Hyperhomocysteinemia stimulates hepatic glucose output and PEPCK expression

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Introduction

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.

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 at the Library of Chinese Academy of Sciences on July 18, 2010 http://abbs.oxfordjournals.org Downloaded from http://abbs.oxfordjournals.org at The Library of Chinese Academy of Sciences on July 18, 2010
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 NaHCO3, 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’-CAGGATCGAAAGGAAGACAGT-3’ (F), and 5’-AAGTCTCT-CTTCCGACATCCAG-3’ (R); β-actin, 5’-AGTGTGACGTTGACATCCGTA-3’ (F), and 5’-GCCAGAGCATCTCCTCTCT-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 a 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
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.

Homocysteine upregulated $\text{PEPCK}$ 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
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)].
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 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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References