Prevention of incipient diabetic cardiomyopathy by high-dose thiamine

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ABSTRACT — Diabetic cardiomyopathy can progress toward overt heart failure with increased mortality. The hexosamine biosynthesis pathway has been implicated in signaling for fibrosis by the kidney. Thiamine (vitamin B₁) is an indispensable coenzyme and required at intracellular glucose metabolism. In this study, we assessed if decrease of flux through the hexosamine biosynthesis pathway induced by high-dose thiamine therapy counteracts diabetes-induced cardiac fibrosis. The diabetes model was the streptozotocin-induced diabetic rat. Normal control and diabetic rats were studied for 2 weeks with and without thiamine, and followings were analyzed; plasma biochemicals (total cholesterol and triglycerides), morphological changes, mRNA abundance relevant to cardiac failure (brain natriuretic peptide) and fibrosis (transforming growth factor-β1, thrombospondine, fibronectin, plasminogen activator-I and connective tissue growth factor) as well as and matrix metalloproteinase activity were investigated. Thiamine repletion prevented diabetes-induced cardiac fibrosis without changes in plasma glucose concentration. This was achieved by prevention of thiamine depletion, increased pro-fibrotic mRNA abundance and decreased metalloproteinase activity in the heart of diabetic rats. O-glycosylated protein was significantly higher in the left ventricular of diabetic rats compared to control rats, which was decreased by thiamine administration. Thiamine repletion prevented diabetes-induced cardiac fibrosis in experimental diabetes, probably by suppression of hexosamine biosynthesis pathway.

Key words: Diabetic cardiomyopathy, Cardiac fibrosis, Thiamine, Hexosamine biosynthesis pathway, O-GlcNAcylation

INTRODUCTION

Mega clinical studies, i.e., Framingham study, studies of left ventricular dysfunction (SOLVD), heart outcome prevention evaluation study (HOPE) and cardiovascular health study (CHS) report that diabetes is a major risk factor of heart failure (William et al., 1974; Ho et al., 1993; Shindler et al., 1996; Bertoni et al., 2003; Piccini et al., 2004). Although cardiac failure in diabetes is mostly due to coronary artery disease relevant ischemic heart disease, the incidence of heart failure in diabetes independent of atherosclerotic coronary lesion is speculated to be 4 to 5 times higher than in normal people. Rubler et al. (1972) propounded the concept of diabetic cardiomyopathy without coronary lesion as diabetic cardiomyopathy, which was accompanied by systolic and diastolic failure due to diabetes per se. The high incidence and poor prognosis of heart failure in diabetic patients have been linked in part to the presence of an underlying diabetic cardiomyopathy characterized by myocardial hypertrophy and myocardial fibrosis (Factor et al., 1980). Experimentally induced diabetes models cause changes in myocardial cellular calcium transport and contractile proteins, which result in subclinical systolic and diastolic dysfunction (Ganguly et al., 1983; Giacomelli and Wiener, 1979). The increased myocardial collagen content associated with diabetic cardiomyopathy further worsens diastolic dysfunction (Poirier et al., 2001).

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Among the possible mechanisms of glucose-induced cellular damage and phenotype changes, four hypotheses have been widely accepted and clinical trials established to study specific inhibitors; (1) increased flux through the polyol pathway; (2) increased formation of advanced glycation end (AGE) product; (3) protein kinase C (PKC) activation and (4) increased flux through the hexosamine biosynthesis pathway (HBP) (Beltramo et al., 2008). Activation of PKC isoforms leads to endothelial dysfunction by activation of NADPH oxidase, enhanced vascular permeability through upregulation of vascular endothelial growth factor, and thrombosis by increasing plasminogen activator inhibitor (PAI)-1 levels (Williams and Nadler, 2007). The activation of the HBP also contributes to the mechanisms of diabetes-induced cellular damages. Stimulatory protein 1 (Sp1), one of the transcriptional factors, can be O-linked β-N-acetylgalcosamine (O-GlcNAC) enzymatic glycosylated. O-GlcNAc enzymatic glycosylation (O-GlcNAcylation) of Sp1 increased Sp1 transactivation and Sp1-dependent expression of both transforming growth factor-B1 (TGF-β1) and PAI-1 (Du et al., 2000; Verrecchia et al., 2001).

Thiamine is an indispensable coenzyme and required at several stages of anabolic and catabolic intermediary metabolism such as intracellular glucose metabolism (glycolysis, Krebs cycle, pentose-phosphate cycle). Although diabetic subjects usually do not manifest the typical clinical manifestation of thiamine deficiency, it was observed that diabetic subjects tend to have lower blood thiamine concentrations than healthy controls, together with a reduced erythrocyte transketolase activity (Saito et al., 1987; Valerio et al., 1999; Jermendy, 2006) and an increased thiamine renal clearance (Thornalley et al., 2007). Recently, high-dose benfotiamine, a lipophilic derivative of thiamine, has been shown to inhibit increase of HBP, diacylglycerol (DAG) pathway and AGE pathway under the hyperglycemia state, accompanied with the prevention of the pathogenesis of diabetic nephropathy and retinopathy in the delay of the diabetes-related complications (Hamnes et al., 2003). In the streptozotocin (STZ) rat model of type I diabetes, diabetes induced renal fibrosis, possibly due to PKC activation, reported to be inhibited by thiamine administration (Koya et al., 1997; Babaei-Jadidi et al., 2003) and α-lipoic acid, another coenzyme of PDH (Melhem et al., 2001). Tanaka et al. (2007) have observed that thiamine attenuates the hypertension and metabolic abnormalities in CD36-defective SHR through rectifying the balance of glucose oxidation to its cellular entry. On the supposition that diabetes might be considered a thiamine-deficient state, if not in absolute terms at least relative to the increased requirements deriving from accelerated and amplified glucose metabolism (Beltramo et al., 2008), we hypothesize that (1) thiamine repletion could activate glucose oxidation and re-orient the glucose flow into the major pathway of glucose metabolism rather than into the minor pathway, and, in turn, (2) it could decrease detrimental metabolic products in each minor pathway.

In this study, we focus on heart failure, especially on cardiac fibrosis, and investigated the following issues, firstly the measurement of thiamine level in blood, secondarily the biological and histological analysis of cardiac fibrosis, and finally effects on cardiac lesion of STZ-induced diabetic rats by thiamine administration.

**MATERIALS AND METHODS**

**Chemicals**
Thiamine hydrochloride was supplied by Kishida Chemical Co., Ltd. (Osaka, Japan). Streptozocin was purchased from Wako Pure Chemical Industries, Japan. Glu Test Sensor was supplied by Sanwa Chemical (Nagoya, Japan). Antibodies used were as follows; anti-O-linked glycosylation antibody (RL-2) from the Affinity BioReagents (Golden, USA), biotinylated goat anti-mouse immunoglobulin and peroxide-conjugated streptavidin from scytek (Logan, Utah 84323, USA), anti-mouse immunoglobulin (H&L, horseradish peroxidase-linked) from Amersham Pharmacia Biotech, (NJ, USA), Sirius red F3BA was supplied by Polysciences, Inc. (Warrington, PA, USA). All other chemicals used were of the highest purity available (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Animals**
Male Wistar-Kyoto rats (6 weeks of age) were used in all experiments. They were the progeny of rats obtained from the Charles River (Kanagawa, Japan) and were maintained in the central animal facility of our university. The animals were provided with a commercial diet and water ad libitum under temperature-, humidity-, and lighting- controlled conditions (22 ± 2°, 55 ± 5%, and a 12:12-hr light-dark cycle, respectively). Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

**Experimental design**
Diabetes was induced by intraperitoneal injection of 70 mg/kg STZ solved in 0.1 M sodium citrate buffer (pH4.5), after overnight fasting. Hyperglycemia was confirmed by a Glu Test Sensor (Sanwa Kagaku, Japan) on tail vein
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![Diagram showing experimental design]

Fig. 1. Experimental design.

blood samples. Only rats with blood glucose levels > 300 mg/dl 3 days after STZ injection were used. Rats treated with a single intraperitoneal injection of vehicle were used as controls. Thiamine was given by water containing 0.2% thiamine.

Rats were placed in one of four study groups as follows; group 1, untreated non-diabetic controls; group 2, untreated diabetic rats; group 3, diabetic rats supplemented with thiamine; group 4, non-diabetic controls supplemented with thiamine. Groups 1 and 2, untreated groups, were freely accessed to tap water. Groups 3 and 4, thiamine treated groups, were freely accessed to the tap water containing 0.2% thiamine. Groups 3 and 4 were started with the supplementation of thiamine 3 days before, and maintained to 15 days after STZ and vehicle injection.

**Measurements of blood pressure and Echocardiographic analysis**

Heart rate and systolic blood pressure were measured by a tail-cuff system (BP98A, Softron) in conscious animals.

Fifteen days after STZ and vehicle treatments, echocardiography was performed on lightly anaesthetized rats (1-2% isoflurane, with spontaneous ventilation). Two-dimensional guided M-mode echocardiography was performed with the use of an echocardiogram equipped with a 10-MHz transducer (Vivid 7 echocardiographic scanner, GE Medical Systems, Horten, Norway). The heart was imaged in a two-dimensional mode in a parasternal short-axis view with a depth setting of 2 cm. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the left ventricle at the level of the papillary muscles. An M-mode image was obtained at a sweep speed of 100 mm/sec. Left ventricular (LV) ejection fraction (EF), LV end-diastolic dimension (Dd), LV end-systolic dimension (Ds) and LV fractional shortening (FS) were measured. The LV systolic function was assessed using the LV ejection fraction (EF).

At the end of the echocardiographic analysis, rats were killed by over-doses of pentobarbital and blood was withdrawn by cardiac puncture. The plasma was immediately separated by centrifugation and frozen at -40°C for later biochemical measurements.

The heart and kidney were removed and weighted. Organ weight was normalized by tibia length. Heart was cut into 3 transverse slices and the middle slice was fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical and Sirius red staining.

**Measurements of blood parameters**

Total plasma cholesterol and triglycerides were assayed enzymatically using the T-Cho E and the TG E commercial kits, respectively, purchased from Wako (Osaka, Japan).

**Measurement of thiamine levels in plasma and red blood cells**

Thiamine levels were determined by the modification described by Marszall et al. (2005) and Lebiedzinska et al. (2007), as follows. Phosphorylated thiamine metabolites were dephosphorylated with diastase and total thiamine status was determined by high-performance liquid chromatography (HPLC) with fluorimetric detection.
(post-column derivatisation to thiochromes). To remove proteins and lipids, 100 μl sample (plasma and erythrocyte hemolysate) was treated with 1 ml of 10% trichloroacetic acid (TCA) and then centrifuged at 5,000 g at 4°C for 10 min. The supernatant (800 μl) was neutralized with 200 μl of 4 mol/l sodium acetate and then added 40 μl of 2% diaisolate solution followed by incubation for 10 hr at 37°C. Aliquots of 20 μl were injected on an Inertsil ODS-3 column (150 x 4.6 mm; 5 μm; GL Sciences Inc., Tokyo Japan), connected to a Shimadzu HPLC system (SCL-10A) coupled to a Shimadzu fluorescence detector (RF-10AXL) (excitation and emission wavelength 375 and 440 nm, respectively). The mobile phase (0.8 ml/min) consisted of methanol and phosphate buffer (0.01 mol/l sodium dihydrogenphosphate, 0.15 mol/l sodium perchlorate (pH 2.2) with the ratio of 1:9. The effluent was derivatized to thiochromes in reaction coil (5 m x 0.33 mm) with 15% (w/v) K3(Fe(CN)6) and 15% (w/v) NaOH (0.4 ml/min).

Quantification was carried out by using the linear range of a standard curve constructed with known amounts of thiamine (Sigma). The calibration curve was linear (r² = 0.998) over thiamine concentrations ranging from 4 to 10-100 nM.

Assessment of total collagen contents with Sirius red staining

Total collagen content of the Sirius Red (Polyscience, Inc., Warrington, PA, USA) stained sections was assessed and quantified by digital image analysis as follows. Sirius Red stained area was assessed using computer software scion image beta 4.03 (scion corporation, USA). At least three high-power (× 400) fields, all available fields (> 30 fields) were measured, including the septum as well as the right and left ventricle.

Assessment of O-glycosylation with immunohistochemistry

For immunohistochemical studies, paraffin-embedded tissue sections (2 μm) were deparaffinized. A mouse monoclonal RL-2 antibody was used as a primary antibody (1:100; overnight at 37°C), followed by a biotinylated goat anti-mouse antibody (1:200) for streptavidin-biotin complex peroxidase staining. Detection was carried out with the use of peroxidase as label and 3,3’ dianinobenzidine as substrate. Negative controls were performed by omitting the primary antibody.

For quantification, immunoreactive area was assessed using computer software scion image beta 4.03 (scion corporation, USA). In each section 3 fields were analyzed. Immunoreactive area was calculated as the area occupied by positive area divided by the total tissue area within the same microscopical view.

Assessment of metalloprotease activity by gelatin zymography

Metalloproteinase 2 (MMP2) activity was determined in the LV tissue by zymography. LV (100 mg) tissues were homogenized in 900 μl extraction buffer (10 mmol/l cacodylic acid, 0.15 mol/l NaCl, 20 mmol/l CaCl₂, 1.5 mmol/l NaN₃, 1 μM ZnCl₂, 0.01% Triton-X, pH7.6). Total homogenates were centrifuged at 15,000 x g for 40 min at 4°C. The supernatant was mixed to sample buffer (pH6.8 Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.0025% bromphenol blue). The proteins (60 μg) were applied without boiling onto a 10% polyacrylamide gel containing 0.1% gelatin and 0.1% SDS.

After electrophoretical separation with 125V constant voltage, gels were washed in renaturing buffer (2.5% Triton-X) for 1 hr and incubated in developing buffer (50 mM Tris-HCl, 5 mM CaCl₂, 50 mM NaCl, 0.05% Brij-35, pH7.6) for 3 overnight at 37°C. Then, gels were stained in 0.1% coomasie blue R-250 for 30 min and destained in 7% acetic acid and 7% methanol in water. MMP2 activities were visualized as clear areas due to lysis of gelatin against a blue background. Quantification was performed by the size-fractionated banding pattern densitometrically by Multi gauge (Fuji Film, Japan). Gelatinase zymography standards (Chemicon international, Inc., USA & Canada) used as positive controls.

RNA preparation and real-time PCR analysis

Left ventricle was cut into 5 mm² and stored at −80°C in RNAlater (Ambion) until RNA preparation. Total RNA was extracted using RNeasy Fibrous Tissue Mini-Kit (Qiagen). The purity and concentration were determined by measuring the optical density at 260 and 280 nm before use. The optical density ratio at 260/280 ranged from 1.8 to 2.1. For reverse transcriptase reactions, 1 μg of total RNA was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Each real-time PCR (RT-PCR) reaction contained 25 ng of cDNA, 250 nM of each primer and 4 μl of LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche) in a total volume of 20 μl. All RT-PCR primers were designed using Primer Express Software (Applied Biosystems, Forster City, CA, USA) based on GenBank sequence data. Primer sequences are described in Table 1. The cDNA was denatured at 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 5 sec, and 65°C for 15 sec followed by 40°C for 30 sec. Each sample was performed in triplicate on a LightCycler and was analyzed using LightCycler3 Front software (Roche). The relative mRNA levels
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Table 1. List of primer sequences used in real-time RT-PCR analysis.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>M25297</td>
<td>Brain natriuretic peptide</td>
<td>5'-GTCAGTCGCTGGGTGGCTGT-3'</td>
<td>5'-AGAGCTGGGAAAGAAGAGC-3'</td>
</tr>
<tr>
<td>NM_022266</td>
<td>Connective tissue growth factor</td>
<td>5'-GCTGACCTAGAGGAAAAATGAAGA-3'</td>
<td>5'-CCGTTAGGCTTCACATGG-3'</td>
</tr>
<tr>
<td>NM_019143</td>
<td>Fibronectin</td>
<td>5'-CAGCCCTGATGGAGTC-3'</td>
<td>5'-TGGTGACCTGAGTGAAC-3'</td>
</tr>
<tr>
<td>M24067</td>
<td>Plasminogen activator inhibitor 1</td>
<td>5'-AGAGCCCAATCACAAAGC-3'</td>
<td>5'-AGGCAAGTGAGGCGTGAAG-3'</td>
</tr>
<tr>
<td>NM_001013062</td>
<td>Thrombospondin</td>
<td>5'-CCGGTGTGGATCGAGTGG-3'</td>
<td>5'-GGTTCGGAAGGTGCAAT-3'</td>
</tr>
<tr>
<td>NM_021578</td>
<td>Transforming growth factor, beta 1</td>
<td>5'-CTTGAAAGGGCTCAAAC-3'</td>
<td>5'-CAGTCTCTGCAGGACACT-3'</td>
</tr>
<tr>
<td>NM_031144</td>
<td>beta actin</td>
<td>5'-CCCGCGATACAACCTT-3'</td>
<td>5'-CGTCATCCATGGCAACT-3'</td>
</tr>
</tbody>
</table>

were calculated by the comparative threshold cycle method using β-actin as an internal control.

Statistical analysis

Results are expressed as means ± S.E. The significance of the differences between the groups was analyzed by Tukey-Kramer test after analysis of variance. P values less than 0.05 were regarded as statistically significant.

RESULTS

Plasma glucose levels in STZ-induced diabetic rats, and effects of thiamine repletion

Plasma glucose levels, determined by the glucose oxidase (GOD) method, were remarkably higher in STZ-administered rats compared with normal controls (164.98+/−13.61 mg/dl in control, 480.94+/−27.88 mg/dl in STZ-treatment; 544.69+/−19.76 mg/dl in STZ-treatment replete with thiamine; p < 0.01, control vs STZ-treatment and STZ-treatment replete with thiamine; 195.89+/−6.11 mg/dl in control rats replete with thiamine) (Fig. 2). Thiamine repletion did not have an effect on plasma glucose levels in all groups, as reported by Babaei-Jadidi et al. (2003).

Plasma and red blood cell thiamine levels in STZ-induced diabetic rats, and effects of thiamine repletion

STZ treatment results in prominent decreases in plasma and red blood cell thiamine concentration (plasma; 298.6+/−60.1 nM in control and 94.1+/−3.1 nM in STZ-treatment, p < 0.01; red blood cell; 0.98+/−0.07 nmol/μg protein in control and 0.49+/−0.05 nmol/μg protein in STZ-treatment, p < 0.01) (Figs. 3A and 3B), in accordance with the result of Babaei-Jadidi et al. (2003). Thiamine repletion counteracts the STZ-induced thiamine deficiency in both plasma and red blood cells (Figs. 3A and 3B). In control rats, no observable changes were noted in thiamine levels in both plasma and red blood cell.

![Fig. 2](image-url) Effect of thiamine on blood glucose levels in rats 15 days after one i.p. injection of streptozotocin (STZ, 70 mg/kg, i.p.). Each value represents the mean ± S.E. of five experiments. *p < 0.01, compared with the control.
Effect of thiamine on heart rate, blood pressure and lipids levels in STZ-induced diabetic rats

Table 2 shows mean heart rate, systolic blood pressure, plasma cholesterol and triglycerides 15 days after STZ injection. STZ-treatment did not have an effect on systolic blood pressure (104.77+/−3.50 mmHg in control and 107.57+/−5.21 mmHg in STZ-treatment), but heart rate was significantly decreased by STZ-treatment (379.40+/−5.44 bpm in control and 305.78+/−1.84 bpm in STZ-treatment, p < 0.05). Thiamine did not rescue the reduction of heart rate in STZ-treated rats (302.90+/−25.9 bpm in STZ-treatment replete with thiamine) (Table 2).

Plasma triglycerides levels were increased in STZ diabetic rats (79.05+/−10.1 mg/dl in control and 367.38+/−61.0 mg/dl in STZ-treatment, p < 0.01). Thiamine repletion significantly improved STZ-induced increases in triglycerides concentration (122.94+/−21.8 mg/dl in STZ-treatment replete with thiamine; p < 0.05, STZ-treatment vs STZ-treatment replete with thiamine). Plasma total cholesterol levels were also increased in STZ-treated rats but were not altered by the thiamine treatment (49.94+/−3.34 mg/dl in control and 98.06+/−10.0 mg/dl in STZ-treatment, p < 0.01; 92.16+/−12.7 mg/dl in STZ-treatment replete with thiamine) (Table 2).

Effect of thiamine on cardiac function in STZ-induced diabetic rats

Echocardiographic analysis revealed the decrease of fractional shortening in STZ-induced diabetic rats (52.26+/−2.33% in control and 40.29+/−1.56% in STZ-treated).
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Table 2. Heart rate, systolic blood pressure, plasma cholesterol and triglycerides concentration in each experimental group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart rate (bpm)</th>
<th>Systolic BP (mmHg)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>379.40 ± 5.44</td>
<td>104.77 ± 3.50</td>
<td>49.94 ± 3.34</td>
<td>79.05 ± 10.1</td>
</tr>
<tr>
<td>STZ-treated</td>
<td>305.78 ± 1.84*</td>
<td>107.57 ± 5.21</td>
<td>98.06 ± 10.0**</td>
<td>367.38 ± 61.0**</td>
</tr>
<tr>
<td>Thiamine-treated STZ</td>
<td>302.90 ± 25.9*</td>
<td>126.55 ± 2.47</td>
<td>92.16 ± 12.7**</td>
<td>122.94 ± 21.8*</td>
</tr>
<tr>
<td>Thiamine-treated</td>
<td>362.05 ± 16.5</td>
<td>111.60 ± 3.79</td>
<td>44.81 ± 3.38</td>
<td>80.63 ± 15.6</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. of four experiments.

**p < 0.01 and *p < 0.05, compared with the control. *p < 0.01, compared with “STZ-treated group”.

treatment, p < 0.05), which was moderately ameliorated by thiamine repletion (47.54+/−1.65% in STZ-treatment replete with thiamine), but failed to reach significance (Table 3). On the other hand, no observable changes were found in LV ejection fraction, LV end-diastolic diameter and LV end-systolic diameter (Table 3).

Effect of thiamine on mRNA abundance of brain natriuretic peptide (BNP) in STZ-induced diabetic rats LV

BNP is known to regenerate in failing heart and has been used as a marker for heart failure. Quantitative real-time RT-PCR revealed significant increase of BNP mRNA abundance in the LV of STZ-treated rats compared with those in the vehicle-treated control group (1.36+/−0.19 relative mRNA levels in control and 2.45+/−0.39 relative mRNA levels in STZ-treatment, p < 0.01), which was almost completely rescued by thiamine repletion (1.27+/−0.09 relative mRNA levels in STZ-treatment replete with thiamine; p < 0.01, STZ-treatment vs STZ-treatment replete with thiamine) (Fig. 4).

Effect of thiamine on organ weight in STZ-induced diabetic rats

Tibial lengths were comparable in each experimental group, suggesting no growth retardation in STZ-induced diabetic rats and thiamine repletion during 15 days of observation frame. The weights of whole heart, ventricle and LV normalized by tibial length were significantly small in STZ-induced diabetic rats (heart; 2.33+/−0.06 g/tibial length (10⁻²) in control and 1.80+/−0.05 g/tibial length (10⁻²) in STZ-treatment, p < 0.01; ventricular; 2.05+/−0.06 g/tibial length (10⁻²) in control and 1.61+/−0.04 g/tibial length (10⁻²) in STZ-treatment, p < 0.01; LV; 1.84+/−0.05 g/tibial length (10⁻²) in control and 1.41+/−0.03 g/tibial length (10⁻²) in STZ-treatment, p < 0.01 (Table 4), and thiamine repletion, no statistical significance but partially, rescued this atrophic change (heart; 1.97+/−0.10 g/tibial length (10⁻²); ventricular; 1.73+/−0.09 g/tibial length (10⁻²); LV; 1.55+/−0.08 g/tibial length (10⁻²) in STZ-treatment replete with thiamine) (Table 4). No observable changes were found in kidney weight of STZ-induced diabetic rats.

Effect of thiamine on cardiac fibrosis in STZ-induced diabetic rats

Fibrosis is a characteristic of diabetic cardiomyopathy. Then the degrees of cardiac fibrosis were assessed by Sirius red staining, with representative pictures shown in Fig. 5. Sirius red, stained fibrotic area, evaluated by digital image, was markedly increased in STZ-induced diabetic rats compared with control rats, which was signifi-

Table 3. Echocardiographic characteristics in each experimental group.

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEF</th>
<th>LVDd (mm)</th>
<th>LVDs (mm)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.89 ± 0.02</td>
<td>6.62 ± 0.50</td>
<td>2.92 ± 0.19</td>
<td>52.26 ± 2.33</td>
</tr>
<tr>
<td>STZ-treated</td>
<td>0.80 ± 0.02</td>
<td>6.33 ± 0.23</td>
<td>3.73 ± 0.20</td>
<td>40.29 ± 1.56*</td>
</tr>
<tr>
<td>Thiamine-treated STZ</td>
<td>0.86 ± 0.01</td>
<td>6.65 ± 0.19</td>
<td>3.06 ± 0.22</td>
<td>47.54 ± 1.65</td>
</tr>
<tr>
<td>Thiamine-treated</td>
<td>0.86 ± 0.01</td>
<td>6.73 ± 0.49</td>
<td>3.48 ± 0.31</td>
<td>48.38 ± 1.52</td>
</tr>
</tbody>
</table>

LVEF indicates left ventricular ejection fraction, LVDd left ventricular end-diastolic diameter, LVDs left ventricular end-systolic diameter, FS Fractional shortening. Each value represents the mean ± S.E. of four experiments. *p < 0.05, compared with the control.
Fig. 4. Effect of thiamine on STZ-induced the increases in mRNA expressions of BNP from the heart left ventricular of diabetic rats. Relative levels of mRNA expression determined by real-time RT-PCR. Expression values were normalized based on the level of b-actin. Each value represents the mean ± S.E. of five experiments. *p < 0.05, compared with the control. "p < 0.01, compared with “STZ-treated group”.

Table 4. Organ weight in each experimental group. Each value represents the mean ± S.E. of five experiments.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tibial length (mm)</th>
<th>Heart (g)</th>
<th>Ventricular (g)</th>
<th>Left ventricular (g)</th>
<th>Kidney (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.6 ± 0.29</td>
<td>2.33 ± 0.06</td>
<td>2.05 ± 0.06</td>
<td>1.84 ± 0.05</td>
<td>3.32 ± 0.08</td>
</tr>
<tr>
<td>STZ-treated</td>
<td>37.63 ± 0.55</td>
<td>1.80 ± 0.05**</td>
<td>1.61 ± 0.04**</td>
<td>1.41 ± 0.03**</td>
<td>3.56 ± 0.09</td>
</tr>
<tr>
<td>Thiamine-treated STZ</td>
<td>36.04 ± 0.25</td>
<td>1.97 ± 0.10*</td>
<td>1.73 ± 0.09*</td>
<td>1.55 ± 0.08*</td>
<td>3.69 ± 0.09</td>
</tr>
<tr>
<td>Thiamine-treated</td>
<td>36.12 ± 0.56</td>
<td>2.36 ± 0.05</td>
<td>2.04 ± 0.05</td>
<td>1.79 ± 0.05</td>
<td>3.11 ± 0.08</td>
</tr>
</tbody>
</table>

**p < 0.01 and *p < 0.05, compared with the control.

Significantly prevented by thiamine repletion (0.8+/−0.3% in control, 4.6+/−0.8% in STZ-treatment, 1.0+/−0.008% in STZ-treatment replete with thiamine; p < 0.01, control vs STZ-treatment; p < 0.01, STZ-treatment vs STZ-treatment replete with thiamine) (Fig. 5E).

Effect of thiamine on mRNA abundance relevant to pro-fibrosis in LV of STZ-induced diabetic rats

Significant fibrosis in STZ-induced diabetic rats demonstrated by Sirius red staining motivated us to evaluate mRANs related to pro-fibrosis, i.e., TGF-β, thrombospondin, fibronectin, PAI-1 and connective tissue growth factor (CTGF). Fig. 6 depicts the abundance of TGF-β, thrombospondin, fibronectin, PAI-1 and CTGF. Thrombospondin, fibronectin, PAI-1 and CTGF mRNA abundance were significantly elevated in the LV of STZ-induced diabetic rats (thrombospondin; 1.31+/−0.15 in control and 3.10+/−0.73 in STZ-treatment, p < 0.01; fibronectin; 1.00+/−0.07 in control and 1.90+/−0.12 in STZ-treatment, p < 0.01; PAI-1; 1.17+/−0.08 in control and 5.50+/−0.26 in STZ-treatment, p < 0.01; CTGF; 1.40+/−0.16 relative mRNA levels in control and 3.29+/−0.39 relative mRNA levels in STZ-treatment, p < 0.01), which were significantly suppressed by thiamine repletion (thrombospondin; 1.42+/−0.13, p < 0.05; fibronectin; 1.32+/−0.11, p < 0.01; PAI-1; 3.20+/−0.25 relative mRNA levels in STZ-treatment replete with thiamine, p < 0.01; STZ-treatment vs STZ-treatment replete with thiamine) (Fig. 6), except for CTGF. CTGF mRNA abundance in the LV of STZ-induced diabetic rats tended to decrease by thiamine administration, but failed to reach significance. There was no significant difference in the expression levels of TGF β mRNA in each group.

Effect of thiamine on MMP2 activity in LV of STZ-induce diabetic rats

Collagen deposition was regulated by synthesis and degradation of collagen. We next examined whether MMP2 played a role in cardiac fibrosis demonstrated in STZ-induced diabetic rats by gelatin zymography. The
Thiamine improves diabetic cardiomyopathy by suppressing HBP

![Thiamine improves diabetic cardiomyopathy by suppressing HBP](image)

**Fig. 5.** Effect of thiamine on STZ-induced cardiac fibrosis in LV. Sirius red stained sections from heart LV in control rats (A), thiamine-treated control rats (B), STZ-treated rats (C), thiamine-treated STZ rats (D). Scale bar, 45 mm. Graphic representation of the fibrosis positive area in the LV (E). Each value represents the mean ± S.E. of four experiments. *p < 0.01, compared with the control. *p < 0.01, compared with “STZ-treated group”.

latent type and active type of MMP2 were decreased in the STZ-treated group, which were significantly reserved by thiamine repletion (Fig. 7).

**Protein O-glycosylation (RL-2) in LV of STZ-induced diabetic rats and effect of thiamine repletion**

The activation of the HBP is known to contribute to the mechanisms to the diabetes-induced cellular damages. Sp1, one of transcriptional factors, can be O-GlcNAcylated. O-GlcNAcylation of Sp1 increased Sp1 transactivation and Sp1-dependent expression of both TGF-β1 and PAI-1 (Du et al., 2000; Verrecchia et al., 2001).

Immunoreactivity was observed in cytosol and nucleus with the RL-2 antibody, which is consistent with previous reports that nuclear and cytosolic proteins are glycosylated by O-GlcNAc transferase (Kreppel et al., 1997; Lubas et al., 1997). RL-2-immunoreactivity was enhanced in the hearts of STZ-induced diabetic rats compared with that of control rats, representative pictures shown in Figs. 8A-8D. The area reacted with RL-2 antibody was significant-
ly higher in rats with STZ-induced diabetes compared to control rats (3.22±0.20% in control and 9.10±0.37% in STZ-treatment; p < 0.01, control vs STZ-treatment) (Fig. 8F). Thiamine treatment prevented the increases of immunoreactivity in STZ-induced diabetic rats (4.46±0.12% in STZ-treatment replete with thiamine; p < 0.01, STZ-treatment vs STZ-treatment replete with thiamine) (Fig. 8F).

DISCUSSION

STZ treatment produced significant increase in plasma glucose and triglyceride levels, and moderate but significant increase in total cholesterol levels, indicating a derangement in the glucose and lipid metabolism. In addition, thiamine level was significantly reduced in plasma and red blood cells, supporting the concept that diabetes might be considered a thiamine-deficient state (Beltramo et al., 2008). Thiamine treatment resulted in significant reduction of triglyceride levels. On the other hand any significant effect on plasma glucose levels was elicited by thiamine treatment as reported by others (Babaei-Jadidi et al., 2003; Ceylan-Isik et al., 2006; Wu and Ren, 2006).

Dysfunction of both contraction and relaxation is regarded as a trait of diabetic cardiomyopathy (Hofmann et al., 1995; Ren and Ceylan-Isik, 2004; Wold et al., 2005). In our study hemodynamic parameters evaluated with echocardiography revealed only marginal but significant reduction of fractional shortening. Nevertheless, biochemical and morphological analyses demonstrated increased BNP, a biochemical surrogate index for heart failure, mRNA abundance and increased collagen deposit in LV tissue. Several mechanisms have been postulated for the mechanical dysfunction in diabetic hearts. Evidence from experimental models of diabetic cardiomyopathy has demonstrated cardiac fibrosis as pathological substrate of diabetic cardiomyopathy (Riva et al., 1998; Tschöpe et al., 2004). Cardiac fibrosis orients to increased LV stiffness and decreased ventricular wall compliance, resulting in systolic and in particular diastolic dysfunction. Thiamine repletion countered these biochemical and morphological disorders produced by STZ diabetes; the reduction of BNP mRNA abundance and reduction of collagen deposit in LV.

Diabetic subjects are predisposed to have lower blood thiamine concentrations than healthy controls, accompanying a reduced erythrocyte transketolase activity (Saito et al., 1987; Valerio et al., 1999; Jermendy, 2006). Thiamine deficiency in diabetic subjects, usually do not recognize due to lack of typical clinical features, is speculated to be bring out by (1) an increased thiamine renal clearance (Thorvalley et al., 2007), (2) a decreased intestinal absorption and membrane transport of thiamine (Patrini et al., 1996), and (3) an increased consumption of thiamine caused by an augmented glucose metabolism (Yui et al., 1980; Seligmann et al., 1991). Additionally, high production of reactive oxygen species (ROS) in disease mellitus could oxidize thiamine, with the production of inactive compounds, such as thiochrome and oxydihydrothiochrome (Stepuro et al., 1997; Obrenovich and Monnier, 2003).

Recently, beneficial effects of high-dose thiamine have been demonstrated in experimental models of diabetic complications, i.e., retinopathy, nephropathy and cardiomyopathy (Babaei-Jadidi et al., 2003; Hammes et al., 2003; Ceylan-Isik et al., 2006). Actually high-dose thia-
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Fig. 7. Effect of thiamine on matrix metalloproteinase (MMP) expressions in the left ventricular of STZ diabetic rats. The latent type and active type of MMP2 bands were zymography using gelatin containing polyacrylamide gel for MMP activity (A) and were quantitated by densitometric scanning (B). Each value represents the mean ± S.E. of five experiments. *p < 0.01, compared with the control. #p < 0.01, compared with “STZ-treated group”.

As thiamine therapy is accepted for diabetic peripheral neuropathy in Europe. Water-soluble thiamine is not stored in the body, but rapidly excreted in the urine and must be replenished every 5-6 hr (Greb and Bitsch, 1998). Although the intestinal thiamine transporter is saturated by relatively low doses of thiamine, there is slow passive diffusion of thiamine at high concentrations (Rindi and Lafarenza, 2000). Accordingly, we reasoned that freely access of a water containing thiamine could be effective to counteract diabetic complications.

The clear-cut mechanisms underlying the pathophysiology of diabetic complications, including cardiomyopathy, remain elusive. Under the high ambient glucose condition, glucose enters into cells via insulin-independent glucose transporter, GLUT1 (Mueckler, 1994). Endothelial cells and pericytes are unable to regulate glucose transport, reaching high levels of intracellular glucose concentrations (Kaiser et al., 1993), in the presence of hyperglycemia. This is probably a rationale of vascular damages generally observed in diabetes mellitus. Increased glucose entry, resulting from hyperglycemia, could exceed the capacity of glucose oxidation, a major pathway of glucose metabolism, and then could orient to a minor pathway of glucose metabolism. Hence, activations of minor pathways have been proposed to potentially contribute to the development of diabetic complications, i.e., activation of DAG pathway with subsequent activation of PKC, activation of the polyol pathway, formation of AGE, increased production of ROS, and activation of the HBP (Du et al., 2000; Brownlee, 2001). Among these contributors, DAG pathway and HBP potentially impinge on gene expressions via posttranslational modification of various transcriptional factors, i.e., phosphorylation and O-glycosylation.

STZ treatment brought on a substantial cardiac fibrosis, accompanied with the increased expression of pro-fibrot-
ic gene and the reduction of collagen degradation relevant MMP2 activity. Posttranslational modification might be accountable for the STZ induced by these phenotype changes. Increased triacylglycerol, a product of DAG pathway, and enhanced immunoreactivity against O-glycosylated proteins, demonstrated in this study, might suggest the activation of DAG pathway and HBP.

Well-established modulation of gene expression is the phosphorylation of transcriptional factors by PKC, which could be, at least partially, accountable for the abundance of mRNA expression shown in this study, i.e., abundance of pro-fibrotic relevant genes including thrombospondin, fibronectin, PAI-1 and CTGF. Although we did not find the change in expression level of TGF-β mRNA, it has been reported that TGF-β activity is enhanced by thrombospondin (Daniel et al., 2004, 2007).

Alternatively, protein O-glycosylation modification could be responsible for the phenotype changes in STZ-induced cardiac fibrosis (Hu et al., 2005). O-GlcNAcylation, which is defined as the covalent attachment of a single O-GlcNAc molecule to serine or threonine residues, is a posttranslational modification of intracellular proteins. This modification affects transcription, translation, nuclear transport, and cell signaling (Comer and Hart, 1999; Yang et al., 2001; Vosseller et al., 2002; Walgren et al., 2003; Wells and Hart, 2003; Wells et al., 2003; Kneass and Marchase, 2005). Similar to phosphorylation, O-GlcNAc addition by O-linked GlcNAc transferase (OGT), or its removal by N-acetylglucosaminidase (O-GlcNAcase, GCA) is a highly dynamic and reversible process that is susceptible to perturbation under certain pathophysiological circumstances (Wells et al., 2002). Sp1, one of transcriptional factors, can be O-GlcNAcylated. O-GlcNAcylation of Sp1 increased Sp1 transactivation and Sp1-dependent expression of both TGF-β1 and PAI-1 (Du et al., 2000; Verrecchia et al., 2001).

Thiamine repletion counteracted the STZ-induced cardiac phenotype. Thiamine acts as an indispensable coenzyme for transketolase and for the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, enzymes which play a fundamental role for intracellular glucose metabolism. Hammes et al. (2003) reported that the lipid-soluble thiamine derivative benfotiamine can inhibit the HBP, the AGE formation pathway and the DAG-PKC pathway, as well as hyperglycemia-associated NF-κB activation, by activating the pentose phosphate pathway enzyme transketolase. In this study, unfortunately, we did not address the enzymatic activities involved in glucose metabolism; nevertheless we believe that thiamine repletion re-orientates the glucose metabolism from the minor pathway to the major pathway. Interestingly,
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The counteraction of thiamine against STZ-induced cardiac fibrosis was achieved without the change of plasma glucose concentration, as shown in our study and other investigators (Babaei-Jadidi et al., 2003; Ceylan-Isik et al., 2006; Wu and Ren, 2006). In the therapy of diabetic complications, it is necessary to control not only plasma glucose concentration but also, we believe, intracellular glucose metabolism.

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