Thiamine Ameliorates Diabetes-Induced Inhibition of Pyruvate Dehydrogenase (PDH) in Rat Heart Mitochondria: Investigating the Discrepancy Between PDH Activity and PDH E1α Phosphorylation in Cardiac Fibroblasts Exposed to High Glucose

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Abstract. The activity of pyruvate dehydrogenase (PDH) is reduced in diabetic patients. Phosphorylation of the PDH E1α subunit by PDH kinase contributes to the suppression of PDH activity. PDH requires thiamine as a coenzyme. We investigated the exact mechanism of diabetes-induced PDH inhibition, and the effect of thiamine in both in vivo and in vitro experiments. Treatment of rats with thiamine significantly, although partially, recovered streptozotocin (STZ)-induced reductions in mitochondrial PDH activity. Nevertheless, we found that PDH E1α phosphorylation in the thiamine-treated STZ group was perfectly diminished to the same level as that in the control group. STZ treatment significantly caused enhancements of the expression of O-glycosylated protein in the rat hearts, which was decreased by thiamine repletion. Next, the rat cardiac fibroblasts (RCFs) were cultured in the presence of high glucose levels. Thiamine dramatically recovered high glucose–induced PDH inhibition. High glucose loads did not alter the phosphorylated PDH E1α. PDH inhibition in RCFs was not accompanied by an increase in the PDH E1α phosphorylation. The O-glycosylated protein was markedly increased in RCFs exposed to high glucose, which was inhibited by thiamine. These results suggest that thiamine ameliorates diabetes-induced PDH inhibition by suppressing the increased expression of the O-glycosylated protein. The O-glycosylation of PDH E1α may be involved in the regulation of the PDH activity.

Keywords: thiamine, pyruvate dehydrogenase (PDH) activity, phosphorylated PDH E1α, O-glycosylated protein, diabetic rat heart

Introduction

Metabolic syndrome can lead to obesity, hypertension, and hyperglycemia. An increase in the incidence of metabolic syndrome worldwide can seriously impair the QOL of patients. Various lifestyle-related diseases (e.g., hyperlipidemia, diabetes, arteriosclerosis) affect the metabolic syndrome, and diabetes causes various complications like retinopathy, nephropathy, neuropathy, and microangiopathy (1).

Intracellular glucose is used for ATP production through the glycolytic pathway, pro-glucose oxidation (TCA cycle), and mitochondrial electron transport chain; these are called the major metabolic pathways. In glucose metabolism, there are minor pathways also, for example, the polyol pathway, hexosamine biosynthetic pathway, and diacylglycerol-protein kinase C pathway, to metabolize a part of the cellular glucose. In diabetes, glucose concentration inside and outside cells remains constant. Excess glucose is glycosylated to proteins via nonenzymatic means; advanced glycation end-products are recognized as factors in the pathogenesis of diabetic complications (2). The development and progression of diabetic complications are due at least in part to the activation of
minor pathways in glucose metabolism under hyperglycemia. If the glucose concentration within the cell becomes too high, a metabolic traffic jam may be generated in the glycolytic pathway or in glucose oxidation. Therefore, a high level of glucose enters in the minor metabolic pathways, and hence, they are efficiently activated.

Activity of pyruvate dehydrogenase (PDH) is reduced in diabetic patients, which damages glucose metabolism (3, 4). PDH activity is regulated by reversible phosphorylation. It is dependent upon PDH kinase (PDK), which phosphorylates the PDH E1α subunit, contributing to suppression of PDH activity. It has been suggested that phosphorylation by PDK renders the PDH inactive (5). PDK is allosterically modulated by increased ratios of acetyl-coenzyme A to CoA, ATP to ADP, and NADH⁺ to NAD⁺. These increased ratios activate PDK (6). Acetyl-CoA is produced not only by PDH, but also by the β-oxidation of fatty acids. During fasting, glucose consumption decreases, which is known to reduce PDH activity due to increased metabolism of fatty acids (Randle cycle) (7). Diabetes is characterized by an increased metabolism of fatty acids due to reduced utilization of glucose. Therefore, reduced PDH activity may be due to phosphorylation by PDK activated with enhancement of fatty acid metabolism (8). In addition, an increase in the uptake of fatty acids into cells activates peroxisome proliferator-activated receptors, transcription factors, and enhances not only protein expression resulting in the uptake and metabolism of fatty acids, but also PDK4 expression (9). Each of these may be caused by an increase in free fatty acids in the circulating blood of diabetic patients.

We posed the question: why do the mechanisms of inhibition of glucose metabolism work despite the presence of excess glucose in the cell? PDH activity decreases before diabetes is clinically evident. Furthermore, a follow-up study of subjects with lowered PDH activity reported that it may subsequently lead to the onset of diabetes (10). Therefore, a mechanism other than enhanced metabolism of fatty acids that lowers PDH activity may be in operation. We predicted that elucidation of these mechanisms would allow us to not only reduce the complications of diabetes, but also to prevent their development.

Thiamine is an indispensable coenzyme that is required for intracellular glucose metabolism. A high dose of thiamine has been reported to be effective in stopping the development of diabetic complications (11 – 16). We have previously reported that thiamine attenuates diabetic cardiomyopathy in streptozotocin (STZ)-treated rats (17). Although thiamine has been reported to activate transketolase and improve the abnormally activated minor metabolic pathway (13, 15, 16), its mechanism of action remains largely unknown. Similar to transketolase, PDH requires thiamine as a coenzyme, but only few studies have investigated the relationship between PDH activity and thiamine in diabetes.

We investigated the mechanism of decreased PDH activity in diabetes and the effect of thiamine on that mechanism in diabetic rats. To exclude increased metabolism of fatty acids and examine the changes under conditions of high glucose concentrations, rat cardiac fibroblasts (RCFs) were cultured in high concentrations of glucose. PDH activity and the effect of thiamine treatment on them were also examined.

Materials and Methods

Chemicals and reagents

Thiamine hydrochloride was supplied by Sigma (St. Louis, MO, USA) and Kishida Chemical Co., Ltd. (Osaka). STZ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). The Glu-test Sensor was supplied by Sanwa Chemical (Nagoya).

Antibodies used were as follows: anti-PDH E1α subunit, anti-β-actin, horseradish peroxidase (HRP)-conjugated anti-mouse, and anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phosphoserine/threonine (Abcam, Cambridge, MA, USA); anti-cytoskeleton c (Cell Signaling Technology, Beverly, MA, USA); and anti-O-linked β-N-acetylglucosamine (O-GlcNAc) (Affinity BioReagents, Golden, CO, USA). [2,14C]Pyruvate (15 MBq/mmol) was purchased from PerkinElmer Japan (Yokohama). Hanks’ balanced salt solution (HBSS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Invitrogen (San Diego, CA, USA). Collagenase was purchased from Worthington (Lakewood, NJ, USA). Fetal bovine serum (FBS) was supplied by Biowest (Nuaillé, France). A solution of penicillin/streptomycin was purchased from MP Biomedicals (Solon, OH, USA). All other chemicals were of the highest purity available (Wako Pure Chemical Industries, Ltd.).

Animals

Male Wistar rats (5 – 6 weeks of age) were used in all experiments. They were progeny of rats obtained from Charles River Laboratories (Kanagawa) and were maintained in the central animal facility of the university.

Rats were provided with a commercial diet and water ad libitum under temperature-, humidity-, and light-controlled conditions (22 ± 2°C, 55 ± 5%, and 12-h–12-h light–dark cycle, respectively). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Experi-
Obtain the mitochondrial pellet. After 2 rinses with ice-cold buffer A, mitochondria were resuspended in 50 mM Tris (pH 7.4), 50 mM KCl, 5 mM MgCl₂, 5 mM ethyleneglycol tetra-acetic acid (EGTA), 50 mM NaF, 5 mM dithiothreitol (DTT), 2.0 mM ethylenediamine tetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4 (buffer A). They were then minced and homogenized in 1.0 ml of homogenization buffer A/100 mg of tissue. The homogenate was centrifuged at 12,000 × g for 15 min at 4°C. The resulting supernatant was centrifuged at 12,000 × g for 15 min at 4°C to obtain the mitochondrial pellet. After 2 rinses with ice-cold buffer A, mitochondria were resuspended in 50 mM Tris (pH 7.4), 50 mM KCl, 5 mM MgCl₂, 5 mM ethyleneglycol tetra-acetic acid (EGTA), 50 mM NaF, 5 mM dichloroacetate, 1 mM DTT, 2.0 mM Na₃, 1 mM PMSF, 250 mM sucrose, and 0.3% Triton-X 100. This mixture was centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was used as the soluble mitochondrial fraction for Western blotting and determination of PDH activity. The purity of the mitochondrial fraction was evaluated by the expression of cytochrome c protein. There was no EGF-receptor protein expression, suggesting no contamination by cytosolic membranes. Protein determinations were performed using Lowry’s method (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

Isolation of RCFs

Cardiac fibroblasts were isolated from ventricular tissue of normal Wistar rats. RCFs were isolated according to a modified protocol described by Simpson and Savion (20). Briefly, rat hearts were isolated and digested with 10 ml of HBSS containing 0.2% collagenase for 45 min at 37°C. After digestion, the medium containing the suspended cells was centrifuged at 500 × g for 2 min. Cell pellets were resuspended in 8 ml of DMEM containing 5.6 mM glucose supplemented with 20% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were then plated on culture dishes at 37°C for 90 min in an atmosphere of 5% CO₂. The medium containing the cardiomyocyte fraction of digested tissue was removed. Dishes were gently rinsed 3 times to remove the remaining cardiomyocytes. The adherent fraction of plated cells consisted of cardiac fibroblasts. The culture medium for cardiac fibroblasts was changed to DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. Cells from the second to third passage were used for the experiments.

Cell culture conditions

RCFs were maintained on passage 3 in low glucose DMEM containing 5.5 mM glucose and supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 150 µM L-proline. Cells were further cultured in high glucose (35 mM) DMEM in the absence or presence of thiamine (200 µM) for 72 h. PDH activity in cell lysates was determined using radiochemical assay. The PDH E1α subunit, phosphorylated PDH E1α, and cytochrome c were analyzed in cell lysates by Western blotting. RCFs were washed with PBS, scraped in ice-cold 50 mM Tris-HCl buffer containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 6% β-mercaptoethanol, and protease inhibitor cocktail. After incubation at 95°C for 10 min, cells were lysed and protein concentrations were determined using Lowry’s method.

Measurement of PDH activity

Measurements of PDH activity were made at 37°C. To determine the active PDH fraction, the assay mixture contained, in a final volume of 97.5 µl, the following: 50 mM Hepes (pH 7.4), 2 mM MgCl₂, 3.35 mM NAD⁺, 0.4 mM TPP, 0.45 mM CoA, 10 mM L-carnitine, 0.5 mM DTT, 0.08 mM EGTA, 2.5 mM NaF, 0.8 units carnitine acetyltransferase, and the above obtained mitochondrial fraction or cell lysate. The reaction was initiated with 2.5 µl of 40 mM [2-14C]pyruvate (specific radioactivity, 15 MBq/mmol; 1.0 mM final pyruvate concentration). Unless specified otherwise, the reaction was terminated after 5 min with 200 µl cold methanol. Exclusion chromatography was employed using an anion exchange resin.
column to separate the positively charged radiolabeled reaction product (acetylcarnitine) from the negatively charged radiolabeled substrate (pyruvate) and the small amount of the reaction intermediate (acetyl-CoA). The entire reaction mixture (300 μl) was applied to the column. Acetylcarnitine was then eluted by washing the column with distilled water (600 μl followed by 1,000 μl). The eluate was collected into scintillation vials and radioactivity was counted. PDH activity was calculated from the specific radioactivity of [14C]acetylcarnitine and expressed in nmol/mg protein per min (21).

Measurement of citrate synthase (CS) activity

The above mitochondrial fraction was preincubated at 37°C for 5 min and then incubated with the assay mixture containing 100 mM Tris/HCl (pH 8.0), 0.1 mM DTNB, and 0.05 mM acetyl-CoA at 37°C for 3 min. The change in extinction by the essential acetylase was removed by this incubation. The enzyme reaction was started by adding 0.1 mM oxaloacetate. The CS activity was calculated from the change in extinction at 405 nm over time.

RNA extraction and real-time PCR analysis

Total RNA was isolated from the left ventricle of the heart and RCFs using RNeasy Fibrous Tissue Mini-Kits (Qiagen, Hilden, Germany). For reverse transcriptase heart and RCFs using RNeasy Fibrous Tissue Mini-Kits RNA extraction and real-time PCR analysis from the change in extinction at 405 nm over time.

Electrophoresis and Western blotting

Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 9% gel. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 0.3% skimmed milk or 5% BSA in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated with the specific primary antibodies; anti-PDH E1α subunit, anti-phosphoserine/threonine, anti-cytochrome c, anti-O-GlcNAc, and anti-β-actin in TBST overnight at 4°C. Membranes were washed 3 times in TBST to remove unbound antibodies. They were then incubated with HRP-conjugated secondary antibody in TBST for 1 h at room temperature. The chemiluminescence was detected by a LAS-3000 machine (Fuji Film Corp., Tokyo) using an enhanced chemiluminescence reagent (Chemi-Lumi One; Nacalai Tesque, Kyoto). The expression of β-actin was used as an internal standard.

Statistical analyses

Data are expressed as the mean ± S.E.M. Statistical analyses of the data from multiple groups were performed by ANOVA followed by Scheffe’s F tests. P < 0.05 was considered significant.

Results

Effect of thiamine on PDH inhibition caused by STZ in rat heart mitochondria

We evaluated if thiamine affected the mitochondrial PDH activity in the diabetic rat heart. The PDH activity in the STZ-administered rats was remarkably lower than that in normal controls. Thiamine repletion significantly recovered STZ-induced reductions in the mitochondrial PDH activity in the hearts of the diabetic rats (Table 1A).

We adjusted the mitochondrial fraction using a density gradient method. It is believed that mitochondrial yield varies according to the mitochondrial fraction during morbidity in the control and STZ-administered groups. The CS activity is routinely used as a marker of mito-

<table>
<thead>
<tr>
<th>Group</th>
<th>PDH activity / CS activity (ratio)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.0 ± 0.057</td>
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<tr>
<td>STZ-treated</td>
<td>0.328 ± 0.067***</td>
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<tr>
<td>Thiamine-treated STZ</td>
<td>0.544 ± 0.054***</td>
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Rats were treated with STZ (70 mg/kg, i.v.) and PDH activity in mitochondria prepared from left ventricle of the heart was determined at 15 days. A) The level of PDH activity expressed in nmol/mg protein per min. B) PDH activity in each experimental group was normalized to citrate synthase (CS) activity and expressed as a ratio. Each value represents the mean ± S.E.M. **P < 0.01 and ***P < 0.001, compared with the control; *P < 0.05, compared with STZ.

Table 1. Effect of thiamine on streptozotocin (STZ)-induced pyruvate dehydrogenase (PDH) inhibition in diabetic rats
PDH Activity and O-Glycosylated Protein

PDH Activity and O-Glycosylated Protein

Activity and O-Glycosylated Protein

chondrial density. In the present study, the PDH activity was normalized to the corresponding CS activity. The PDH activity normalized to the CS activity in the STZ-administered rats was also remarkably lower than that in normal controls. Thiamine repletion significantly recovered STZ-induced reductions in the mitochondrial PDH activity, normalized to the CS activity, in the diabetic rat hearts (Table 1B).

PDH mRNA levels in STZ-induced diabetic rat hearts and effects of thiamine

Next, we examined the effect of high blood glucose on PDH production. There were no significant differences in the expression levels of PDH mRNA in each group (Fig. 1).

Effect of thiamine on mitochondrial phosphorylated PDH E1α and PDH E1α subunits in diabetic rat hearts

The level of PDH E1α and phosphorylated PDH E1α in the rat heart mitochondria were investigated in the STZ-treated rats. The STZ treatment caused a significant increase in the level of the phosphorylated PDH E1α subunit in the diabetic rat hearts. A significant difference was not observed in the level of PDH E1α subunit in the STZ-administered group. The effect of thiamine on the increase in PDH E1α phosphorylation caused by diabetes was investigated in the STZ-treated rats. A significant inhibition of mitochondrial PDH E1α phosphorylation was observed with thiamine repletion (Fig. 2A). There was no significant change observed in the level of the mitochondrial PDH E1α subunit in the presence of thiamine (Fig. 2B).

Correlation between mitochondrial PDH E1α phosphorylation and PDH activity in diabetic rat hearts

We examined the correlation between PDH E1α phosphorylation and the PDH activity in the diabetic rat heart mitochondria. Even though the PDH activity was significantly recovered by thiamine in the STZ group, it did not recover to the same level as that in the control group. No differences in the level of PDH E1α expression were observed as compared with each group. PDH E1α phosphorylation in the thiamine-treated STZ group decreased

Fig. 1. Pyruvate dehydrogenase (PDH) mRNA expressions in streptozotocin (STZ)-induced diabetic rats and effects of thiamine. Rats were treated with STZ (70 mg/kg, i.v.) and PDH mRNA in diabetic rat heart was determined at 15 days. Total RNA was analyzed using real time PCR. PDH mRNA was normalized to the corresponding β-actin. Each value represents the mean ± S.E.M.

Fig. 2. Effect of thiamine on phosphorylation of pyruvate dehydrogenase (PDH) E1α (A) and the E1α subunit of PDH (B) in mitochondria prepared from the left ventricle of the heart of streptozotocin (STZ)-induced diabetic rats. PDH E1α, phosphorylated PDH E1α, and cytochrome c protein bands were detected by Western blotting using each specific antibody and quantified by densitometric scanning. Phosphorylated PDH E1α was normalized to the corresponding PDH E1α. PDH E1α was normalized to the corresponding cytochrome c. Each value represents the mean ± S.E.M. ***p < 0.001, compared with the control; **P < 0.01, compared with STZ.
to nearly the same level as that in the control group, but
the PDH activity was significantly lower (Fig. 3). There
was a discrepancy between the PDH activity and the
degree of PDH E1α phosphorylation; it was considered
that a mechanism other than phosphorylation may be
related to the regulation of the PDH activity.

Expression of O-glycosylated protein in diabetic rat
heart mitochondria and the effects of thiamine

It has been suggested that crosstalk exists between O-
GlcNAcylation and phosphorylation (22, 23). We have
previously reported that level of the O-glycosylated
protein is increased in diabetic rats (17). The STZ treat-
ment caused marked enhancements of the O-glycosylated
protein, which was attenuated by thiamine repletion (Fig.
4). O-GlcNAcylated protein antibody recognized specific
bands with a molecular mass of approximately 43 kD,
which likely corresponds to the band of PDH E1α.

Effect of thiamine on PDH inhibition caused by high
glucose conditions in RCFs

It has been reported that the PDH activity is reduced in
diabetic patients, although high values of free fatty acids
in the blood were not observed (10). We hypothesized
that a mechanism other than enhanced fatty acid metabo-
lism may lower the PDH activity. To exclude increased
fatty acid metabolism and assess the direct effect of high
glucose conditions on the PDH activity, in vitro experi-
ments were performed using RCFs. We incubated RCFs
in a medium containing 35 mM glucose for 72 h and ana-
lyzed the PDH activity. Treatment of RCFs with high
concentrations of glucose reduced the PDH activity re-
markably. Thiamine dramatically recovered the high
glucose–induced PDH inhibition (Table 2). There was no
significant change in the PDH activity in the presence of
mannitol (35 mM).

PDH mRNA levels in RCFs under high glucose condi-
tions and the effects of thiamine

We cultured RCFs in glucose loading conditions using
low glucose (LG: 5.5 mM) and high glucose (HG: 35
mM) concentrations for 72 h. The effect of glucose load
to induce PDH mRNA expression was investigated in
RCFs. High glucose loads did not alter the level of PDH
mRNA expressions in RCFs (Fig. 5).

Effect of thiamine on the level of phosphorylated PDH
E1α and the PDH E1α subunit in RCFs under high-glucose
conditions

High glucose loads did not alter the level of phospho-
ylated PDH E1α and PDH E1α (Fig. 6: A and B).

Correlation between PDH E1α phosphorylation and
PDH activity under high-glucose conditions in RCFs

We examined the correlation between PDH E1α phos-
phorylation and the PDH activity in RCFs. PDH inhibi-
tion under high-glucose conditions in RCFs was not ac-
accompanied by an increase in phosphorylation of the PDH
E1α subunit (Fig. 7).
Table 2. Effect of thiamine on high glucose–induced pyruvate dehydrogenase (PDH) inhibition in rat cardiac fibroblasts (RCFs)

<table>
<thead>
<tr>
<th>Group</th>
<th>PDH activity (pmol/mg per min)</th>
<th>PDH activity / CS activity (ratio)</th>
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<tr>
<td>LG (5.5 mM glucose)</td>
<td>221.1 ± 19.2</td>
<td>1.0 ± 0.040</td>
</tr>
<tr>
<td>HG (35 mM glucose)</td>
<td>169.3 ± 20.0**</td>
<td>0.755 ± 0.053**</td>
</tr>
<tr>
<td>HG + thiamine (200 μM)</td>
<td>346.1 ± 38.5**</td>
<td>1.398 ± 0.108**</td>
</tr>
<tr>
<td>Mannitol (35 mM)</td>
<td>243.2 ± 29.7</td>
<td>1.020 ± 0.069</td>
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</table>

RCFs were cultured for 72 h in the presence of low glucose (LG: 5.5 mM), high glucose (HG: 35 mM), HG with thiamine (200 μM), or mannitol (35 mM). A) The level of PDH activity expressed in pmol/mg protein per min. B) PDH activity in each experimental group was normalized to citrate synthase (CS) activity and expressed as a ratio. Each value represents the mean ± S.E.M. **P < 0.01, compared with LG; *P < 0.01 and ***P < 0.001, compared with HG.

Expression of O-glycosylated protein in RCFs and the effects of thiamine

In RCFs, high-glucose conditions caused prominent enhancements of the levels of O-glycosylated protein, which was decreased by thiamine (Fig. 8). O-GlcNAcylated protein antibody recognized remarkable bands with a molecular mass of approximately 43 kD, which corresponds to the band of PDH E1α.

Discussion

A high dose of thiamine has been reported to be effective in the treatment of diabetic complications (11 – 17). Increased expression of transketolase, which utilizes thiamine as a coenzyme, has been shown to be the mechanism of action (13, 15, 16). Reduced PDH activity has been reported (5, 6, 10) in diabetes but studies evaluating the effect of thiamine on PDH inhibition in diabetes have not
been conducted. PDH also utilizes thiamine as a coenzyme. We investigated the mechanism of PDH inhibition in diabetes and the influence of thiamine on the PDH activity.

In the present study, the PDH activity was significantly reduced in the heart mitochondria of the STZ-induced diabetic rats as compared to the control group. There were no differences in the level of PDH mRNA and PDH E1α expression between the STZ and control groups, suggesting that high blood glucose did not affect the PDH production. PDH E1α phosphorylation significantly increased in the STZ group. The PDH activity was significantly recovered by thiamine administration in the STZ group. However, the PDH activity was markedly lower; it did not recover to the level observed in the control group. Nevertheless, PDH E1α phosphorylation in the thiamine-treated STZ group was reduced to nearly the same level as that in the control group. There was a discrepancy between the PDH activity and the degree of PDH E1α phosphorylation. It is considered that a mechanism other than phosphorylation may be related to the regulation of the PDH activity.

In recent years, serine and threonine residues, which are phosphorylated, have been reported to receive dynamic post-translational modification by O-GlcNAc transferase (OGT) and O-GlcNAcase (24). O-GlcNAc modification influences the enzyme activity and the stability and transcription of proteins. It is, therefore, speculated to be involved in diabetes mellitus and the development of diabetic complications (25). We reported that the hexosamine biosynthesis pathway is activated and the level of O-glycosylated protein in the diabetic rat hearts and RCFs are increased (17, 26). OGT is present in mitochondria (27); therefore, PDH can be a substrate of OGT. STZ is an analog of glucosamine and also reported to act as an inhibitor of O-GlcNAcase (28). It has been reported that there is crosstalk between GlcNAcylation and phosphorylation (22, 23). Therefore, O-GlcNAc proteins were investigated in the diabetic rat hearts. In the present study, the O-glycosylated protein was markedly expressed in the heart mitochondria of the diabetic rats and was decreased by thiamine repletion. PDH inhibition may be mediated in part by O-glycosylation of PDH E1α.

The PDH activity is reduced in diabetic patients. A follow-up study of subjects with reduced PDH activity reported that it may lead to a future onset of diabetes, although no high value of free fatty acid in the blood was observed (10). A mechanism other than enhanced fatty acid metabolism may lower the PDH activity. We examined the effect of thiamine on the PDH activity using a culture system, that is, cells cultivated in high glucose concentration, because we wanted to exclude the effect of increased levels of free fatty acids in blood. In the in vivo experiments, we obtained data showing that blood glucose levels in the rats 15 days after STZ injection were remarkably higher in the diabetic rats compared to normal controls (approximately 100 mg/dl in the con-

![Fig. 7. Correlation between pyruvate dehydrogenase (PDH) enzyme activity and PDH E1α phosphorylation in rat heart mitochondria under low glucose (LG) (circle), high glucose (HG) (square), HG + thiamine (triangle), or mannitol (diamond). Each value represents the mean ± S.E.M. (n = 5 – 7).](image)

![Fig. 8. Effects of thiamine on O-glycosylated protein in rat cardiac fibroblasts (RCFs). RCFs were cultured for 72 h in the presence of low glucose (LG: 5.5 mM), high glucose (HG: 35 mM), HG with thiamine (200 μM), or mannitol (35 mM). O-GlcNAcylated and pyruvate dehydrogenase (PDH) E1α protein bands were detected by Western blotting using each specific antibody.](image)
trols, 600 mg/dl in the diabetic rats), suggesting that high glucose levels are maintained until after the experiment has been completed. Therefore, we selected the 5.5 mM low-glucose and 35 mM high-glucose models in vitro. In the in vitro experiments, the PDH activity was decreased by high levels of glucose; the high-glucose condition directly influenced the PDH activity. Thiamine addition in the high glucose–concentration group significantly prevented PDH inhibition. A high-glucose condition and thiamine addition did not show significant differences in the PDH E1α expression and in the degree of phosphorylation. In the in vitro study, the O-glycosylated protein also showed marked expression in RCFs, which was reduced by thiamine. From these findings, it is observed that thiamine ameliorates diabetes-induced PDH inhibition by suppressing the increased expression of the O-glycosylated protein. PDH inhibition may be caused by post-translational modification: O-GlcNAc modification of PDH E1α. We presumed that O-glycosylation of PDH E1α results in reduced PDH activity.

In the in vivo study, phosphorylation of PDH E1α significantly increased in diabetic heart mitochondria. It has been reported that PDH phosphorylation may be caused by PDK, which is activated with enhancement of fatty acid metabolism. We obtained data that revealed increased expression of the free fatty acids in the diabetic rats. In the in vitro study, there were no significant changes in the level of PDH E1α phosphorylation in RCFs exposed to high glucose, suggesting that fatty acid metabolism cannot be enhanced in RCFs. In this study, the different results of PDH phosphorylation between in vivo and in vitro experiments are probably due to the fatty acid metabolism.

In the diabetic rat in vivo model, thiamine repletion significantly recovered the PDH activity, but not completely. Thiamine also markedly decreased the O-glycosylated protein expression, but partially. In the culture model, thiamine recovered the PDH activity and perfectly suppressed the O-glycosylated protein expression. Modification of the PDH activity by thiamine may be involved in the degree of O-glycosylated protein expression.

Preventing and treating diabetes is an urgent issue worldwide. In this study, PDH inhibition in diabetes was related not only to an increase in PDH E1α phosphorylation with the enhancement of fatty acid metabolism, as previously reported, but O-GlcNAc modification of PDH E1α were also suggested to be associated. Details regarding the mechanism with respect to PDH inhibition or the degree of involvement were not revealed in this study. Discovering the exact mechanisms that lead to a reduction in the PDH activity may enable the development of new drugs to prevent diabetes and diabetic complications.

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