Original Communication

Abnormality in Expression Levels of Gluconeogenesis-Related Genes by High-Dose Supplementation with Pyridoxamine in Mice

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Abstract: Pyridoxamine supplementation caused the alteration of the expression of genes encoding six gluconeogenesis-related proteins. The expression levels of phosphoenolpyruvate carboxykinase, pyruvate kinase, and pyruvate dehydrogenase kinase 4 in the pyridoxamine-supplemented mice were higher than those in the control mice. In contrast, the pyridoxamine supplementation caused lower expression levels of peroxisome proliferator-activated receptor-γ coactivator-1α, carbohydrate response element-binding protein, glucocorticoid receptor, and glucose-6-phosphatase. The pyridoxamine-supplemented mice showed significantly low glucose clearance in a glucose tolerance test, but they showed no symptoms of diabetes, which was estimated according to the levels of hemoglobin A1c and blood glucose. Pyruvate challenge testing suggested that pyridoxamine supplementation enhanced gluconeogenic activity from pyruvate. The results showed that a high-dose of pyridoxamine may require a careful inquiry concerning its validity.

Key words: pyridoxamine, gene expression, gluconeogenesis, phosphoenolpyruvate carboxykinase, diabetes, diabetic complications

Introduction

Pyridoxamine is one of the family of vitamin B_6 compounds, which consists of six compounds; i.e. pyridoxal, pyridoxamine, pyridoxine, pyridoxal 5´-phosphate, pyridoxamine 5´-phosphate, and pyridoxine 5´-phosphate. Pyridoxal 5´-phosphate is the coenzyme form for many enzymes involved in amino acid and carbohydrate metabolism, and plays a key role in the nutritional function of vitamin B_6. The other forms show the same nutritional efficiency because they are converted into pyridoxal 5´-phosphate [1]. Pyridox-

amine inhibits specifically the formation of advanced glycation end products (AGEs) and toxic effects of reactive oxygen species, and scavenges reactive carbonyl compounds [2]. Thus, its pharmacological potential for treatment of diabetic nephropathy [3], diabetic retinopathy [4], and hyperlipidemia [5] has been demonstrated.

Vitamin B_6 supplementation has been shown to be beneficial in treating several disorders like atherosclerosis [6] and gyrate atrophy [7]. The risk of toxicity of vitamin B_6 supplementation has generally been thought to be low. However, it was found that pyridoxine hydrochloride caused neuropathy at intakes of 200 mg per day or more, which is about 140-fold higher than the daily intake from foods, when it was used to alleviate symptoms of carpal tunnel syndrome [8]. In some cases, intakes of 100–300 mg per day caused neurotoxicity. An *in vitro* study with a model system of dorsal root ganglion neurons in culture showed that very high doses of pyridoxamine, pyridoxine, and pyridoxal are neurotoxic in humans and other animals [9]. Ataxia developed in rats fed diets containing 5.9 mg of pyridoxine/g by the 15th week [10]. Pyridoxine caused neuropathy in a human at intakes of 1000 mg per day or more, which is about 800 times the daily intake from foods [11]. The toxicity studies on animals and humans have usually been performed using pyridoxine hydrochloride as a source of vitamin B_6. Little is known about the mechanism of vitamin B_6 toxicity. Because pyridoxine is converted into pyridoxal 5´-phosphate, which modulates the expression of a variety of genes that respond to hormones [12], a high concentration of pyridoxine may change the expression levels of many genes. Pyridoxamine is also converted into pyridoxal 5´-phosphate, and may change the expression level of genes. As far as we know, no study has reported about the effect of pyridoxamine on the expression level of genes. Because pyridoxamine is better than aminoguanidine as an AGE inhibitor in terms of safety [13], and can be used in large amounts to alleviate symptoms of diabetic complications as described above, its effect on gene expression should be elucidated.

Here, for the first time, we have studied the effect of pyridoxamine supplementation on the expression levels of gluconeogenesis-related genes in mice, and found that it changed their expression levels, and decreased the glucose clearance in the blood of the animals without an incidence of the symptoms of diabetes.

Materials and methods

Animals

Experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee. Male mice of the C57BL/6 genetic background were housed in specific pathogen-free conditions in the animal facility at Kochi University. The mice were kept in the same room at 22–25 ºC with a regular 12-hour light/dark cycle and fed with a standard rodent chow (NMF; Oriental Yeast, Tokyo, Japan) and water *ad libitum.*

Reagents and treatments

Pyridoxamine dihydrochloride (4-aminomethyl-3-hydroxy-2-methyl-5-hydroxymethylpyridine dihydrochloride) was purchased from Tokyo Chemical Company, Ltd. (Tokyo, Japan). Ten and six male mice, respectively, were given drinking water with and without pyridoxamine dihydrochloride (1 g/L) for 3 months, starting at 2 months of age. The mice consumed 3–5 mg of pyridoxamine dihydrochloride per day. The value is equivalent to 4.5–7.5 g of pyridoxamine in humans weighing 60 kg.

Biochemical characterization

The hemoglobin A1c (HbA1c) levels were measured at age of 5 months by immunoassay (DCA 2000 system, Bayer Diagnostics, Elkhart, IN). Glucose levels in blood were measured using Glutest Ace and Glutest Sensor (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan).

Glucose tolerance, insulin tolerance and pyruvate challenge tests

Mice were fasted for 16 hours overnight with full access to water. The glucose tolerance test (GTT) was done with the mice as described previously [14]. In brief, glucose dissolved in a physiological saline solution was injected intraperitoneally (1 mg/g of body weight). Glucose levels in tail bloods, collected by vein nicks at 0, 15, 30, 60, 90, and 120 minutes after the injection, were measured. Insulin sensitivity was assessed using an insulin tolerance test (ITT) as described previously [14]. For the pyruvate challenge test, the mice were injected intraperitoneally with

pyruvate (2 mg/g of body weight) dissolved in saline, and then glucose concentrations in tail bloods were collected by vein nicks at 0, 15, 30, 60, 90, and 120 minutes after the injection were measured [15]. The mice were injected intraperitoneally with human recombinant insulin (Novo Nordisk Pharma Ltd) at a dose of 0.75 mU/g of body weight, and then glucose concentrations in tail bloods were measured at 0, 10, 30, 60 and 120 minutes.

Statistical analysis

All data are presented as mean ± SD. The statistical significance was examined by the independent-measures *t*-test. The Excel software was used. P values less than 0.05 were considered as statistically significant.

Quantitative real-time PCR (qRT-PCR) with SYBR green

Total RNA was prepared from the livers by the method of Zarlenga and Gamble [16]. The total RNA (1 μg) was reverse-transcribed using the M-MLV Superscript™ II Reverse Transcriptase (Life Technologies Japan Ltd. Tokyo, Japan) for first-strand cDNA synthesis with 0.8 μM oligo (dT) primer (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's instructions. In brief, the RNA and the primer were mixed, and then the mixture was incubated at 65 °C for 10 minutes, followed by cooling on ice for 5 minutes. The first-strand cDNA synthesis was started by addition of a transcription mixture to the cooled mixture, and then the mixture was incubated at 37 °C for 1 hour for the reverse transcription reaction. All of the reaction mixture containing the

Table I: Primers for quantitative real-time PCR.

Proteins (ID/Version)	Primers
Carbohydrate response element-binding protein (ChREBP NM_021455.3)	
forward	CTGGGGACCTAAACAGGAGC
reverse	GAAGCCACCCTATAGCTCCC
Cyclophilin A (NM_008907.1)	
forward	ATGGCACTGGCGGCAGGTCC
reverse	TTGCCATTCCTGGACCCAAA
Glucocorticoid receptor (NM_008173.3)	
forward	AAAGAGCTAGGAAAAGCCATTGTC
reverse	TCAGCTAACATCTCTGGGAATTCA
Glucose 6-phosphatase (Glc-6-Pase NM_008061.3)	
forward	TGAAACTTTCAGCCACATCCG
reverse	GCAGGTAGAATCCAAGCGCGAA
Peroxisome proliferator-activated receptor γ coactivator 1-α (Pgc-1α NM_008904)	
forward	CCCTGCCATTGTTAAGACC
reverse	TGCTGCTGTTCCTGTTTTC
Phosphoenolpyruvate carboxykinase (PEPCK NM_011044.2)	
forward	CACCAACGTGGCTGAGACTA
reverse	CTACGGCCACCAAAGATGAT
Pyruvate dehydrogenase kinase 4 (Pdk4 NM_013743.2)	
forward	TTTCTCGTCTCTACGCCAAG
reverse	GATACACCAGTCATCAGCTTCG
Pyruvate kinase (Pklr NM_013631.2)	
forward	TACCACCGCCAG TTG TTTG
reverse	GCGGCCAGTCTTTGTCAGC

synthesized cDNA was diluted 1:5 with H_2O and stored at –20 °C.

Quantitative PCR (qRT-PCR) was done with a Thermal Cycler Dice® real time system (TaKaRa Bio Inc., Shiga, Japan) using SYBR®Premix ExTaq ™II mix (TaKaRa Bio Inc., Shiga, Japan). The PCR reaction mixture (20 μL) contained 2 μL cDNA (8 ng/μL), 1 μL (10 μM) of each primer shown in Table I, 10 μL Premix ExTaq ™II mix, and H_2O. After initial denaturation (one cycle at 95 °C for 30 seconds), 40 cycles of amplification (95 °C for 5 seconds, 60 °C for 30 seconds) were performed. The production of a single amplified product was confirmed by a dissociation protocol according to the manufacturer's instruction and an electrophoretic analysis on 10 % polyacrylamide gels [17]. Relative gene expression levels were calculated with the comparative CT method using cyclophilin A (peptidyl-prolyl cis-trans isomerase A) as an internal reference gene. The expression levels in the control mice were set as 1. Differences between the control and the pyridoxamine-supplemented mice were presented as fold-differences.

Results

Biochemical parameters

The levels of HbA1c and body weight at age of 5 months in the control group were 4.2 ± 0.4 % and 34.4 ± 1.8 g, respectively. Those in the pyridoxamine-supplemented group were 4.1 ± 0.2 % and 33.8 ± 1.0 g, respectively. There were no significant differences between these values. The mean levels of fasting blood glucose at the age of 5 months in the control (88.9 ± 8.6 mg/dL) and pyridoxamine-supplemented groups (99.3 ± 5.7 mg/dL) were not significantly different either. The results showed that the prolonged supplementation with pyridoxamine caused no symptom of diabetes.

Expression of mRNA for enzymes involved in gluconeogenesis

Liver mRNA levels of six gluconeogenesis-related proteins in the control and pyridoxamine-supplemented mice were compared as shown in Table II. The mRNA level for phosphoenolpyruvate carboxykinase (PEPCK), which plays an important role in gluconeogenesis from non-sugar carbon [18], was 1.38-fold higher than the control mice. The expression level for pyruvate kinase (Pklr), which is involved in glycolysis, converts phosphoenolpyruvate into pyruvate, and thus inhibits gluconeogenesis, was also 1.22-fold higher in the liver of the pyridoxamine-supplemented mice. The expression levels of Pgc-1α and glucocorticoid receptor (GR) in the pyridoxamine-supplemented mice were 0.66- and 0.75-fold lower than that of the control mice, respectively. Pgc-1α is a member of the PPARγ coactivator-1 (Pgc-1) family and plays a central role in a regulatory network governing mitochondrial biogenesis [19]. The lower expression level of Pgc-1α suggested that mitochondrial biogenesis and/or energy production modestly declined in the liver via pyridoxamine supplementation. The complex of Pgc-1α, GR, and hepatocyte nuclear factor 4α (HNF4α) has been shown to be involved in augmentation of the expression level of PEPCK [20]. The results suggested that pyridoxamine changed the metabolism of glucose in mice, although it caused no incidence of diabetes.

Table II: Expression of mRNA for gluconeogenesis-related proteins.

Proteins	Fold differences[a]
Phosphoenolpyruvate carboxykinase	1.38 ± 0.11[b]
Pyruvate kinase	1.22 ± 0.11[b]
Pyruvate dehydrogenase kinase 4	1.10 ± 0.20
Glucose 6-phosphatase	0.88 ± 0.11
Glucocorticoid receptor	0.75 ± 0.05[b]
Carbohydrate response element-binding protein	0.74 ± 0.02[b]
Peroxisome proliferator-activated receptor γ coactivator 1-α	0.66 ± 0.09[b]

[a]Fold differences in expression levels of mRNA between the control (1.00) and the pyridoxamine-supplemented mice.
[b]Significantly different from the control.

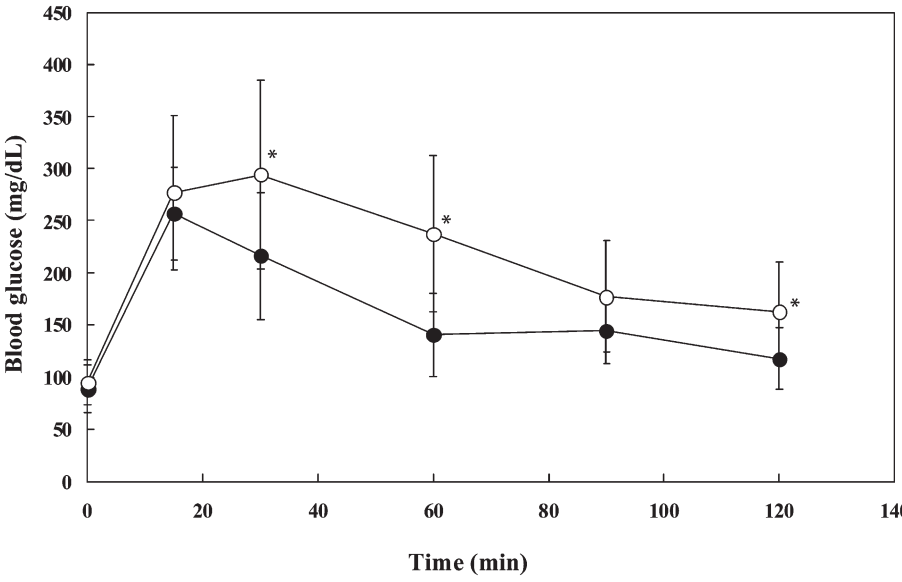

Figure 1: Glucose tolerance test. Blood glucose concentrations in the control (closed circles) and pyridoxamine-supplemented (open circles) mice were determined, after the glucose injection.
*$p < 0.05$ versus the corresponding value for the control mice.

Glucose tolerance, insulin tolerance, and pyruvate challenge tests

The effect of pyridoxamine-supplementation on glucose intolerance was examined. The pyridoxamine-supplemented mice showed a symptom of glucose intolerance, with a significantly higher glucose level from 30 minutes to 120 minutes after the glucose injection (Figure 1). In contrast, the pyridoxamine-supplemented mice did not show differences in insulin sensitivity, and thus had the same pattern of time-dependent changes in the blood glucose level as shown in Figure 2. Interestingly, administration of pyruvate, a substrate for gluconeogenesis, resulted in a significant increase in blood glucose concentration at 120 minutes in the pyridoxamine-supplemented mice (Figure 3). The results clearly showed that pyridoxamine supplementation deteriorated glucose clearance in blood.

Discussion

As pyridoxamine is implicated to have the pharmacological potential for treatment of complications associated with diabetic mellitus [2–5], effects of its

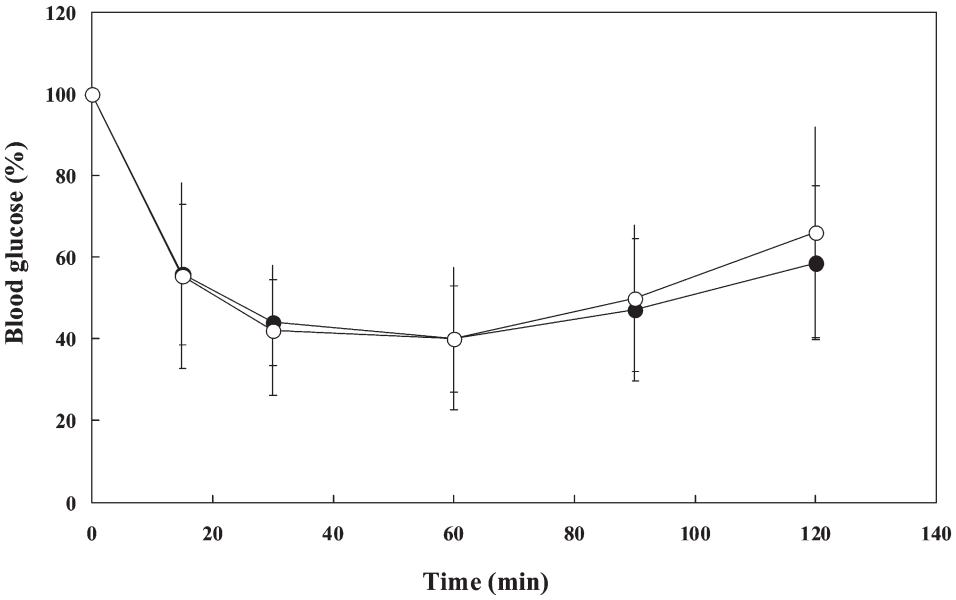

Figure 2: Insulin tolerance test. The changes in blood glucose concentrations in the control (closed circles) and pyridoxamine-supplemented (open circles) mice were determined after the insulin injection. The glucose concentration in the mice just before the injection was 100 %.

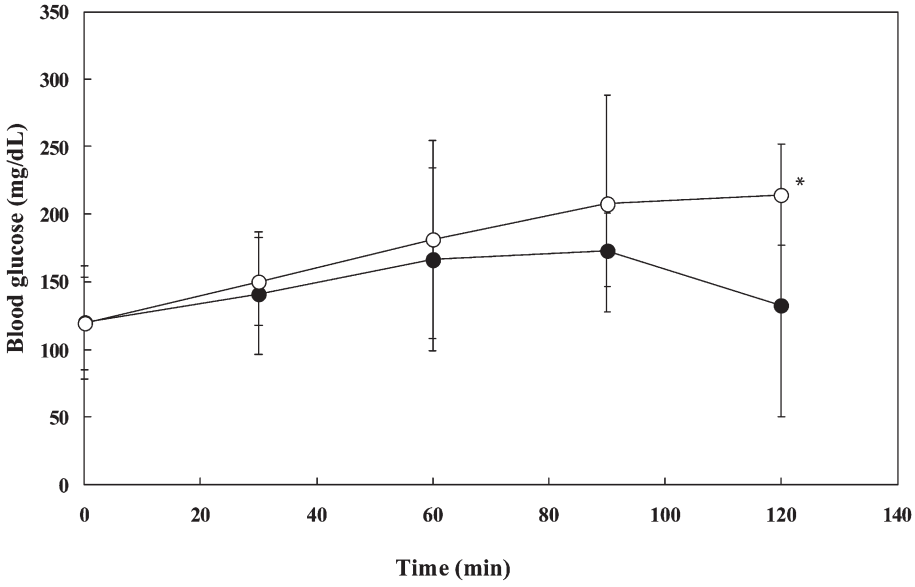

Figure 3: Pyruvate challenge test. Blood glucose concentrations in the control (closed circles) and pyridoxamine-supplemented (open circles) mice were determined after the pyruvate injection. *$p < 0.05$ versus the corresponding value for the control mice.

long-term high-dose administration were evaluated on hepatic gene expression levels as well as on serum glucose levels. The analysis revealed marginal effects on the genes related to glucose metabolism; 1.2~1.3 fold enhancement in the mRNA levels for PEPCK and PK, whereas decreases by 25~35 % for ChREBP, Pgc-1α, and GR over the control levels, respectively. Physiological relevance of these subtle effects on the mRNA levels remains to be elucidated, although the differences are statistically significant. Apparently additional studies are required to elucidate the mechanism of modulation of gene expression by pyridoxamine. However, pyridoxal 5´-phosphate produced from pyridoxamine [1] in the liver may play a key role because it acts as a modulator of both steroid hormone receptor-mediated gene expression and albumin gene expression [21]. Thus, it would be valuable to examine whether pyridoxine-supplementation also modulates the gluconeogenesis-related genes examined here.

The supplementation with pyridoxamine did not cause symptoms (based on the levels of blood glucose and HbA1c) of diabetes in the mice in accordance with the results reported previously [22]. The results of the insulin tolerance test also supported this phenomenon. However, it was found that the pyridoxamine-supplemented mice showed a slower clearance of glucose in blood after glucose injection than the control mice. Based on the results of glucose tolerance test (GTT) and insulin tolerance test (ITT) analysis, we speculate that pyridoxamine might cause impairment in pancreatic function, including synthesis/secretion of insulin or sensory activity to serum glucose levels. Furthermore, pyridoxamine-supplemented mice showed higher blood glucose levels at 120 minutes after the injection of pyruvate, suggesting that gluconeogenic activity from pyruvate was enhanced. Because pyridoxal 5´-phosphate shows considerable inhibition of cathepsins at high concentrations [23], it is plausible that gluconeogenic activity using pyruvate as a substrate might be enhanced to meet systemic demands for glucose in the pyridoxamine-supplemented mice. Taken together, our present study thus indicates that supplementation with pyridoxamine for the long term may decrease whole-body glucose disposal and/or glucose uptake function in tissue sites such as muscle and adipose.

The results showed that the pyridoxamine-supplementation changed the expression pattern of gluconeogenesis-related genes examined. The nutrigenomic approach will indicate the alteration of gene expression patterns of many other functional genes. In this study, the dose of pyridoxamine was very high (the equivalent of 4.5–7.5 g for humans weighing 60 kg). Because, the toxicity of high-dose pyridoxine-supplementation is well known [11], supplementation of such a high amount of pyridoxamine to humans is beyond the realm of possibility. However, a high dose of pyridoxamine over a long period of time to alleviate symptoms of diabetic complications may require a careful inquiry concerning its validity, although pyridoxamine-supplementation is beneficial for treatment of complications associated with diabetic mellitus [2–5].

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