Vitamin B1 Analog Benfotiamine Prevents Diabetes-Induced Diastolic Dysfunction and Heart Failure Through Akt/Pim-1–Mediated Survival Pathway

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Background—The increasing incidence of diabetes mellitus will result in a new epidemic of heart failure unless novel treatments able to halt diabetic cardiomyopathy early in its course are introduced. This study aimed to determine whether the activity of the Akt/Pim-1 signaling pathway is altered at critical stages of diabetic cardiomyopathy and whether supplementation with vitamin B1 analog benfotiamine (BFT) helps to sustain the above prosurvival mechanism, thereby preserving cardiomyocyte viability and function.

Methods and Results—Untreated streptozotocin-induced type 1 or leptin-receptor mutant type 2 diabetic mice showed diastolic dysfunction evolving to contractile impairment and cardiac dilatation and failure. BFT (70 mg/kg/d) improved diastolic and systolic function and prevented left ventricular end-diastolic pressure increase and chamber dilatation in both diabetic models. Moreover, BFT improved cardiac perfusion and reduced cardiomyocyte apoptosis and interstitial fibrosis. In hearts of untreated diabetic mice, the expression and activity of Akt/Pim-1 signaling declined along with O-N-acetylglycosamine modification of Akt, inhibition of pentose phosphate pathway, activation of oxidative stress, and accumulation of glycation end products. Furthermore, diabetes reduced pSTAT3 independently of Akt. BFT inhibited these effects of diabetes mellitus, thereby conferring cardiomyocytes with improved resistance to high glucose-induced damage. The phosphoinositide-3-kinase inhibitor LY294002 and dominant-negative Akt inhibited antiapoptotic action of BFT-induced and Pim-1 upregulation in high glucose-challenged cardiomyocytes.

Conclusions—These results show that BFT protects from diabetes mellitus-induced cardiac dysfunction through pleiotropic mechanisms, culminating in the activation of prosurvival signaling pathway. Thus, BFT merits attention for application in clinical practice. (Circ Heart Fail. 2010;3:294-305.)

Key Words: diabetes mellitus ■ cardiomyopathy ■ diastolic dysfunction ■ benfotiamine ■ apoptosis

Diabetes mellitus (DM) is a potent and prevalent risk factor for heart failure independent of coronary artery disease or hypertension.1 Diabetic cardiomyopathy has an insidious onset and remains, therefore, undiagnosed and untreated in a large number of patients. Furthermore, recent studies have shown evidence of diastolic dysfunction in up to 75% of young, asymptomatic patients with type 1 or type 2 DM.2 The association of diastolic dysfunction and microangiopathy synergistically increases the risk of heart failure, thus pointing out the urgent need of early mechanistic treatment.3,4

Clinical Perspective on p 305

A variety of molecular alterations have been associated with diabetic cardiomyopathy, including defects in calcium homeostasis5 and substrate metabolism,6 accumulation of advanced glycation end products (AGE),7 activation of the hexosamine pathway,8 and oxidative stress leading to cardiomyocyte apoptosis.9 However, early stage mechanisms remain mostly unknown.

The pivotal role of the phosphoinositide-3-kinase (PI3K)/Akt/proviral integration site for the Moloney murine leukemia virus-1 (Pim-1) signaling pathway in the control of cardiac contractile function and cardiomyocyte growth and survival is well established.10–12 Of note, the hearts of mice with long-standing DM show decreased levels of activated Akt,13 and intriguingly, signal transducer and activator of transcription 3 (STAT3 [an upstream modulator of Pim-1]-deficient mice spontaneously develop a form of dilated cardiomyopathy similar to that occurring in diabetic mice.14 However, to the best of our knowledge, no information exists on whether altered glucose metabolism may dampen the activity of the STAT3/Akt/Pim-1 trio from early phases of cardiomyopathy and whether pharmacological manipulation

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of such kinases could help to halt DM-induced cardiac damage.

The conversion of glucose to pentose is hampered in DM because of the inhibition of pivotal enzymes of the pentose phosphate pathway, such as transketolase and glucose-6-phosphate dehydrogenase, resulting in depletion of reducing agents and accumulation of glycolysis end products.\textsuperscript{15,16} Inhibition of transketolase activity was ascribed to deficit of its coenzyme thiamine. This situation is further aggravated by the fact that increased reactive oxygen species induces compensatory activation of the DNA-repairing enzyme poly(ADP-ribose) polymerase, which in turn inhibits the activity of GAPDH, a crucial enzyme of glycolysis. Intermediate metabolites of glycolysis consequently are forced down to alternative pathways, including AGE formation and hexosamine pathway, which are responsible for protein modification and inactivation and cardiovascular damage.\textsuperscript{17,18}

The aim of this study was 2-fold: (1) to verify whether high glucose might alter STAT3/Akt/Pim-1 expression and activity from early stages of cardiomyopathy and (2) to determine whether benfotiamine (BFT) supplementation can sustain prosurvival signaling and prevent cardiac dysfunction in DM. The rationale of BFT supplementation is to correct thiamine deficit and thereby provide a shunt for glucose through the pentose pathway. Results of this study show the chronological alterations in the prosurvival signaling during the progression of diabetic cardiomyopathy and its correction by treatment with BFT.

Methods
Details are provided in the online-only Data Supplement in Expanded Materials and Methods.

Ethics
Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (the Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol. Type 1 diabetes was induced in Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol. Type 1 diabetes was induced in male C57BL/6J mice by injection of streptozotocin (40 mg/kg body weight IP per day for 5 days).\textsuperscript{19} Gender- and age-matched healthy (for type 1 DM) or similarly randomized to treatments starting from 9 weeks of age. The water starting at 4 weeks from DM induction (last day of streptozotocin injection) throughout the study. Type 2 DM mice were water started at 4 weeks from DM induction (last day of streptozotocin injection) throughout the study. Type 2 DM mice were assigned to receive BFT or vehicle. After an additional 24 hours, cells were used for caspase-3/7 activity of infection.\textsuperscript{25} In a separate study, HG-treated HL-1 cardiomyocytes were collected for measurement of caspase-3/7 activity in 6 wells per condition and repeated 3 times.

Biochemical Measurements
Confirmation of DM was achieved by measurements of blood glucose levels through the study (supplemental Figure II). Transketolase, glucose-6-phosphate dehydrogenase, and GAPDH activities in peripheral blood erythrocytes and left ventricular (LV) tissue were measured at the time of euthanizing as described previously (n=5 mice per group).\textsuperscript{19,17,21} AGE levels were measured by ELISA (n=5 mice per group).\textsuperscript{22}

Echocardiography
Dimensional and functional parameters were measured using a high-frequency, high-resolution echocardiography system. LV chamber dimensions, LV ejection fraction, and LV fractional shortening were determined as described previously.\textsuperscript{23}

Measurement of LV Pressures and Cardiac Dimensions
LV pressure was measured in anesthetized mice (n=10 to 12 per group) before killing, using a high-fidelity 1.4F transducer-tipped catheter.

Measurement of Blood Flow
Myocardial blood flow was assessed by the use of fluorescent microspheres, which were injected into the LV cavity (n=4 to 6 mice per group).\textsuperscript{24}

Immunohistochemistry
Immunohistochemical techniques were used to identify myocardial endothelial cells (isolectin B4 staining) and smooth muscle cells (α-smooth muscle actin), fibrosis (Sirius red), apoptosis (TUNEL), and hydroxyl radicals (8-hydroxydeoxygenosine). Superoxides were revealed using dihydroethidium staining of LV cryostat sections.

In Vitro Studies
Prosurvival Effect of BFT on High Glucose-Challenged Cardiomyocytes
HL-1 cardiomyocytes (a gift from Prof William Claycomb, Louisiana State University Medical Center, New Orleans, La) were cultured in the presence of high D-glucose (HG, 30 mmol/L) or normal glucose (5 mmol/L) added with 25 mmol/L D-mannitol as osmotic control. After 72 hours, cells were supplemented with either BFT (150 μmol/L) or vehicle (1 mmol/L HCl) for a further 24 hours. Optimal BFT concentration was decided on the basis of pilot titration studies (supplemental Figure IIIA). In addition, a fixed dosage of BFT was tested to contrast the apoptotic effect of increasing doses of D-glucose (supplemental Figure IIIB). At the end of the experiments, cardiomyocytes were collected for measurement of caspase-3/7 activity performed in 6 wells per condition and repeated 3 times.

Inhibition of PI3K, Akt, or STAT3
To verify the involvement of PI3K/Akt in BFT-induced prosurvival effects, we used either the PI3K inhibitor LY-294002 (50 μmol/L) or hemagglutinin-tagged dominant negative mutated form of Akt (Ad.DN-Akt, K179 mol/L) or control Ad.Null (both at 100 multiplicity of infection). In a separate study, HG-treated HL-1 cardiomyocytes were exposed for 30 minutes to STAT3 inhibitor peptide (Ac-PpYLKTK-OH, 1 mmol/L), a cell permeable compound that reduces the levels of active STAT3 and inhibits DNA-binding activity of STAT3 by forming an inactive STAT3-peptide complex.\textsuperscript{26} This exposure was followed by treatment with BFT or vehicle. After an additional 24 hours, cells were used for caspase-3/7

Inhibition of PI3K, Akt, or STAT3
activity and Western blot analyses as described earlier. Western blot analyses were performed in triplicates and repeated 3 times, and caspase-3/7 activity was performed in 6 wells per each condition and repeated 3 times.

**Expressional Studies**

Western blot analyses were performed on LV samples and HL-1 cardiomyocytes to verify the effects of DM, HG, and treatment on total and pSTAT3 (Tyr705), protein phosphatase 2A (PP2A), total and p-endothelial nitric oxide synthase (p-eNOS), total and p-Akt (Ser473), Pim-1, total and p-Bad (Ser112), Bcl-2, total and p-forkhead box O-1 (p-FOXO-1), and cleaved caspase-3 expression.

To assess the O-Acetylglucosamine (O-GlcNAc) modification of Akt, LV extracts were immunoprecipitated with anti-Akt antibody.27 The precipitate was then immunoblotted with antibodies for O-GlcNAc (1:10 000, a gift from Prof G.W. Hart, Johns Hopkins University School of Medicine, Baltimore, Md)28 and for total and p-Akt. Akt activity in protein extracts was measured with a commercial kit.

Quantitative reverse transcription polymerase chain reaction for mouse Pim-1 and 18s rRNA (for normalization) was performed in a LightCycler. All the expressional analyses were performed on 4 biological replicates.

**Statistical Analysis**

Results are presented as mean±SD. The hemodynamic and echocardiographic measurements were compared by the use of repeated measures 2-way ANOVA (factorial design, 2 independent variables [treatment and presence or absence of diabetes]), followed by pairwise comparison using the Holm-Sidak method. For the histological, biochemical, and morphometric analyses,
differences between multiple groups were analyzed with 1-way ANOVA and differences between 2 groups with t test (paired or unpaired as appropriate). For blood flow and molecular expression studies, when normality test fails, differences between groups were analyzed using Siegel-Tukey test. Survival curves were analyzed by the Kaplan–Meier method, and comparisons were made with the Gehan-Breslow log-rank test using SigmaStat statistical software. A $P<0.05$ is considered statistically significant for all the parameters.

**Results**

**BFT Attenuates DM-Induced Cardiac Remodeling and Dysfunction**

The murine models used in this study showed distinct phases in the evolution of cardiomyopathy. The initial phase consisted of diastolic dysfunction, as indicated by the significant decrease in Doppler E/A ratio at 4 weeks from type 1 DM induction (Figure 1A). A similar figure was observed in 9-week-old type 2 diabetic mice (Figure 2A). BFT partially rescued the reduced E/A ratio and prevented the further deterioration of diastolic function with duration of DM in both diabetic models (Figures 1A and 2A, $P<0.01$ versus healthy controls for both comparisons).

The second phase consisted of impaired contractile performance and cardiac dilatation. In type 1 diabetic mice, systolic function started decreasing from 12 weeks of DM, as indicated by the progressive reduction in LV ejection fraction and LV fractional shortening (Figure 1B). The late stage was characterized by LV chamber enlargement, LV wall thinning, and critical fall in cardiac output, which are typical features of heart failure (Figure 1B and Figure 2).
supplemental Figure IVA). Analysis of LV pressure indices and pressure-volume loops further verified the marked dysfunction of diabetic hearts (Figure 1C). In type 2 diabetic mice, systolic dysfunction was precocious, being already apparent at 9 weeks of age and rapidly advancing toward heart failure (Figure 2 and supplemental Figure IVB). Importantly, BFT treatment resulted in the global improvement of LV performance, pressure indices, and volumes in both diabetic models (Figures 1 and 2 and supplemental Figure IV, \( P<0.01 \) versus vehicle-treated diabetic mice for all parameters) but did not affect cardiac function in healthy mice (data not shown). Inotropic and lusitropic responses to adrenergic stimulation were blunted in type 1 diabetic mice (\( P<0.01 \) versus healthy controls) but improved by BFT (\( P<0.01 \) versus vehicle-treated diabetic mice, supplemental Figure V).

### BFT Improves Survival Rate

The survival rate for type 1 diabetic mice was less than that for healthy controls but was remarkably improved by BFT (supplemental Figure VI, \( P<0.001 \) versus vehicle-treated diabetic mice).

### BFT Improves Myocardial Blood Flow

As shown in Figure 3A, myocardial perfusion was reduced by DM, with this defect being prevented by BFT in both diabetic models (\( P<0.01 \) versus vehicle-treated diabetic mice). Concordantly, capillary density was reduced in the LV of diabetic mice (\( P<0.001 \) versus healthy controls) and partially conserved by BFT (\( P<0.01 \) versus vehicle-treated diabetic mice, Figure 3B). Fractional analysis of arteriole density revealed a marked decrease in small (\(<30 \mu m\) diameter) arterioles in the LV of both diabetic models (\( P<0.001 \) versus healthy controls).
for both comparisons), which was inhibited by BFT ($P<0.001$ versus vehicle-treated diabetic mice for both comparisons, Figure 3C).

**Histological Validation of BFT Effects**
Cardiomyocyte cross-sectional area was reduced in type 1 diabetic mice ($66\pm8$ $\mu m^2$ versus $83\pm6$ $\mu m^2$ in healthy controls; $P<0.01$) and preserved by BFT ($79\pm7$ $\mu m^2$; $P<0.01$ versus vehicle-treated diabetic mice). BFT remarkably prevented cardiomyocyte apoptosis and interstitial fibrosis in both diabetic models (Figure 4; $P<0.01$ for both comparisons).

**BFT Activates the Pentose Phosphate Shunt Pathway and Reduces Oxidative Stress**
We found that the activity of transketolase, glucose-6-phosphate dehydrogenase, and GAPDH is reduced in diabetic hearts (Figure 5A through 5C) and associated with marked increase in AGE (Figure 5D) and O-GlcNAc protein modification (see later). BFT prevented these effects and avoided the increase in reactive oxygen species levels in the heart of both diabetic models (Figure 5E and supplemental Figure VII; $P<0.01$ versus vehicle-treated diabetic mice for both comparisons).

**BFT Prevents DM-Induced Downregulation of STAT3/Akt/Pim-1**
Surprisingly, at initial stages, type 1 diabetic hearts showed increased pAkt and peNOS levels but low Pim-1 protein (Figure 6) and mRNA expression (supplemental Figure VIIIa). Importantly, Pim-1 continuously decreased with progression of cardiomyopathy in parallel with accruing changes in its upstream modulators. In temporal sequence, the first change consisted of the decrease in the activated form of STAT3 (STAT3-p-Tyr705), which reportedly induces Pim-1 expression.29 This decrease was followed by an increase in PP2A, which is known to induce Pim-1 mRNA downregulation and protein degradation (data not shown).30 At a later stage, when heart failure was overtly manifested, the diabetic myocardium showed reduced pAkt (Figure 6B) and Akt activity (Figure 6J) and increased O-GlcNAc modification of Akt, which was previously associated with Akt inhibition (Figure 6K).31 Levels of peNOS and pFOXO-1 also were decreased in the failing heart of type 1 diabetic mice (Figure 6C and 6D).
Previous studies showed that both Akt and Pim-1 phosphorylates the proapoptotic Bad, resulting in the formation of Bad-(14 to 3-3) protein homodimer, which leaves Bcl-XL and Bcl-2 free to inhibit apoptosis.11,32,33 We found that downregulation of Akt/Pim-1 signaling by type 1 DM is associated to reduced pBad (Ser 112) and Bcl-2 levels and increased cleaved caspase-3 (Figure 6G through 6I).

The heart of type 2 diabetic mice exhibited a marked decrease in STAT3/Pim-1 and modification of Pim-1 downstream effectors but at variance with type 1 DM, also showed an early reduction in Akt phosphorylation and activity (Figure 7). The global depression of STAT3/Akt/Pim-1 was mirrored by a more rapid evolution of cardiomyopathy.

Importantly, BFT prevented the downregulation of STAT3 and Pim-1 and the O-GlcNAc modification of Akt, thereby preserving Akt activity and downstream targets in both diabetic models (Figures 6 and 7). In contrast, myocardial PP2A levels remained increased in BFT-treated diabetic mice (data not shown).

Finally, to verify the direct action of BFT on cardiomyocytes, we performed in vitro assays in which adult cardiomyocytes were cultured in HG or normal glucose in the presence of BFT or vehicle. Consistent with in vivo experiments, HG increased cardiomyocyte apoptosis, this effect being prevented by BFT (supplemental Figure IXA). The antiapoptotic action of BFT was paralleled by conservation of Pim-1 at mRNA (supplemental Figure IXB) and protein level (supplemental Figure IXC) and inhibition of HG-induced effects on pBad and Bcl-2 (supplemental Figure IXD). Furthermore, BFT contrasted HG-induced decreases in pSTAT3 (supplemental Figure IXE), pAkt and Akt activity (supplemental Figure IXF and IXG) and peNOS and pFOXO-1 (supplemental Figure IXH and IXI). Of note, HG reduced the nuclear localization of Akt, which is necessary for Akt to induce Pim-1 expression and phosphorylate or inhibit FOXOs,34 but BFT restored proper nuclear Akt levels (supplemental Figure IJ).

Infection of cardiomyocytes with Ad.DN-Akt, verified by assessing the expression of hemagglutinin tag (data not shown), abrogated the stimulating action of BFT on pAkt without affecting STAT3. Furthermore, Ad.DN-Akt significantly inhibited the antiapoptotic action of BFT under HG and contrasted the effects of BFT on Pim-1, pBad, and Bcl-2 (supplemental Figure XA through XF). Prevention of apoptosis by BFT was similarly reduced by the PI3K inhibitor...
TREATMENT OF CARDIOMYOCYTES WITH STAT3 INHIBITOR REDUCED BASAL AND BFT-STIMULATED pSTAT3 LEVELS WITHOUT AFFECTING pAkt (SUPPLEMENTAL FIGURES XG AND XI). STAT3 INHIBITION POTENTIATED PIM-1 DOWNREGULATION AND CARDIOMYOCYTE APOPTOSIS UNDER HG (SUPPLEMENTAL FIGURES XJ AND XM). IT ALSO CONTRASTED BFT IN PRESERVING PIM-1 EXPRESSION AND TO A LESSER EXTENT pBAD AND BC1-2 (SUPPLEMENTAL FIGURES XJ THROUGH XL) BUT WAS INEFFECTIVE IN INHIBITING THE ANTIAPOTOTIC ACTION OF BFT, SUGGESTING THAT pAkt INDUCTION BY BFT COULD COMPENSATE FOR STAT3/PIM-1 DEFICIT TO SUPPORT CARDIOMYOCYTE VIABILITY UNDER HG CONDITIONS.

LY294002 (SUPPLEMENTAL FIGURE XG). TREATMENT OF CARDIOMYOCYTES WITH STAT3 INHIBITOR REDUCED BASAL AND BFT-STIMULATED pSTAT3 LEVELS WITHOUT AFFECTING pAkt (SUPPLEMENTAL FIGURES XH AND XI). STAT3 INHIBITION POTENTIATED PIM-1 DOWNREGULATION AND CARDIOMYOCYTE APOPTOSIS UNDER HG (SUPPLEMENTAL FIGURES XJ AND XM). IT ALSO CONTRASTED BFT IN PRESERVING PIM-1 EXPRESSION AND TO A LESSER EXTENT pBAD AND BC1-2 (SUPPLEMENTAL FIGURES XJ THROUGH XL) BUT WAS INEFFECTIVE IN INHIBITING THE ANTIAPOTOTIC ACTION OF BFT, SUGGESTING THAT pAkt INDUCTION BY BFT COULD COMPENSATE FOR STAT3/PIM-1 DEFICIT TO SUPPORT CARDIOMYOCYTE VIABILITY UNDER HG CONDITIONS.

FIGURE 6. BFT STIMULATES PROSURVIVAL SIGNALING IN TYPE 1 DM. REPRESENTATIVE BLOTS (A) AND BAR GRAPHS (B THROUGH I) SHOWING THE LEVELS OF pAkt, peNOS, pFOXO-1, pSTAT3, Pim-1, pBad, Bcl-2, AND CLEAVED CASPASE-3 (n=4 PER GROUP). BAR GRAPHS SHOWING MYOCARDIAL Akt ACTIVITY (J). REPRESENTATIVE IMMUNOBLOTS AND BAR GRAPHS SHOWING O-GlcNAc MODIFICATION OF Akt IN DIABETIC HEARTS (K) (n=4 PER GROUP). VALUES ARE EXPRESSED AS n-FOLD CHANGES TOWARD AGE-MATCHED HEALTHY CONTROLS. STZ INDICATES STREPTOZOTOCIN. SIEGEL-TUKEY TEST DETECTED STATISTICAL DIFFERENCES AS FOLLOWS: *P<0.01 AND **P<0.001 Versus Healthy Controls; #P<0.01 AND ##P<0.001 Versus Vehicle-Treated Diabetic Mice.

DISCUSSION

IN A PREVIOUS REPORT IN STREPTOZOTOCIN MICE, A 2-WEEK ADMINISTRATION OF BFT PRESERVED IN VITRO CONTRACTILE PROPERTIES AND INTRACELLULAR Ca2+ KINETICS OF CARDIOMYOCYTES.35 TO THE BEST OF OUR KNOWLEDGE, HOWEVER, THIS IS THE FIRST STUDY TO SHOW THE THERAPEUTIC POTENTIAL OF LONG-TERM TREATMENT WITH BFT FOR PREVENTION OF CARDIOMYOPATHY IN MODELS OF TYPES 1 AND 2 DM, WHICH IS PARTICULARLY RELEVANT IN LIGHT OF THE PREVALENT INCIDENCE OF CARDIOMYOPATHY IN PATIENTS WITH TYPE 2 DM. THE EFFECT OF BFT WAS DOCUMENTED BY ECHOCARDIOGRAPHY FOLLOW-UP OF LV FUNCTION, MILLAR TRANSDUCER MEASUREMENT OF LV PRESSURE, AND CARDIAC MORPHOMETRY. FURTHERMORE, WE...
BFT stimulates prosurvival signaling in type 2 DM. Representative blots (A) and bar graphs (B through I) showing the levels of pAkt, peNOS, pFOXO-1, pSTAT3, Pim-1, pBad, Bcl-2, and cleaved caspase-3 (n=4 per group). Bar graphs showing myocardial Akt activity (J). Representative immunoblots and bar graphs showing O-GlcNAc modification of Akt in diabetic hearts (K) (n=4 per group). Values are expressed as n-fold changes toward age-matched healthy controls. Siegel-Tukey test detected statistical differences are as follows: *P<0.01, **P<0.001, and ***P<0.001 versus db/db mice; #P<0.01 and ##P<0.001 versus vehicle-treated db/db mice.
newly reported that BFT prevents myocardial microvascular rarefaction, thereby improving myocardial perfusion of diabetic hearts. The role of microcirculation in the pathogenesis of diabetic cardiomyopathy has been highlighted by a study in which gene therapy with vascular endothelial growth factor-A prevented capillary rarefaction and cardiac dysfunction in mice. Of note, BFT, which reportedly reduces the glucose flux to the hexosamine pathway, abrogated DM-induced O-GlcNAc modification of transcription factors and proteins involved in cardiomyocyte function. We show for the first time the combination of reduced Akt activity and increased O-GlcNAc modification of Akt in myocardium of diabetic mice. Inactivation of Akt may account for increased cardiomyocyte apoptosis in failing diabetic hearts because Akt directly controls the phosphorylation and sequestration of the Bad and FOXO transcription factors. Of note, BFT, which reportedly reduces the glucose flux to the hexosamine pathway, abrogated DM-induced O-GlcNAc modification of Akt, thereby restoring Akt activity and Bad and FOXO-1 phosphorylation levels.

One intriguing aspect of our results consists of increased Akt activity at early stages of cardiomyopathy in type 1 diabetic mice, which is possibly a compensatory attempt to combat DM-induced damage. On the other hand, chronic Akt activation could have detrimental effects on the heart through inhibition of insulin receptor substrate/PI3K signaling and disruption of the coordinated association between adaptive cardiac hypertrophy and microvascular growth. Of note, while confirming the reduction of microvascular density in failing diabetic hearts, our data show no evidence of hypertrophic remodeling during initial increase of Akt in type 1 diabetic hearts. The time duration of Akt activation might have been too short to sustain LV hypertrophy in our experimental setting. Furthermore, the dual action of Akt on cardiomyocyte survival and growth could depend on the specific intracellular location of activated Akt.

Many of the cardiac actions of Akt are ascribed to be Pim-1 dependent. For instance, Akt activation induces Pim-1 expression, but forced expression of Akt failed to protect the infarcted myocardium of Pim-1-deficient mice. Pim-1-induced cardioprotection is reportedly mediated by upregulation of Bcl-2 and Bcl-XL as well as phosphorylation and inactivation of Bad. Our in vitro findings showing that PI3K/Akt inhibition abrogates the action of BFT on cardiomyocyte survival under HG and contrasts the stimulatory effect of BFT on Pim-1 and Bcl-2 indicate that Akt is a crucial facet of cardioprotection exerted by this compound. This concept is reinforced by the finding that BFT-induced Akt upregulation in HG-challenged cardiomyocytes is sufficient for the prevention of apoptosis under STAT3 inhibition.

In conclusion, our results illustrate fundamental mechanisms that are involved in the pathogenesis of diabetic cardiomyopathy and could be prevented by BFT (summarized in supplemental Figure XI). Implications of the present preclinical study to the clinical field should be evaluated cautiously in the light of similarities and differences of cardiomyopathy developing in patients with diabetes and diabetic animal models. Furthermore, the dosage and time schedule of BFT supplementation represent a crucial issue as for other preventive treatments, with the additional caveat that sustained activation of proto-oncogenes, like Akt and Pim-1, might increase the risk of cancer. This concern however is not supported by our results showing no evidence of adverse effects but rather improved survival of diabetic mice treated with a high dose of BFT.

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Disclosures

None.

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Diabetes mellitus is a potent and prevalent risk factor for heart failure, independently of coronary artery disease or hypertension. Importantly, diabetes mellitus can affect cardiac structure and function independently of coexisting hypertension or ischemic heart disease, resulting in a condition named diabetic cardiomyopathy. Diabetic cardiomyopathy has an insidious onset, manifesting with isolated diastolic dysfunction, which progresses with time to global heart failure. The present study unravels distinct molecular signatures of the progression of diabetic cardiomyopathy from early and probably reversible stages to more advanced heart remodeling and dilatation. In particular, we clarified a possible link between the metabolic disorder and epigenetic changes that make the prosurvival Akt/Pim1 duo dysfunctional, thereby compromising the heart’s performance. This discovery opens new windows of therapeutic opportunity to combat this increasingly harmful complication of diabetes. Importantly, vitamin B1 analogue benfotiamine is able to beneficially interfere with the Akt/Pim1 disturbed signaling and thus prevents the mismatch between angiogenesis and cardiomyocyte remodeling in the diabetic heart. Whether benfotiamine is therapeutically useful needs now to be demonstrated by clinical trials. Furthermore, because the main action of benfotiamine is to boost the activity of transketolase, a key enzyme of the pentose phosphate pathway, and because transketolase activity is reduced in diabetes, it is important to determine whether patients with reduced transketolase activity are susceptible to develop an accelerated form of cardiomyopathy. If this is proved true, patients with low transketolase would become the best candidates to benfotiamine treatment.
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SUPPLEMENTAL MATERIAL

Expanded Methods

Echocardiography
Measurements of dimensional and functional parameters were performed at baseline, before mice entered the treatment, and every 4 weeks thereafter, using a high-frequency, high resolution echocardiography system (Vevo 660, Visual Sonics, Toronto, Canada). Briefly, mice were anesthetized using tribromo-ethanol and transferred to an imaging stage equipped with a warming pad for controlled maintenance of mouse body temperature at 37° C and a built-in electrocardiography system for continuous heart rate (HR) monitoring. Standard B mode (2D) images of the heart and pulsed Doppler images of the mitral valve inflow were acquired. The thickness of the left ventricle (LV) was measured at the level of the papillary muscles in parasternal short axis at end-systole and end-diastole. LV ejection fraction (LVEF) and fractional shortening (LVFS) were determined as described by De Simone et al.\(^1\)

Measurement of intra-ventricular pressure
Terminal measurement of left ventricular pressure (LVP) was made at 20 weeks after DM induction in type-1 diabetic mice (n=12 in each group) and at 17 weeks of age in type-2 diabetic mice (n=10 in each group). The body temperature of the mice was maintained between 36º and 37º C throughout the experiment using a homeothermic blanket warming system. A tracheotomy was made and the mouse was intubated using the 23 gauge catheter, secured in place with 6-0 silk suture.
A high-fidelity 1.4F transducer tipped catheter (Millar Instruments, Houston, TX, USA) was zeroed in 37ºC saline. Calibration of the transducer was verified using a mercury manometer, as suggested by the manufacturer. The right carotid artery was isolated, and tow ties were gently pulled back, using hemostats, to block blood flow from vessel. When pulsatile flow was no longer visible, a small cut was made just below the distal tie, and the catheter was placed inside the carotid artery and secured in place. The transducer was advanced into the heart, where its
position was confirmed by the rapid deflection of the diastolic pressure wave without any change in systolic pressure. Mice were allowed to stabilize for 10min. After stabilization, baseline data were collected, including the HR, Peak LV systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), and maximal rates of LV pressure rise (dP/dt\text{max}) and fall (dP/dt\text{min}). For the pressure volume relationship, the recording from Millar catheter was synchronized with echocardiography measurements as per manufacturer instructions.

**Measurement of transketolase activity**

Frozen heart tissue was defrosted, chopped finely and a 10% homogenate was prepared with 0.1M Tris-HCl buffer (pH 8.0) and centrifuged at 3,000 x g for 10 min. The lysate was kept on ice until used. For erythrocytes, peripheral blood samples were centrifuged (2,000 x g, 5 min) and the plasma and white blood cells were removed. The packed erythrocytes pellet was washed three times with PBS and lysed with ddH\textsubscript{2}O and membrane fragments sedimented (10,000 x g, 10 min, 4°C). The activity of transketolase in myocardial tissue homogenate and erythrocytes lysate was determined by the method of Chamberlain et al.\textsuperscript{3, 4} Aliquot (200 µl) of substrate cocktail (14.8 mM R-5-P, 253 µM NADH, 185 U/ml TPI, and 70 µl of 21.5 U/ml GDH in 250 mM Tris/HCl buffer, pH 7.8, all from Sigma Chemical, UK) was added to the wells of a 96-well microplate and 20 µl of a 6-fold dilution of erythrocyte lysate or tissue homogenate was added. The absorbance at 340 nm was monitored at 10 min intervals for 120 min and the rate of decrease in absorbance between 10 to 80 min was used to deduce the rate of oxidation of NADH in the GDH catalyzed reaction, which is rate limited by the transketolase catalyzed conversion of R-5-P and xylulose-5-phosphate to sedoheptulose-7-phosphate and GA3P under these conditions.

**Measurement of glucose-6-phosphate dehydrogenase (G6PD) activity**

G6PD activity was determined by measuring the rate of production of NADPH as previously described.\textsuperscript{5, 6} In brief, the samples were prepared in a similar manner explained above for transketolase activity. Aliquot (250µl) of substrate cocktail (50 mM glyglycine, pH 7.4, 2 mM D-glucose-6-phosphate, 100mM 6-phosphogluconic acid, 670 µM βNADP and 10mM MgCl\textsubscript{2})
was added to the wells of a 96-well microplate and 12.5 µl of a 6-fold dilution of myocardial tissue homogenate or erythrocyte lysate was added. The absorbance at 340 nm was monitored at 1min intervals for 5min. A second 12.5µl of a 6-fold dilution of erythrocyte lysate or tissue homogenate was added to a separate substrate cocktail (250µl) without D-glucose-6-phosphate and the absorbance was measured for 5 min. G6PD activity was calculated by subtracting the rate of change of absorbance with or without D-glucose-6-phosphate to eliminate the contribution of 6-phosphogluconate dehydrogenase (6PGD) to total NADPH production, as 6PGD also produces NADPH. Protein content in each sample was measured by BioRad assay with commercially available kit (BioRad, UK). Data are represented as units/min/ml for erythrocytes and units/min/mg of protein for myocardial homogenates.

**Measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity**
GAPDH activity was measured using the cytosolic fraction of myocardial tissue homogenate, which is prepared by centrifuging the myocardial tissue lysate at 100,000 x g at 4°C for 30 min. Enzyme activity was measured as described earlier. Briefly, 1µg of cytosolic protein was added to 200µl of assay buffer (100mM Tris/HCl pH 8.6, 1.5mM NAD, 3mM dithiothreitol, 5mM sodium arsenate, 1.5mM glyceraldehydes-3-phosphate) at room temperature. NADH formed was determined by monitoring the increase in absorbance at 340 nm at 10 sec interval for 1 min and then every minute for 60 min. Activity was expressed as units/sec/mg of protein.

**Measurement of blood flow using fluorescent microspheres**
Myocardial perfusion was measured using fluorescent microspheres. A polyethylene (PE10) catheter was inserted through the right carotid artery for the reference blood withdrawal. Microspheres, 0.02µm in diameter (Molecular Probes, CA, USA) were injected into the LV cavity over 1 min and flushed with 0.15 ml of 0.9% NaCl. Reference blood was collected via the carotid catheter starting 15 sec before to 1 min after the microsphere injection. The animals were sacrificed 2 min later and the heart was removed and separated into LV, RV and septum. The kidneys were also collected and analyzed as internal control organs to demonstrate homogenous distribution of the microspheres throughout the bloodstream. Each sample was weighed, cut into
small pieces and digested in 10 ml of 2 M ethanolic KOH containing 0.5% Tween 80 at 60°C for 48h with constant shaking. After complete digestion of tissues, the microspheres were collected by centrifugation at 2,000 x g for 20 min and sequential washing with 10 ml of deionized water with or without 0.25% Tween 80. Finally, microspheres were dissolved in 3 ml of 2-ethoxyethylacetate and the fluorescence intensity was determined using a fluorophotometer (Fluostar Optima, BMG labtech). Regional blood flow was calculated as the absolute blood flow in ml/min/g of tissue as described earlier.\(^8\)

**Assessment of myocardial capillary and arteriole densities**

LV sections (3µm) were deparaffinized and incubated with biotinylated Isolectin B4 (Invitrogen, Molecular Probes) followed by streptavidin Alexa Fluor 488 (Invitrogen, Molecular probes) for measurement of capillary density. To measure arteriole density, the sections were incubated with anti-mouse \(\alpha\)-smooth muscle cell actin antibody (Sigma chemicals) conjugated with Alexa Fluor 488 (Invitrogen, Molecular probes). Capillaries and arterioles were calculated in at least 20 fields at X400 magnification and the final data expressed as the number of capillaries or arterioles per square millimeter. Arterioles were also categorized according to their luminal size.\(^9\)

**Assessment of myocardial fibrosis**

Myocardial fibrosis was analyzed by Sirius red staining followed by morphometric analysis using the Image Pro analysis software (MediaCybernetics, USA) and the data expressed as the ratio between intensity of staining and area examined.

**TUNEL staining**

Apoptosis was quantified on paraffin embedded LV sections (3µm) by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (in situ cell death detection kit Fluorescein, Roche applied science, USA). Following treatment of slides with proteinase K (20 µg/ml, 30min at 37°C), TUNEL assay was performed according to the manufacturer’s instruction. The same sections were then stained with DAPI to recognize nuclei. To recognize cardiomyocytes, sections were also stained with mouse monoclonal primary
antibody for the cardiomyocyte marker α-sarcomeric actin (Dako, 1: 50, overnight at 4°C), which was revealed by counterstaining with the secondary antibody conjugated to fluorophore (Alexa 568, Invitrogen, Molecular probes). Twenty fields were randomly evaluated in each section at X400 magnification. The fraction of TUNEL positive nuclei over total cardiomyocyte nuclei was then calculated.10

**In situ detection of reactive oxygen species**

*Dihydroethidium staining for detection of superoxide*

Superoxide production in the myocardium was determined using the fluorescent dye dihydroethidium (DHE, Invitrogen, Molecular probes). LV cryosections (10µm) were incubated with 5 µmol/L DHE, at 37°C for 30 min, in a humidified chamber. Images (X100 magnification) were captured on an Olympus fluorescence microscope fitted with camera (Media cybermatics) and the mean DHE fluorescence intensity of myocyte nuclei was calculated by dividing the combined fluorescence value of the pixels by the total number of pixels in 15 randomly selected field using Image-Pro advanced software.11

**8-OHGD staining for detection of hydroxyl radicals**

Myocardial production of hydroxyl radicals was determined by immunofluorescent staining of the deparaffinized LV sections (3µm) using the primary antibody for 8-hydroxy-2'-deoxyguanosine (8-OHGD, Cosmo Bio, Japan). The nuclear localization of 8-OHGD was detected using the goat anti-mouse secondary antibody conjugated to fluorophore (Alexa 568, Invitrogen, Molecular probes) and counterstaining the cardiomyocytes with α-sarcomeric actin and DAPI to recognize nuclei. Images (X1000 magnification) were captured using an Olympus fluorescence microscope fitted with camera (Media cybernetics, USA) and data expressed as percentage of 8-OHGD positive nuclei.12

**Immunocytochemical analysis for Akt localization**

Effect of HG on localization of Akt was detected using immunocytochemical analysis. For this purpose, HL-1 cells were fixed with 4% paraformaldehyde following exposure to HG for 24h or 72h with or without treatment with BFT. After repeated washing and subsequent blocking with
serum, the cells were incubated with primary antibody against Ser473- phospho-Akt (Cell Signaling, 1:1000) at 4°C overnight. The nuclear or cytoplasmic localization of pAkt was detected using the goat anti-rabbit secondary antibody conjugated to fluorophore (Alexa 488, Invitrogen, Molecular probes). Images (X400 magnification) were captured using an Olympus fluorescence microscope fitted with camera (Media cybernetics, USA).

**Western blot analyses**

Proteins were extracted from LV using ice-cold RIPA buffer. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). Detection of proteins by western blot analysis was done following separation of whole tissue / cell extracts (50µg) on SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Amersham-Pharmacia) and probed with the following antibodies: anti-mouse Ser 1177-phospho-eNOS (Cell Signaling, 1:1000), eNOS (Cell Signaling, 1:1000), Ser473-phospho-Akt (Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), Ser256- phospho-FOXO-1 (Upstate, UK), FOXO-1 (Cell Signaling, 1:1000), Tyr705 phospho-STAT3 (Cell signaling, 1:500), STAT3 (Cell Signaling, 1:1000), Pim-1 (Santacruz biotechnology, 1:250), Ser112-phospho-Bad (Cell Signaling, 1:1000), Bad (Cell Signaling, 1:1000), Bcl-2 (Cell Signaling, 1:1000) and cleaved-caspase-3 (Cell Signaling, 1:1000). Actin (Cell Signaling, 1:1000) was used as loading control. For detection, secondary antibody goat anti-rabbit or anti-mouse conjugated to horseradish peroxidase (both from Amersham Pharmacia, 1:5000) were used, followed by chemiluminescence reaction (ECL, Amersham Pharmacia).

**RT-PCR**

Total RNA was isolated from LV samples as well as from HL-1 cells (Trizol, Invitrogen, UK) and reverse transcribed (Sensiscript reverse transcriptase, Qiagen). Quantitative PCR (qPCR) was performed in a LightCycler (Roche, Burgess Hill, UK) using Platinum taq polymerase (Qiagen) and the primer pairs listed below. For quantification, mRNA amount of the respective gene was normalized to the amount of 18S rRNA using the 2−DDCT method. Each reaction was performed in triplicate.\textsuperscript{13}
In vitro inhibition of PI3K and Akt

To verify the involvement of PI3K/Akt in BFT-induced pro-survival effects, we used two protocols. In the first protocol, HG-treated HL-1 cardiomyocytes were exposed to the PI3K inhibitor LY-294002 (50µM, Sigma Aldrich)\textsuperscript{10} for 24h followed by treatment with BFT (150µM) or vehicle (1mM HCl). In the second protocol, HG-treated HL-1 cardiomyocytes were infected with an adenovirus carrying a HA-tagged dominant negative mutated form of Akt (Ad.DN-Akt, K179M) or control Ad.Null (both at 100MOI).\textsuperscript{10} After 24h, the medium was replaced with a fresh one supplemented with either BFT or vehicle. After additional 24h, cells were used for measurements of caspase-3/7 activity (6 wells per each condition and repeated 3 times) and western blot (WB) analyses (n=4 samples per group).
References


10. Caporali A, Sala-Newby GB, Meloni M, Graiani G, Pani E, Cristofaro B, Newby AC,


Supplemental Figure 1A - Experimental protocol with type-1 DM mice

Basal Echocardiography (n=140)

Injection of streptozocin (STZ, 40mg/kg/ip for 5 days)

Pre-treatment Echocardiography at 4 weeks after the last STZ injection (n=120)*

Randomization

Vehicle (n=60) at 4 weeks after STZ (1mMol/l HCl in drinking water) Benfotiamine (n=60) at 4 weeks after STZ (in drinking water, 70mg/kg/day)

Randomization

Echocardiography at 8, 12, 16 and 20 weeks after STZ Collection of heart for molecular biology at 8, 12 and 20 weeks after STZ

Randomization

Measurement of intraventricular pressure by pressure tip catheter (n=at least 12 in each group). Evaluation of cardiac perfusion using fluorescent microspheres (n=6). Sampling for histology and immunohistochemistry (n=7).
Supplemental Figure 1B - Experimental protocol with type-2 DM mice

Pre-treatment Echocardiography (n=28, 9 weeks of age)

Randomization

Vehicle (n=14)  
(1mMol/l HCl in drinking water)  

Benfotiamine (n=14)  
(in drinking water, 70mg/kg/day)

Randomization

Echocardiography at 13 and 17 weeks of age

Collection of heart for molecular biology at 13 and 17 weeks of age

Randomization

Measurement of intraventricular pressure by pressure tip catheter (n=10, 17 weeks of age)

Evaluation of cardiac perfusion using fluorescent microspheres (n=4, 17 weeks of age)

Sampling for histology and immunohistochemistry (n=6, 17 weeks of age)
Supplemental Figure 2 - Serum glucose levels

A. Type-1 DM

B. Type-2 DM
Supplemental Figure 3 - Caspase-glo 3/7 Assay

A. Titration of Benfotiamine dosage

B. Benfotiamine inhibits the apoptotic action of increasing doses of glucose
Supplemental Figure 4 - Cardiac function

A. Type-1 DM

- **End systolic LV chamber internal diameter**
- **End diastolic LV chamber internal diameter**
- **LV posterior wall (end diastole)**
- **Cardiac output**

B. Type-2 DM

- **End systolic LV chamber internal diameter**
- **End diastolic LV chamber internal diameter**
- **LV posterior wall (end diastole)**
- **Cardiac output**

Legend:
- Healthy
- Diabetic
- Diabetic-BFT

**db/+**
- db/db
- db/db-BFT
Supplemental Figure 5

A

B

![Graph A](image1)

![Graph B](image2)
Supplemental Figure 7

DHE staining for superoxides in type-1 DM heart

![Image of healthy and diabetic heart staining]

![Intensity/Area ratio graph]

Healthy  Diabetic  Diabetic-BFT

**  *  ##
Supplemental Figure 8

A: Pim-1 mRNA expression in type-1 DM heart

B: Pim-1 mRNA expression in type-2 DM heart

C: Pim-1 mRNA expression in HL-1 cells exposed to high glucose
Supplemental Figure 9
Molecular changes in HL-1 cells exposed to high glucose

A. Caspase-glo 3/7 Assay

B. Phospho-L1 (Actin)

C. Phospho-Bad (Bad)

D. Bcl-2 (Actin)

E. Phospho-STAT3 (STAT3)

F. Phospho-Akt (Akt)

G. Akt kinase assay

H. Phospho-eNOS (eNOS)

I. Phospho-Foxo-1 (Foxo-1)

J. pAkt localization

pAkt DAPI
Supplemental Figure 10 (i)

Inhibition of benfotiamine-induced expressional effects by Ad.DN-Akt

G. Inhibition of benfotiamine-induced pro-survival effects by LY294002
Supplemental Figure 10 (ii)
Inhibition of benfotiamine-induced expressional effects by STAT3 Inhibitor peptide

H

I

J

K

L

M

Caspase-glo 3/7 Assay

Vehicle

STAT3 inhibitor
Supplemental Figure 11

Diabetes Mellitus/ Hyperglycemia

BFT → STAT3

PP2A

Akt ← BFT

Pim-1

Phosphorylation of Bad

Preservation of Bcl-2

Inhibition of apoptosis

Cell survival
Legends to Supplemental Figures

Supplemental Figure 1
Illustration of experimental protocols in type-1 (A) and type-2 (B) diabetic mice. Animals were randomized in subsequent phases for: (1) treatment with BFT or vehicle (4 weeks after the last STZ injection throughout duration of the study), (2) echocardiography/survival follow up or molecular biology assessment at indicated time points and (3) terminal measurement of intra-ventricular pressure or measurement of cardiac perfusion, or sampling of the heart for histology. *In type-1 DM protocol, 120 mice showing overt glycosuria entered the study, while the remaining 20 were discarded because of unsuccessful induction of DM.

The same protocol was carried out in parallel in (1) age-matched mice (healthy controls of type-1 DM), which were injected with the STZ-vehicle and 4 weeks later were randomly allocated to treatment with BFT or its vehicle (n=40 each) and (2) age-matched lean mice (BKS.Cg-m+/+Lepr db/OlaHsd, db/+)) which were randomly allocated to treatment with BFT or its vehicle at 9 weeks of age (n=14 each)

Supplemental Figure 2
Scatter plots show the serum glucose levels in type-1 (A) and type-2 (B) diabetic mice during treatment with BFT or vehicle. **P<0.001 versus respective control healthy mice in type-1 DM or db/+ mice in type-2 DM; #P<0.05 or ##P<0.01 versus pre-treatment (time 0, corresponding to 9 weeks of age) in db/db mice.

Supplemental Figure 3
Bar graphs show the levels of activated caspase-3/7 in cultured adult cardiomyocytes.
A. Cardiomyocytes were cultured in normal glucose (NG) or high glucose (30mM) with different concentrations of benfotiamine (B) or vehicle (V). Values are expressed as relative units (RLU) and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG; #P<0.01 versus V.
B. Cardiomyocytes were cultured in normal (5mM D-glucose) or high glucose (15 - 35mM
D-glucose) with 150µM benfotiamine or vehicle. Values are expressed as relative units (RLU), and are mean ± standard deviation. **P<0.001 versus 5mM D-glucose and $P<0.01$ versus corresponding vehicle group.

Each experiment was performed in 6 wells per each condition and repeated 3 times.

**Supplemental Figure 4**

Echocardiographic assessment of cardiac function in type-1 (A, n=16 in each group) and type-2 diabetic mice (B, n=10 in each group). Upper panels show linear measures of LV cavity captured at end-systole and end-diastole. Lower panels show LV posterior wall thickness at end-diastole and cardiac output. Values are mean ± standard deviation. BFT did not affect cardiac parameters in healthy mice (data not shown). Results of pair-wise comparison are illustrated: *P<0.01, **P<0.001 and ***P<0.0001 versus vehicle-treated healthy mice in type-1 DM or db/+ mice in type-2 DM; #P<0.01 and ##P<0.001 versus vehicle-treated diabetic mice in type-1 DM or db/db mice in type-2 DM. End systolic LV chamber internal diameter values of healthy and diabetic-BFT mice from 4 and 12 weeks overlapped and are therefore expressed by the same line.

**Supplemental Figure 5**

(A) Line graphs show changes in the maximum (inotropic) and minimum (lusitropic) rates of developed pressure in response to adrenergic stimulation (epinephrine 1mg/kg/IV) in healthy and type-1 diabetic mice, given BFT or vehicle. (B) Average response to adrenergic stimulation is expressed as area under the curve. Values are mean ± standard deviation. Results of pair-wise comparison are illustrated: *P<0.01 versus vehicle-treated healthy mice; $P<0.01$ versus vehicle-treated diabetic mice. Each group consisted of 6 mice.

**Supplemental Figure 6**

Line graph shows the effect of BFT on survival of type-1 diabetic mice. Mice were followed until 20 weeks after STZ (n=32 vehicle-treated or BFT-treated diabetic mice) or STZ-vehicle injection (n=40 healthy mice). Diabetic mice showed an increased mortality (P<0.001 versus
healthy mice), which was prevented by BFT (P<0.001 versus vehicle-treated diabetic mice). The survival rate of type-2 diabetic mice was not determined because of the limited group size.

**Supplemental Figure 7**

Representative microphotographs and bar graphs show the effect of BFT on superoxide levels in myocardium at 20 weeks from STZ or STZ-vehicle injection in type-1 diabetic mice. Each group consisted of 5 mice. Values are mean ± standard deviation. Results of pair-wise comparison are illustrated: *P<0.001 and **P<0.001 versus healthy mice; ##P<0.001 versus vehicle-treated diabetic mice. Scale bars are 100µm.

**Supplemental figure 8**

(A-B) Bar graphs show the levels of Pim-1 gene expression in LV of type-1 (at 8, 12 and 20 weeks from STZ or STZ-vehicle injection, A) or type-2 diabetic mice (at 13 and 17 weeks of age, B). Each group consisted of 4 mice. Values are expressed as n-fold changes toward vehicle-treated healthy mice and are mean ± standard deviation. *P<0.01 and **P<0.001 versus healthy mice in type-1 DM or db/+ mice in type-2 DM; #P<0.01 and ##P<0.01 versus vehicle-treated diabetic mice in type-1 DM or db/db mice in type-2 DM. (C) Bar graphs showing the levels of Pim-1 gene expression in cultured adult cardiomyocytes. Cardiomyocytes were cultured in normal (NG) or high glucose (HG) in the presence of benfotiamine (HGB) or vehicle. Each experiment was repeated three times in triplicate. Values are expressed as n-fold changes toward NG and are mean ± standard deviation. *P<0.01 versus NG; #P<0.01 versus HG.

**Supplemental Figure 9**

(A-I) Bar graphs show the levels of activated caspase-3/7 (A), Pim-1 (B), pBad (C), Bcl-2 (D), pSTAT3 (E), pAkt (F), Akt kinase activity (G), peNOS (H) and pFOXO-1 (I) in cultured adult cardiomyocytes. Cardiomyocytes were cultured in normal (NG) or high glucose (HG) in the presence of benfotiamine (NGB and HGB) or vehicle. Each experiment was repeated three times in triplicate. Values are expressed as n-fold changes toward NG for all parameters, except caspase 3/7 which is expressed as relative units (RLU), and are mean ± standard deviation.
Siegel-Tukey test detected statistical differences as illustrated: *P<0.01 and **P<0.001 versus NG; *P<0.01 and #*P<0.001 versus HG. (J) Representative microphotographs showing the pAkt intracellular localization in cardiomyocytes cultured in normal (NG) or high glucose (HG) for 24 or 72 h in presence of benfotiamine (HGB) or vehicle. Scale bars are 50 µm.

Supplemental Figure 10

(A-F) Bar graphs show the levels of pSTAT3 (A), pAkt (B), Pim-1 (C), pBad (D) Bcl-2 (E) and activated caspase-3/7 (F). Cardiomyocytes cultured in high glucose (HG) or normal glucose (NG) were infected with Ad.DN-Akt or Ad.Null followed by another 24h culture in the presence of benfotiamine (HGB) or vehicle. Each experiment was performed in triplicate and repeated three times. For caspase-3/7 activity, assay was performed in 6 wells per each condition and repeated three times. Values are expressed as n-fold changes toward Ad.Null NG for all parameters, except for caspase 3/7, which is expressed as relative units (RLU), and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG within Ad.DN-Akt or Ad.Null groups; *P<0.01 and #*P<0.001 versus HG within Ad.DN-Akt or Ad.Null groups. $P<0.01 versus the corresponding treatment of the Ad.Null group. (G) Bar graphs show the levels of activated caspase-3/7 in cultured adult cardiomyocytes exposed to high glucose in the presence of LY 294002 (50µM) or vehicle. Each experiment was performed in 6 wells per each condition and repeated three times. Values expressed as relative units (RLU), and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG within vehicle or LY 294002 groups; *P<0.001 versus HG within vehicle group. $P<0.01 versus corresponding treatment of the vehicle group. (H-M) Bar graphs show the levels of pSTAT3 (H), pAkt (I), Pim-1 (J), pBad (K) Bcl-2 (L) and activated caspase-3/7 (M) in cultured adult cardiomyocytes. Cardiomyocytes cultured in HG or NG were preincubated with STAT3 inhibitor peptide (1mM) or vehicle for 30 min, followed by another 24 h culture in the presence of benfotiamine (HGB) or vehicle. Values are expressed as n-fold changes toward NG (vehicle) for all parameters, except for caspase 3/7, which is expressed as relative units (RLU), and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG within STAT3 inhibitor peptide or vehicle groups; *P<0.01 and #*P<0.001 versus HG within STAT3 inhibitor peptide or vehicle groups. $P<0.01 versus corresponding treatment of
Supplemental Figure 11
Schematic illustration showing mechanisms implicated in BFT induced cardio-protection in the setting of diabetes.

Diabetes or hyperglycemia inhibits the phosphorylation of STAT3, which is known to activate cell survival Pim-1 by binding to its promoter. Furthermore, diabetes or hyperglycemia induces O-GlcNAC modification of Akt, resulting in inhibition of Akt activity. BFT acts on both arms of this pathway which converges into Pim-1. We found that BFT preserves STAT3 phosphorylation in cardiomyocytes, with this effect, together with Pim-1 upregulation, being abolished by STAT3 inhibitor peptide. In addition, BFT prevents O-GlcNAC modification of Akt, thereby restoring Akt activity and Pim-1 expression. The effect of BFT on Pim-1 expression is abolished by treating cardiomyocytes with dominant negative form of Akt or PI3-kinase inhibitor LY294002. On the other hand, diabetes or hyperglycemia induced the activation of protein phosphatase 2A (PP2A), which is known to dephosphorylate and destabilize Pim-1. However, the upregulation of PP2A was not affected by BFT, suggesting that BFT mainly acts through positive regulators of Pim-1.