High-Dose Benfotiamine Rescues Cardiomyocyte Contractile Dysfunction in Streptozotocin-Induced Diabetes Mellitus

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Running Title: Benfotiamine and diabetic cardiomyopathy

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Diabetic cardiomyopathy is characterized by cardiac dysfunction. This study was designed to examine the effect of benfotiamine, a lipophilic derivative of thiamine, on streptozotocin (STZ)-induced cardiac contractile dysfunction in mouse cardiomyocytes. Adult male FVB mice were made diabetic with a single injection of STZ (200 mg/kg, i.p.). Fourteen days later, control and diabetic (fasting plasma glucose > 13.9 mM) mice were put on benfotiamine therapy (100 mg/kg/d, i.p.) for another 14 days. Mechanical and intracellular Ca\(^{2+}\) properties were evaluated in left ventricular myocytes using an IonOptix MyoCam system. The following indices were evaluated: peak shortening (PS), time-to-PS (TPS), time-to-90\% relengthening (TR\(_{90}\)), maximal velocity of shortening/relengthening (± dL/dt), resting and rise of intracellular Ca\(^{2+}\) in response to electrical stimulus, sarcoplasmic reticulum (SR) Ca\(^{2+}\) load and intracellular Ca\(^{2+}\) decay rate (tau). Two or 4 weeks STZ treatment led to hyperglycemia, prolonged TPS and TR\(_{90}\), reduced SR Ca\(^{2+}\)-load, elevated resting intracellular Ca\(^{2+}\) level and prolonged tau associated with normal PS, ± dL/dt and intracellular Ca\(^{2+}\) rise in response to electrical stimulus. Benfotiamine treatment abolished prolongation in TPS, TR\(_{90}\) and tau as well as reduction in SR Ca\(^{2+}\)-load without affecting hyperglycemia and elevated resting intracellular Ca\(^{2+}\). Diabetes triggered oxidative stress, measured by GSH/GSSG ratio and formation of advanced glycation end-product (AGE) in the hearts. Benfotiamine treatment alleviated oxidative stress without affecting AGE or protein carbonyl formation. Collectively, our results indicated benfotiamine may rescue STZ-induced cardiomyocyte dysfunction but unlikely AGE formation in short-term diabetes.

**Key words:** benfotiamine, diabetes, ventricular myocyte, contraction, AGE.
INTRODUCTION

Diabetes mellitus leads to high cardiovascular morbidity and mortality as a result of functional and morphological damage in diabetic hearts (20; 21). A unique myopathic alteration, namely diabetic cardiomyopathy, has been established independent of macro- or micro-vascular diseases commonly seen in diabetes (20). The most prominent defects of diabetic cardiomyopathy are prolonged duration of contraction and relaxation, as well as reduced cardiac compliance (19-21). The etiology of diabetic cardiomyopathy is rather complex and involves metabolic derangements, depressed autonomic function and abnormalities in certain hormones or proteins which regulate intracellular ion homeostasis, particularly Ca$^{2+}$ (8; 16; 20). The high mortality of diabetic heart complications warrants stringent and aggressive treatment against hyperglycemia, hyperinsulinemia, dyslipidemia and oxidant damage. Classical therapeutic agents against diabetic heart diseases include angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor antagonists, digoxin, diuretics, β-blockers, Ca$^{2+}$ antagonists and spironolactone. Both enzymatic and non-enzymatic antioxidants have been proven to be effective against diabetic heart complications (15; 24; 29). Insulin-sensitizing or lipid reducing agents such as thiazolidinediones and peroxisome proliferators-activated receptors (PPARs) agonists have also shown promises for various types of diabetic complications (18). In addition to pharmacological interventions, lifestyle changes such as smoking cessation, weight control, exercise and dietary restriction have all been included in the primary care for diabetes mellitus (14). Nevertheless, none of these therapeutic strategies may be considered the panacea for optimal and ultimate management of high morbidity and mortality of diabetic heart complications.

Recent evidence has indicated that treatment of diabetic rats with high doses of thiamine (vitamin B1) prevents diabetic retinopathy through inhibition of advanced glycation end-product
(AGE) formation and other signaling or metabolic pathways (10). AGE accumulation occurs under hyperglycemic and diabetic environments where elevated oxidative stress is usually present (10). AGE binds to its cell-surface receptor, RAGE, resulting in the activation of post-receptor signaling, generation of intracellular reactive oxygen species and alteration of gene expression (13; 23). The finding that thiamine interrupts AGE formation (10) may be related to a so-called "AGE-breaker" effect; that is, the ability to cleave dicarbonyl bonds formed during advanced glycation (26). The clinical potential of thiamine in diabetic therapeutics is consistent with the notion that this vitamin may be depleted as a result of diabetes-induced oxidative stress or glucose autoxidation where thiamine is oxidized into the biologically nonfunctional products thiochrome and oxodihydrothiochrome (17). Deficiencies in B series vitamins and folic acid are among the key causative factors leading to diabetic organ damage, consistent with the high incidence of idiopathic dilated cardiomyopathy in patients with vitamin-deficiency (25). Group B vitamins, which may be acquired through dietary intake, are important water-soluble vitamins essential for DNA synthesis and repair. To further examine the impact of thiamine supplementation on cardiomyocyte function in diabetes, type 1 experimental diabetes was induced with a single injection of streptozotocin (STZ) in FVB albino mice. Both control and diabetic mice received 14-day oral treatment of benfotiamine, a lipophilic derivative of thiamine. State-of-the-art cell physiological techniques were employed to examine the mechanical and intracellular Ca\(^{2+}\) properties of isolated ventricular myocytes.
MATERIALS AND METHODS

Experimental Animals and Benfotiamine Therapy

The procedures described here were approved by the Institutional Animal Care and Use Committee of University of Wyoming (Laramie, WY) and were in accordance with NIH animal care standards. Eight week-old male FVB albino mice (16 – 20 g) were injected with a single dose of streptozotocin (STZ, 200 mg/kg., i.p. in 0.01 M citrate buffer with a pH of 4.3) (9). Non-diabetic control mice (weight-matched) received citrate buffer only. Fasting plasma glucose was examined after 3 and 14 days of STZ injection and diabetes was confirmed by fasting plasma glucose value of 13.9 mM or higher using ACCU-CHEK Advantage Glucometer (Boehringer Mannheim Diagnostics, Indianapolis, IN). On day 15 of STZ or citrate injection, both diabetic and nondiabetic control mice were randomly divided into two experimental groups with only one group being gavaged with benfotiamine (100 mg/kg/d) for two weeks. Selection of the benfotiamine dose (100 mg/kg/d) and duration of treatment were chosen based on previously published data for the thiamine derivative (2; 3; 10). Mice were maintained on a 12/12- light/dark cycle and were allowed access to food and water ad libitum. A cohort of confirmed diabetic mice (without benfotiamine treatment) was sacrificed on day 15 to evaluate cardiomyocyte function. All other mice were sacrificed four weeks after STZ or citrate injection. Plasma glucose levels were measured again at the time of sacrifice and were included in table 1.

Ventricular Myocytes Isolation Procedures

Hearts were rapidly removed from anesthetized mice and immediately mounted on a temperature-controlled (37°C) Langendorff perfusion system. After perfusion with modified Tyrode solution (Ca²⁺ free) for 2 min, the heart was digested for 10 min with 0.9 mg/ml collagenase D
(Boehringer Mannheim Biochemicals, Indianapolis, IN) in modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, MgCl₂ 1.0, HEPES 10, NaH₂PO₄ 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO₂-95% O₂. The digested heart was then removed from the cannula and the left ventricle was cut into small pieces in the modified Tyrode solution. These pieces were gently agitated and the pellet of cells was resuspended in modified Tyrode solution and allowed to settle for another 20 min at room temperature during which time extracellular Ca²⁺ was added incrementally back to 1.20 mM. Isolated myocytes were used for experiments between 1 and 8 hours after isolation. Only rod-shaped myocytes with clear edges were selected for recording of mechanical properties and intracellular Ca²⁺ transients as described (8).

**Cell Shortening/Relengthening**

Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA) (8). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70, Olympus Optical Corporation, Tokyo, Japan) and superfused (~1 ml/min at 25°C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz, 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (FHC Incorporation, Bowdoinham, ME). The polarity of stimulating electrodes was reversed frequently to avoid build up of electrolyte by-products. The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera, which rapidly scans the image every 8.3 msec. A SoftEdge software (IonOptix) was used to capture changes in cell length during shortening and relengthening.
The sub-physiological stimulus frequency (0.5 Hz) was chosen to best exemplify contractile properties including peak shortening (PS) amplitude, maximal velocity of shortening/relengethening (± dL/dt), time-to-peak shortening (TPS) and time-to-90% relengthening (TR90). Higher stimulus frequencies (> 1.0 Hz) cannot provide recording of TPS and TR90 with an excellent fidelity limited by the 8.3 msec time interval between two adjacent data points.

**Intracellular Fluorescence Measurement and Sarcoplasmic Reticulum Ca\(^{2+}\) Load**

A separate cohort of murine myocytes was loaded with fura-2/AM (0.5 µM) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix) as described (8). Myocytes were placed on an Olympus IX-70 inverted microscope (Olympus Incorporation, Tokyo, Japan) and imaged through a Fluor x 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter (bandwidths were ± 15 nm), while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480-520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_i) were inferred from the ratio of the fluorescence intensity at two wavelengths. SR Ca\(^{2+}\) loading capacity was assessed by rapid puff of caffeine (10 mM)-induced intracellular Ca\(^{2+}\) transient intensity in fura-2-loaded ventricular myocytes. Caffeine triggers release of Ca\(^{2+}\) from SR, the major pool of Ca\(^{2+}\) available to contractile proteins in rodent cardiac muscle. Multiple applications of caffeine were given at 5 min intervals to ensure steady state (11).
**Protein Carbonyl Assay**

To assess cardiac oxidative protein damage, the carbonyl content of protein was extracted from mitochondria and was lysed to prevent proteolytic degradation. Nucleic acids were eliminated by treating the samples with 1% streptomycin sulfate for 15 min, followed by a 10 min centrifugation (11,000 x g). Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) to protein (0.5 mg) and centrifuged for 1 min. The TCA solution was removed and the sample resuspended in 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) solution. Samples were incubated at room temperature for 15-30 min. 500 µl of 20% TCA was added and samples centrifuged for 3 min. The resultant supernatant was discarded, the pellet was washed in ethanol:ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min and the ethanol:ethyl acetate steps were repeated twice more. The precipitate was resuspended in 6 M guanidine solution, centrifuged for 3 min and insoluble debris was removed. The maximum absorbance (360-390 nm) was read against blank (2 M HCl) and the carbonyl content was calculated using the molar absorption coefficient of 22,000 M⁻¹ cm⁻¹ (11).

**Glutathione and glutathione disulfide (GSH/GSSG) assay**

Glutathione levels were determined and the ratio of GSH/GSSG was used as an indicator for oxidative stress. In brief, samples were homogenized in 4 volumes (w/v) of 1% picric acid. Acid homogenates were centrifuged at 16,000 x g (30 min) and supernatant fractions collected. Supernatant fractions were assayed for total GSH and GSSG by the standard recycling method. The procedure consisted of using one-half of each sample for GSSG determination and the other half for GSH. Samples for GSSG determination were incubated at room temperature with 2 µl of
4-vinyl pyridine (4-VP) per 100 µl sample for 1 hr after vigorous vortexing. Incubation with 4-VP conjugates any GSH present in the sample so that only GSSG is recycled to GSH without interference by GSH. The GSSG (as GSHx2) was then subtracted from the total GSH to determine actual GSH level and GSH/GSSG ratio (22).

**Western Blot Analysis of AGE and Phospholamban**

Tissue samples from ventricles were removed and homogenized in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Samples were then sonicated for 15 sec and centrifuged at 12,000x g for 20 min at 4°C. The protein concentration of the supernatant was evaluated using Protein Assay Reagent (Bio-Rad, Hercules, CA). Equal amounts (50 µg protein/lane) of the protein from the tissue extraction, or prestained molecular weight markers (Gibco-BRL, Gaithersburg, MD) were separated on 10% or 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad); then were transferred electrophoretically to Nitrocellulose membranes (0.2 µm pore size, Bio-Rad Laboratories, Inc, Hercules, CA). Membranes were incubated for 1 hr in a blocking solution containing 5% milk in Tris-buffered saline (TBS), then membranes were washed briefly in TBS and incubated overnight at 4°C with mouse anti-AGE monoclonal (1:1000) (Trans Genic Inc., Japan) and mouse anti-phospholamban monoclonal (1:2000, provided by Dr. Steven Cala from Wayne State University, Detroit, MI) antibodies. After washing blots to remove excessive primary antibody binding, blots were incubated for 1 hr with horseradish peroxidase (HRP)–conjugated secondary antibody (1:5,000). The membrane was then exposed to 2 ml of a mixture of luminol plus hydrogen peroxide under alkaline conditions (SuperSignal® West Dura Extended Duration Substrate, Pierce, Rockford,
IL) for 1 min, and the resulting chemiluminescent reaction was detected by Kodak X-OMAT AR Film (Eastman Kodak, Rochester, NY) (8).

**Statistical Analyses**

An average of 5-7 mice was used per group (control and diabetic with or without benfotiamine) for mechanical and intracellular Ca\(^{2+}\) recordings. For each experimental series, data were presented as Mean ± SEM. Statistical significance (p < 0.05) for each variable was estimated by analysis of variance (ANOVA) using Dunnett’s test as the *post hoc* analysis.
RESULTS

General Features of Experimental Animals

Four weeks of STZ treatment significantly increased fasting plasma glucose levels and reduced body weight gain. The absolute organ (heart, liver and kidney) weight or organ-to-body weight ratio was comparable between control and STZ-induced diabetic groups. Two weeks of benfotiamine treatment (100 mg/kg/d) did not elicit any significant effect on fasting plasma glucose levels, body, organ weight or organ-to-body weight ratio in either control or STZ diabetic mouse group (Table 1). Two weeks of STZ treatment produced biometric change similar to those found in 4 weeks STZ mice when compared with their age-matched non-diabetic controls (data not shown).

Cell Shortening and Relengthening Properties of Myocytes

There was no significant difference in resting cell length of ventricular myocytes from control, 2-week STZ or 4-week STZ diabetic mice with or without benfotiamine treatment. Peak shortening (PS) amplitude normalized to resting cell length was similar in ventricular myocytes from all five mouse groups examined. Myocytes from diabetic mice (either 2 or 4 weeks of STZ treatment) demonstrated significantly prolonged time-to-peak shortening (TPS) and time-to-90%-relengthening (TR90) compared to those from control groups, consistent with our previous findings (8; 29). Interestingly, 2 weeks of benfotiamine treatment completely blunted diabetes-induced prolongation of TPS and TR90. Neither the maximal velocity of shortening (+ dL/dt) nor relengthening (- dL/dt) was significantly affected by STZ (2 or 4 weeks treatment) or benfotiamine treatment (Fig. 1).

Intracellular Ca^{2+} Transient Properties and SR Ca^{2+} Load
We used the membrane permeant form of fura-2/AM to evaluate the properties of intracellular Ca\(^{2+}\) transients in cardiomyocytes from control, 2-week STZ or 4-week STZ diabetic mice. The time course of fluorescence signal decay was fitted to a single exponential equation, and the time constant (tau) was calculated as a measure of intracellular Ca\(^{2+}\) clearing rate. The fluorescence measurements revealed that both 2 and 4 weeks STZ-induced diabetes significantly elevated resting intracellular Ca\(^{2+}\) level, reduced SR Ca\(^{2+}\) release and prolonged intracellular Ca\(^{2+}\) decay rate without affecting rise of intracellular Ca\(^{2+}\) in response to electrical stimulus. Benfotiamine treatment restored diabetes-induced reduction in SR Ca\(^{2+}\) release and intracellular Ca\(^{2+}\) clearing rate without affecting resting intracellular Ca\(^{2+}\) level. Last but not the least, benfotiamine itself suppressed rise of intracellular Ca\(^{2+}\) in response to electrical stimulus in control myocytes (Fig. 2).

**Effect of Benfotiamine on Oxidative Stress, Protein Carbonyl Formation and AGE Accumulation in Control and Diabetic Mouse Hearts**

Diabetes is often associated with enhanced oxidative stress leading to irreversible damage of membrane proteins or lipids (27). The glutathione (GSH) and glutathione disulfide (GSSG) levels are commonly used markers for oxidative stress. A low GSH/GSSG ratio suggests increased oxidative stress. Result in Fig. 3 indicates that 4 weeks STZ treatment-induced diabetes displayed significantly lowered GSH/GSSG ratio, suggesting enhanced cardiac oxidative stress. Protein carbonyl formation, an indicative of protein damage, was not altered by 4 weeks STZ-induced diabetes or benfotiamine. Two weeks STZ treatment also lowered GSH/GSSG ratio without affecting protein carbonyl formation, similar to those elicited by 4 weeks STZ treatment (data not shown). Since AGE accumulation may serve as a main source for oxidative stress (13;
23), the levels of AGE was evaluated by immunoblot. In agreement with elevated oxidative stress in STZ-induced diabetic group, the level of AGE formation was increased significantly in 4 weeks STZ treatment-induced diabetic hearts. However, benfotiamine treatment failed to alter AGE accumulation in either control or 4 weeks STZ treatment-induced diabetic group (Fig. 4). We also evaluated the effect of benfotiamine on expression of phospholamban, a key intracellular Ca^{2+} regulatory protein. Our data shown in Fig. 4 indicated that expression of phospholamban was not affected by 4 weeks STZ treatment. Benfotiamine treatment itself did not affect expression of phospholamban in control mice although it significantly attenuated expression of the cardiac regulatory protein under diabetes (Fig. 4). Two weeks of STZ elicited similar influence on AGE accumulation or phospholamban expression, comparable to those found after 4 weeks STZ treatment (data not shown).
DISCUSSION

Our study reported for the first time beneficial effects of the lipophilic derivative of thiamine - benfotiamine on diabetic cardiac dysfunction. In addition, we found that benfotiamine-elicited beneficial effects for diabetic heart dysfunction may be related to, at least in part, reduced oxidative stress but less likely AGE accumulation in diabetic hearts. Our results did not favor any major role of cardiac protein damage or Ca\(^{2+}\) regulatory protein phospholamban in benfotiamine-exerted beneficial effects in diabetes-associated cardiac dysfunction.

Prolonged duration of both contraction and relaxation duration is considered a hallmark of diabetic cardiomyopathy (12; 20; 27). Somewhat similar to our earlier observations using chemically-induced or genetic models of diabetes (8; 19; 21), our present study demonstrated prolonged duration of shortening (TPS) and relengthening (TR\(_{90}\)) associated with normal peak shortening (PS) amplitude and maximal rate of shortening and relengthening (± dL/dt) after only two weeks of diabetes induction by STZ. These mechanical dysfunctions, which persist through 4 weeks of STZ treatment seen in our study, are consistent with our findings of impaired intracellular Ca\(^{2+}\) homeostasis shown as reduced intracellular Ca\(^{2+}\) clearing rate and SR Ca\(^{2+}\) load in diabetic cardiomyocytes. These mechanical defects may be reversed with a 2-week treatment of benfotiamine while the thiamine derivative itself exerted little effect on cardiac mechanics in control cardiomyocytes. Several mechanisms have been postulated for the mechanical and intracellular Ca\(^{2+}\) defects in diabetic hearts. Diabetes has been demonstrated to compromise myofilament Ca\(^{2+}\) sensitivity (12; 19) and function of SR Ca\(^{2+}\)-ATPase (SERCA), phospholamban or Na\(^+\)/Ca\(^{2+}\) exchange (6; 8). Although the mechanism(s) behind the reduced rise in intracellular Ca\(^{2+}\) in response to electrical stimuli in control mouse group (Fig. 2B) is unknown, it may be speculated that benfotiamine is capable of promoting myofilament Ca\(^{2+}\) responsiveness since it
maintains peak myocyte shortening amplitude despite of reduced intracellular Ca\textsuperscript{2+} rise in control myocytes (Fig. 2B). Although short-term STZ treatment has been shown to transiently up- or down-regulate certain intracellular Ca\textsuperscript{2+} regulatory proteins to either diminish or reconcile cardiac contractile function in diabetes (7), our data failed to observe any effect of either diabetes or benfotiamine treatment on phospholamban expression (although the reason behind benfotiamine-induced reduction of phospholamban expression in diabetic hearts is unclear at the present time). Nevertheless, involvement of other Ca\textsuperscript{2+} regulatory proteins in the beneficial effects of benfotiamine should not be excluded at this time.

Our present study revealed that benfotiamine treatment counters diabetes-induced cardiac mechanical dysfunction at cellular level associated with reduction in oxidative stress but unlikely AGE formation or cardiac protein carbonyl formation. This apparent discrepancy in benfotiamine-elicited action on AGE formation and oxidative stress (GSH/GSSG ratio), seems to indicate that other mechanism(s) may predominantly contribute to diabetes-induced oxidative stress and cardiac contractile dysfunction in current experimental setting. Possible candidates may include alteration in glucose metabolism and protein kinase C activation (5; 20) although further study is warranted to verify involvement of these signaling pathways and beneficial effects of benfotiamine against diabetic cardiomyopathy. In addition, benfotiamine may directly participate in glucose metabolic regulation. Thiamine and benfotiamine were demonstrated to activate the pentose phosphate pathway enzyme transketolase, facilitating conversion of glyceraldehyde-3-phosphate and fructose-6-phosphate into pentose-5-phosphates (10; 17). Finally, the observation that benfotiamine treatment failed to produce any overt cardiac hypertrophic response should indicate that ventricular remodeling is unlikely a concern for the clinical application of benfotiamine. It is noteworthy that we failed to observe cardiac...
hypertrophy following STZ treatment in our current study. Although STZ has been reported to induce cardiac hypertrophy and does so largely through genomic adaptive or maladaptive process (7), the period of diabetes employed in our present study (4 weeks) may not be long enough to induce such cardiac remodeling process.

**Experimental limitations:** Our study employed a short-term (2 to 4 weeks) STZ-induced type 1 diabetic model, this diabetic model may not represent the most prevalent chronic type 2 diabetes. Further work using *ob/ob* and *db/db* type 2 diabetic models is warranted to provide convincing evidence regarding the therapeutic effectiveness of benfotiamine against diabetic cardiomyopathy. In addition, the potential direct cardiac toxicity of STZ (28) may jeopardize the understanding of therapeutic value of the thiamine derivative in diabetic heart complications. Our current short-term diabetic setting may obscure long-term or delayed effect of benfotiamine on cardiac AGE accumulation. Longitudinal effect of benfotiamine on diabetic complications should markedly increase our understanding of the therapeutic potential of this thiamine derivative. Therefore no precise and conclusive statement should be drawn at this point regarding the role of AGE accumulation in benfotiamine treatment regimen. Finally, mechanical and intracellular Ca^{2+} properties were measured in isolated ventricular myocytes while others (protein carbonyl formation, GSH/GSSG and immunoblots) were assessed in ventricular tissue. Inclusion of endothelial cells and fibroblasts in ventricular tissues may make it rather difficult to compare data obtained from tissues with those from isolated cardiomyocytes.

In summary, our findings revealed that benfotiamine treatment antagonizes impaired cardiomyocyte contractile function in STZ-induced diabetic mouse hearts, associated with reduced oxidative stress but is unlikely dependent upon AGE formation or cardiac protein damage. Given what we know about the ability of benfotiamine, thiamine and other B series
vitamins to promote cell survival and cardiac performance (1-4; 17), the in-depth mechanism of action and clinical value of employing benfotiamine in the treatment of diabetic heart diseases warrants further investigation.
ACKNOWLEDGMENTS

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**FIGURE LEGEND**

*Fig 1.* Contractile properties of ventricular myocytes from control, 2-week STZ diabetic mice and 4-week STZ diabetic mice with or without benfotiamine (BT) treatment at a dose of 100 mg/kg/d for 2 weeks. A. Resting cell length; B. Peak shortening (PS as % of resting cell length); C. Time-to-peak shortening (TPS); D. Time-to-90% relengthening (TR90); E. Maximal velocity of shortening (+ dL/dt); F. Maximal velocity of relengthening (-dL/dt). Mean ± SEM, n = 85-86 myocytes from 5 – 7 mice per group, * p < 0.05 vs. corresponding control group, # p < 0.05 vs. 4-week STZ diabetic group.

*Fig 2:* Intracellular Ca$^{2+}$ properties of ventricular myocytes from control, 2-week STZ diabetic mice and STZ diabetic mice with or without benfotiamine (BT, 100 mg/kg/d for 2 weeks) treatment. A. Resting intracellular Ca$^{2+}$ levels; B. Increase in intracellular Ca$^{2+}$ in response to electrical stimuli; C. Intracellular Ca$^{2+}$ transient decay rate (tau); D. SR Ca$^{2+}$ release. Mean ± SEM, n = 57 – 58 myocytes from 5- 7 mice per group (n = 17-18 cells per group for panel D), * p < 0.05 vs. corresponding control group, # p < 0.05 vs. 4-week STZ diabetic group.

*Fig 3:* Protein carbonyl (A) and GSH/GSSG ratio (B) in ventricular tissues from control or STZ-induced diabetic mice with or without benfotiamine (BT, 100 mg/kg/d for 2 weeks) treatment. Mean ± SEM, n = 7 – 12 samples per group, * p < 0.05 vs. corresponding control group.

*Fig 4:* Western blot analysis of AGE (A) and phospholamban (B) in ventricular tissues from control or STZ-induced diabetic mice with or without benfotiamine (BT, 100 mg/kg/d for 2 weeks) treatment. Insets: actual gel blotting using anti-AGE and anti-phospholamban (PLB)
antibodies. C: control; C+B: control+BT; D: diabetic; D+B: diabetic+BT; Mean ± SEM, n = 5 – 7 samples per group, * p < 0.05 vs. control group.
Table 1: General features of control or STZ-induced diabetic mice with or without benfotiamine treatment (100 mg/kg/day for 14 days).

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Control (8)</th>
<th>Diabetic (11)</th>
<th>Control-Benfotiamine (8)</th>
<th>Diabetic-Benfotiamine (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>25.0 ± 0.5</td>
<td>22.0 ± 1.2*</td>
<td>24.9 ± 0.3</td>
<td>21.4 ± 1.2*</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>140 ± 23</td>
<td>113 ± 22</td>
<td>140 ± 24</td>
<td>105 ± 20</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>5.49 ± 0.83</td>
<td>4.92 ± 0.69</td>
<td>5.59 ± 0.94</td>
<td>4.68 ± 0.59</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>1.53 ± 0.10</td>
<td>1.40 ± 0.14</td>
<td>1.47 ± 0.11</td>
<td>1.43 ± 0.14</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>61.1 ± 3.3</td>
<td>62.4 ± 3.3</td>
<td>59.1 ± 4.0</td>
<td>65.4 ± 4.2</td>
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<tr>
<td>Kidney Weight (g)</td>
<td>0.448 ± 0.040</td>
<td>0.443 ± 0.080</td>
<td>0.469 ± 0.050</td>
<td>0.473 ± 0.057</td>
</tr>
<tr>
<td>KW/BW (mg/g)</td>
<td>17.8 ± 1.3</td>
<td>19.2 ± 2.4</td>
<td>18.8 ± 1.9</td>
<td>22.1 ± 1.4</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>4.88 ± 0.43</td>
<td>25.51 ± 1.03*</td>
<td>4.83 ± 0.44</td>
<td>24.87 ± 1.74*</td>
</tr>
</tbody>
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HW = heart weight; LW = liver weight; KW = kidney weight. Mean ± SEM, * p < 0.05 vs. corresponding control groups, (n): number of animals.
Ceylan-Isik et al., Fig. 2

**A.**
Baseline 360/380 Ratio

**B.**
Rise in Intracellular Ca$^{2+}$ (360/380)

**C.**
Rise in Intracellular Ca$^{2+}$ (360/380)

**D.**
SR Ca$^{2+}$ Load (360/380)
Ceylan-Isik et al., Fig. 3
A. AGE Expression (Arbitrary Density)

B. Phospholamban Expression (Arbitrary Density)

Ceylan-Isik et al., Fig. 4
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13. **Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA and Schmidt AM.** Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-


