolism. We demonstrated that the levels of insulin were significantly higher in mice with hyperhomocysteinemia than those in the normal mice after administration of glucose. The effect of insulin on glucose output was significantly blocked in the homocysteine-treated hepatocytes. In addition, the expression of phosphoenolpyruvate carboxykinase (PEPCK) gene was elevated in the liver of mice with hyperhomocysteinemia and primary mouse hepatocytes treated with homocysteine. The action of homocysteine was suppressed by H89, a protein kinase A (PKA) inhibitor. Thus, hyperhomocysteinemia may be considered as a risk factor that contributes to the development of insulin resistance with respect to elevated glucose output and upregulation of PEPCK, probably via the PKA pathway. Our study provides a novel mechanistic explanation for the development of insulin resistance in hyperhomocysteinemia.

Keywords homocysteine; insulin resistance; phosphoenolpyruvate carboxykinase (PEPCK); protein kinase A

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Introduction

Homocysteine is an intermediate in the sulfur amino acid metabolism. High plasma homocysteine levels (called hyperhomocysteinemia) are correlated with cardiovascular disease [1], neurodegenerative diseases [2,3], and liver fibrosis [4,5]. Epidemiological studies also suggested that there might be links between hyperhomocysteinemia and insulin resistance. Elevated levels of homocysteine have been observed in a variety of patients with type 2 diabetes [6], metabolic syndrome [7], and obesity [8–10]. The plasma homocysteine levels are significantly higher in the fructose-induced hyperinsulinemic rats than in the normal rats [11]. On the other hand, several studies have indicated that hyperhomocysteinemia caused insulin resistance. For example, a prolonged folate treatment decreased not only homocysteine levels but also reduced insulin levels, thus improving insulin resistance in patients with metabolic syndrome [12]. The administration of homocysteine in rats increases insulin resistance index [13]. Furthermore, *in vitro* studies have shown that homocysteine thiolactone inhibits insulin receptor tyrosine kinase activity, phosphorylation of phosphatidylinositol 3-kinase (PI3K), and glycogen synthase kinase-3 (GSK-3), leading to inhibition of glycogen synthesis [14,15]. However, the mechanism underlying the homocysteine mediated-insulin resistance still remains unclear.

In this study, we investigated the effect of homocysteine on insulin resistance *in vitro* and *in vivo*. Results revealed that homocysteine promoted elevated glucose output and the expression of phosphoenolpyruvate carboxykinase (PEPCK) gene, probably via the protein kinase A (PKA) pathway.

Materials and Methods

Induction of hyperhomocysteinemia

Adult BALB/c mice were obtained from Baiyao Pharmacological Co. (Kunming, China). The animals were fed one of the two diets: (i) control diet (LM-485 chow; Harlan Teklad, Madison, USA); (ii) high methionine diet (LM-485 chow with drinking water

supplemented with 0.5% L-methionine) for 3 months. Then mice were sacrificed. The protocol of the experiments was approved by the Animal Care and Use Committee at Kunming Medical College. Homocysteine levels in plasma of mice were determined by using an ELISA kit (Axis-Shield, Cambridgeshire, UK).

Isolation of mouse hepatocytes

Mouse hepatocytes were isolated as described previously [16]. The cells were grown in Williams E media (W-4125) (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS), 2.2 g/L $NaHCO_3$, 15 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 mg/ml gentamicin. When experiments were initiated, the cells were changed to DMEM (Gibco, Gaithersburg, USA) without FBS and maintained overnight.

Biochemical assay

Blood samples were taken for the measurement of fasting plasma glucose and insulin. Plasma glucose levels were measured by the glucose oxidase method. Insulin levels were measured by using radioimmunoassay kit (Linco, St. Charles, USA).

Quantitative real-time PCR analysis

Total RNA from cells and liver tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, USA). Random-primed cDNAs were generated by reverse transcription of total RNA samples with SuperScript II (Invitrogen). A real-time PCR analysis was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, USA) using SYBR® Premix-Ex Tag™ (TaKaRa, Dalian, China). All results were standardized to the levels of β-actin. The primers used for PCR were as follows: *PEPCK*, 5′-CAGGATCGAAAGCAAGACAGT-3′ (F), and 5′-AAGTCCTCTTCCGACATCCAG-3′ (R); *β-actin*, 5′-AGTGTGACGTTGACATCCGTA-3′ (F), and 5′-GCCAGAGCAGTAATCTCCTTCT-3′ (R).

Western blotting analysis

Cells were lysed on ice for 30 min in the lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Total protein concentrations were determined by the Bradford method. Proteins (20 μg per lane) were electrophoretically separated by SDS-PAGE, and transferred onto nitrocellulose membrane. Primary antibodies were anti-PEPCK antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) (1:5000 dilution) and anti-β-actin antibodies (Santa Cruz Biotechnology) (1:10,000 dilution). The secondary antibody was a peroxidase-coupled anti-rabbit IgG (Amersham Biosciences, Piscataway, USA) (1:10,000 dilution). The membrane was exposed to ECL Hyperfilm (Amersham Biosciences), and the film was developed.

Metabolic studies

For glucose tolerance tests, mice were fasted overnight and administered the bolus glucose (1.5 mg/g) by oral gavage. Blood glucose was measured through the tail tip before and after injection at the time course indicated by using the OneTouch (Lifescan, Milpitas, USA) glucose-monitoring system. Insulin levels were measured by using radioimmunoassay kit.

Glucose output assay

Primary mouse hepatocytes were cultured in 6-well plates in the Williams E media (W-4125) supplemented with 10% FBS. When experiments were initiated, the cells were changed to DMEM lacking FBS and maintained overnight. Then the medium was replaced with glucose-free DMEM, without phenol red, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate. After the cells were incubated with 100 nM insulin for 3 h, the glucose level in the medium was determined by using a colorimetric glucose assay kit (Sigma). Readings were corrected for total protein content using the BCA protein assay (Bio-Rad, Hercules, USA).

Statistical analysis

Data from experiments were expressed as the mean ± SD. Statistical difference between the groups was analyzed using one-way ANOVA, followed by a Student–Newman–Keuls test. *P*-values of <0.05 were considered statistically significant.

Results

Hyperhomocysteinemia-induced hyperinsulinemia

To investigate whether hyperhomocysteinemia affects insulin resistance in mice, we induced hyperhomocysteinemia in mice fed a diet enriched in methionine for 3 months. Mice treated with methionine demonstrated an approximately 5-fold increase in the plasma level of homocysteine compared with the control mice fed normal diet (**Fig. 1**). We then performed glucose-tolerance test on these two types of mice. As shown in **Fig. 2(A)**, glucose response curves were indistinguishable between the normal mice and mice with

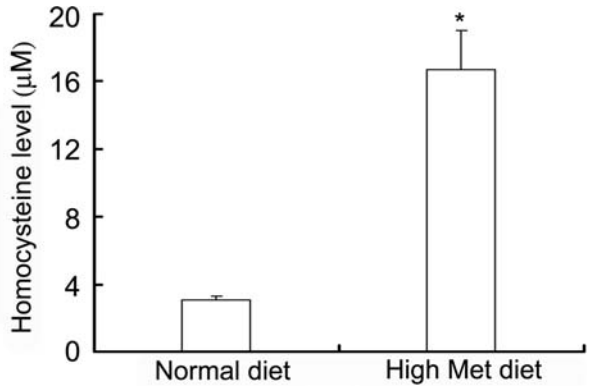

Figure 1 Induction of hyperhomocysteinemia in mice Adult BALB/c mice were fed with normal and high methionine (Met) diets for 3 months. Then mice were sacrificed. Levels of plasma total homocysteine were determined using the ELISA method. Data are presented as the mean ± SD ($n = 10$). *$P < 0.05$ vs normal diet.

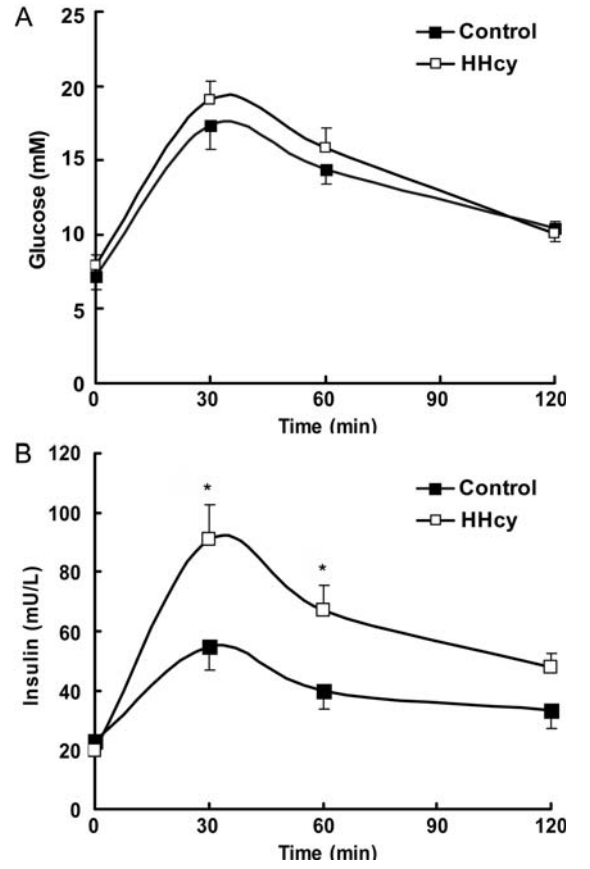

Figure 2 Hyperhomocysteinemia (HHcy) results in an increase in the plasma levels of insulin Blood glucose (A) and plasma insulin (B) concentrations during the oral glucose tolerance test in the normal mice (control) and mice with HHcy were shown. Data are presented as the mean ± SD ($n = 10$). *$P < 0.05$ vs control.

hyperhomocysteinemia. The fasting insulin levels were similar between the normal mice and mice with hyperhomocysteinemia. However, insulin response curves after the glucose challenge were different between the two groups of mice. The levels of plasma insulin were significantly higher in mice with hyperhomocysteinemia than in the normal mice after administration of glucose for 30 and 60 min, respectively [**Fig. 2(B)**].

Homocysteine suppressed insulin-dependent glucose metabolism

To examine the effect of homocysteine on insulin-dependent hepatic glucose metabolism, we measured glucose production in the primary mouse hepatocytes. The cells were pretreated with homocysteine (0.1 mM) for 6 h prior to stimulation for 3 h with 100 nM insulin. As shown in **Fig. 3**, insulin reduced the glucose output by approximately 50% in the control cells. The effect of insulin on the glucose output was significantly blocked in homocysteine-treated cells.

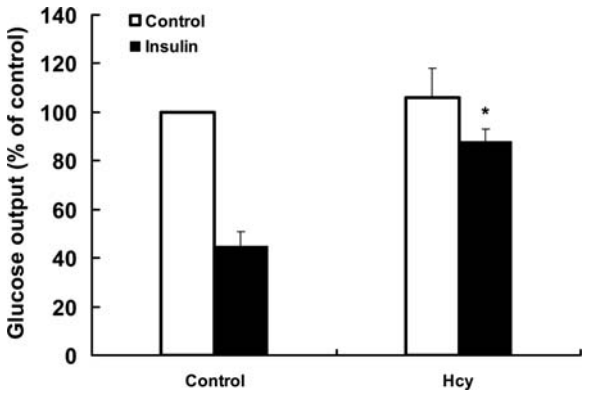

Figure 3 Homocysteine suppresses the inhibitory effect of insulin on glucose output Primary mouse hepatocytes were pre-incubated with 0.1 mM homocysteine (Hcy) for 6 h. Then the cells were incubated with 100 nM insulin in the glucose production buffer consisting of glucose-free DMEM for 3 h, and glucose levels in the medium were determined by using a colorimetric glucose assay. *$P <$ 0.05 vs control (without Hcy).

Homocysteine upregulated *PEPCK* expression

Elevated plasma insulin probably resulted from an increase in gluconeogenesis. Therefore, we tested the effects of homocysteine on PEPCK, a key gluconeogenic enzyme, which catalyzes the rate-limiting step in the hepatic gluconeogenesis [17]. Results showed that the mRNA [**Fig. 4(A)**] and protein [**Fig. 4(B)**] levels of PEPCK were significantly higher in the liver of mice with hyperhomocysteinemia than in the normal mice in the re-fed state. To further confirm these results, primary

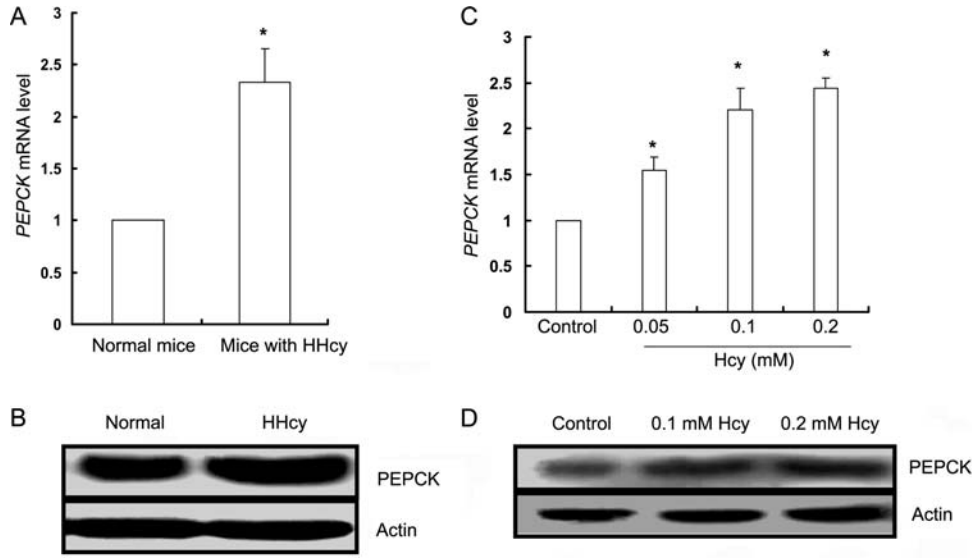

Figure 4 Homocysteine upregulates the expression of PEPCK Mice were sacrificed in the re-fed state. (A) The mRNA levels of *PEPCK* in the liver of mice were detected by real-time PCR. All results are standardized to the levels of β-actin and are the mean ± SD of five independent experiments. $*P < 0.05$ vs normal mice. HHcy, hyperhomocysteinemia. (B) Total proteins from the liver extract were separated by SDS-PAGE. PEPCK and actin were detected using specific antibodies as described in the 'Materials and Methods' section. (C) Primary cultured cells were incubated with homocysteine (Hcy) for 6 h. The mRNA levels of *PEPCK* were detected by real-time PCR. All results are standardized to the levels of β-actin. Data are shown as the mean ± SD of five independent experiments. $*P < 0.05$ vs control. (D) Total proteins from cell lysates were separated on SDS-PAGE. PEPCK and actin were detected using specific antibodies, respectively.

cultured hepatocytes were treated with homocysteine (0.05, 0.1 or 0.2 mM). As shown in **Fig. 4 (C,D)**, homocysteine upregulated the mRNA and protein levels of PEPCK in the primary cultured hepatocytes, respectively.

Homocysteine showed its effects via the PKA-dependent pathway

It has been shown that homocysteine could promote hepatic cAMP levels and PKA activity [18]. To clarify the mechanisms underlying homocysteine-induced *PEPCK* expression, we tested the effect of H89, a PKA inhibitor, on homocysteine-induced expression of PEPCK. As shown in **Fig. 5(A)**, H89 significantly repressed the activity of PEPCK induced by homocysteine.

As described above, the insulin-mediated inhibition of the glucose output was significantly attenuated by homocysteine. In this study, we found that H89 also significantly suppressed the effect of homocysteine on the insulin-mediated inhibition of the glucose output [**Fig. 5(B)**].

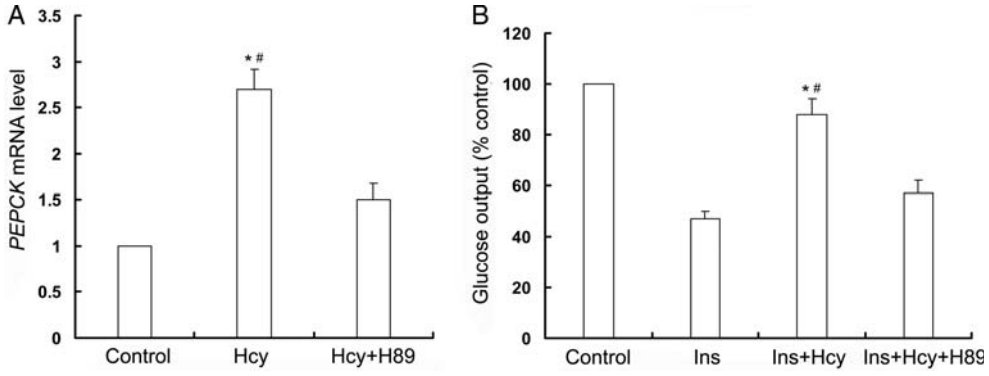

Figure 5 PKA inhibitor H89 inhibits the action of homocysteine Primary cultured hepatocytes were pre-treated with 5 μM H89 for 30 min. (A) The cells were incubated with 0.1 mM homocysteine (Hcy) for 6 h. The mRNA levels were detected by real-time PCR. All results are standardized to the levels of β-actin. Data are the mean ± SD of five independent experiments. $*P < 0.05$ vs control (without Hcy); $^{\#}P < 0.05$ vs Hcy + H89. (B) Then the cells were pre-incubated with homocysteine for 6 h. After cells were incubated with 100 nM insulin for 3 h, glucose levels in the medium were determined by using a colorimetric glucose assay. $*P < 0.05$ vs insulin alone; $^{\#}P < 0.05$ vs insulin + Hcy + H89.

Discussion

Although the relationship between the hyperhomocysteinemia and the insulin resistance has been reported, there is still debate over the direction of causality in this association. Increasing evidence suggests that hyperhomocysteinemia causes insulin resistance. For instance, the treatment of folate decreases the homocysteine levels and improves the insulin resistance in patients with metabolic syndrome [12]. Elevated insulin resistance index has been observed in rats after administration of homocysteine [13]. These results suggest that homocysteine causes insulin resistance. However, the underlying molecular mechanisms to link homocysteine to insulin resistance remain to be elucidated.

In this study, we found that the levels of insulin were significantly higher in mice with hyperhomocysteinemia than in the normal mice after administration of glucose. These results suggest that homocysteine causes hyperinsulinemia. One may speculate that homocysteine probably disrupts insulin-signaling cascade, resulting in secretion of more insulin by the β-cells of the pancreas after administration of glucose. Several studies support this hypothesis. For instance, *in vitro* studies have shown that homocysteine thiolactone inhibits the glycogen synthesis through blocking the PI3K/GSK-3 [14,15]. More recently, Li *et al*. reported that homocysteine upregulated the expression and secretion of resistin, a mediator of insulin resistance, from adipose tissue [19].

In this study, we demonstrated that homocysteine treatment abolished the inhibitory effect of insulin on glucose output, indicating that homocysteine may disturb glucose metabolism by impairing the insulin signaling. PEPCK catalyzes the first committed step in hepatic gluconeogenesis. It has been shown that elevations in the *PEPCK* gene expression attenuate insulin signaling and hepatic insulin sensitivity [20]. Transgenic mice overexpressing the *PEPCK* gene developed severe obesity and were more hyperinsulinemic, glucose intolerant, and insulin resistant than controls [21]. We found that the expression of *PEPCK* gene was elevated in the liver of mice with hyperhomocysteinemia and primary mouse hepatocytes treated with homocysteine. We do not know whether elevated *PEPCK* expression is the result or the reason of hyperhomocysteinemia-induced insulin resistance. However, the elevation in the *PEPCK* expression would aggravate insulin resistance. Thus, the upregulation of the *PEPCK* expression plays a critical role in the homocysteine-induced insulin resistance.

Since no allosteric modifiers of PEPCK have been reported, *PEPCK* expression is primarily regulated at the transcriptional levels. Previous studies demonstrated that cAMP response element-binding (CREB) protein is involved in the regulation of *PEPCK* gene expression [22,23]. In the current study, PKA inhibitor H89 inhibited homocysteine-induced *PEPCK* expression, suggesting a role of PKA in this process. Indeed, homocysteine could promote hepatic cAMP levels and the PKA activity in the liver and primary cultured hepatocytes [24]. Moreover, CREB-DNA binding activity is significantly elevated in the liver of hyperhomocysteinemic rat as well as in the homocysteine-treated hepatocytes [18]. In addition, H89 significantly attenuated the effect of homocysteine on insulin-mediated inhibition of glucose output. Thus, homocysteine disturbs the glucose metabolism probably through the cAMP/PKA/CREB pathway.

In summary, the current study demonstrated that hyperhomocysteinemia might be considered as a risk factor for the development of insulin resistance with respect to elevated glucose output and upregulation of PEPCK in a PKA-dependent manner. These results will further enhance our understanding of the link between hyperhomocysteinemia and the molecular events that result in insulin resistance.

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