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Therapeutic inhibition of mitochondrial reactive oxygen species with mito-TEMPO reduces diabetic cardiomyopathy

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Abstract

Aims—The mitochondria are important sources of reactive oxygen species (ROS) in the heart. Mitochondrial ROS production has been implicated in the pathogenesis of diabetic cardiomyopathy, suggesting that therapeutic strategies specifically targeting mitochondrial ROS may have benefit in this disease. We investigated the therapeutic effects of mitochondria-targeted antioxidant mito-TEMPO on diabetic cardiomyopathy.

Methods—The mitochondria-targeted antioxidant mito-TEMPO was administered after diabetes onset in a mouse model of streptozotocin-induced type-1 diabetes and type-2 diabetic db/db mice. Cardiac adverse changes were analyzed and myocardial function assessed. Cultured adult cardiomyocytes were stimulated with high glucose, and mitochondrial superoxide generation and cell death were measured.

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Appendix A. Supplementary material: Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2015.11.013>.

Results—Incubation with high glucose increased mitochondria superoxide generation in cultured cardiomyocytes, which was prevented by mito-TEMPO. Co-incubation with mito-TEMPO abrogated high glucose-induced cell death. Mitochondrial ROS generation, and intracellular oxidative stress levels were induced in both type-1 and type-2 diabetic mouse hearts. Daily injection of mito-TEMPO for 30 days inhibited mitochondrial ROS generation, prevented intracellular oxidative stress levels, decreased apoptosis and reduced myocardial hypertrophy in diabetic hearts, leading to improvement of myocardial function in both type-1 and type-2 diabetic mice. Incubation with mito-TEMPO or inhibition of Nox2-containing NADPH oxidase prevented oxidative stress levels and cell death in high glucose-stimulated cardiomyocytes. Mechanistic study revealed that the protective effects of mito-TEMPO were associated with down-regulation of ERK1/2 phosphorylation.

Conclusions—Therapeutic inhibition of mitochondrial ROS by mito-TEMPO reduced adverse cardiac changes and mitigated myocardial dysfunction in diabetic mice. Thus, mitochondria-targeted antioxidants may be an effective therapy for diabetic cardiac complications.

Keywords

Diabetic cardiomyopathy; ERK1/2; Mitochondria; Mito-TEMPO; Reactive oxygen species

1. Introduction

Globally, the number of adults affected with diabetes is rapidly growing and it is estimated to increase to nearly 400 million by 2030 [1]. Both type-1 and type-2 diabetes induce complications including visual impairments and blindness, nerve and kidney damage [2]. However, the greatest challenges lie in cardiovascular complications, accounting for up to 80% diabetes-related morbidity and mortality [3]. Diabetes can directly affect the heart, a condition described as diabetic cardiomyopathy. Diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs in the absence of changes in blood pressure and coronary artery disease [4]. So far, there is no specific therapy available for this disease.

Oxidative damage induced by reactive oxygen species (ROS) has been implicated in the pathogenesis of diabetic cardiomyopathy [5–7]. ROS is mainly produced by mitochondria, NADPH oxidase and xanthine oxidase in the heart [8,9]. Inhibition of xanthine oxidase or NADPH oxidase reduces diabetic cardiomyopathy [10–12]. There is convincing evidence that mitochondrial ROS production is increased in type-1 and type-2 diabetic hearts [13–17]. Transgenic over-expression of manganese superoxide dismutase (MnSOD) and mitochondrial catalase inhibit mitochondrial ROS and reduce cardiac hypertrophy, preserves cardiac structures and improves function in a mouse model of type-1 diabetes and in insulin-resistant and obese Ay mice, respectively [18,19]. These studies suggest a critical role of mitochondrial ROS in diabetic cardiomyopathy. Given the fact that commonly employed antioxidants have proven ineffective in clinical trials, it is possible that these agents may not be adequately delivered to the sub-cellular sites of ROS production. Because the mitochondria are important sources of ROS, we hypothesized that therapeutic inhibition of mitochondrial ROS by a mitochondrial targeted antioxidant (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride, monohydrate (mito-TEM-PO) might be beneficial in the setting of diabetic cardiomyopathy.

Mito-TEMPO is a physicochemical compound as one of SOD mimics. It has an ability to pass through lipid bilayers easily and accumulate selectively in mitochondria [20]. Both in vitro and in vivo studies have confirmed that mito-TEMPO is a mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties [20–22]. In vitro study showed that incubation with mito-TEMPO prevented cell death in adult cardiomyocytes induced by a pharmacological MnSOD inhibitor [23]. In vivo studies revealed that administration of mito-TEMPO improved cardiac function in a mouse model of pressure over-load heart failure [24], and reduced diabetes-attributable cardiac injury and mortality after myocardial infarction [25]. In this study, we demonstrated that therapeutic inhibition of mitochondrial ROS using mito-TEMPO prevented oxidative stress and reduced cardiomyopathic changes in mouse models of type-1 and type-2 diabetes. Our data strongly indicate that mitochondria-targeted antioxidants have therapeutic effects on diabetic cardiac complications.

2. Material and methods

2.1. Animals and cardiomyocytes culture

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). All experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada (2008-079). Breeding pairs of C57BL/6 mice and db+/- mice were purchased from the Jackson Laboratory. Transgenic mice over-expressing a circularly permuted yellow fluorescent protein in the mitochondrial matrix of cells (Tg-mtcpYFP) were kindly provided by Dr. Wang Wang (University of Washington, Seattle) [26]. A breeding program for mice was implemented at our animal care facilities.

Adult mouse ventricle cardiomyocytes were isolated from C57BL/6 mice, and cultured as described in our recent studies [27].

2.2. Experimental protocol

Type-1 diabetes was induced in adult male mice (2-month old) by consecutive intraperitoneal injection of streptozotocin (STZ, 50 mg/kg/day) for 5 days [11]. Seventy-two hours after the last injection of STZ, random blood glucose levels were measured using the OneTouch Ultra 2 blood glucose monitoring system (Life Scan, Inc., CA, USA). Mice were considered diabetic and used for the study only if they had hyperglycemia (> 15 mM) 72 h after STZ injection. Citrate buffer-treated mice were used as non-diabetic control (blood glucose < 12 mM). Thirty days after diagnosis, diabetic mice (6–8 in each group) received daily injection of mito-TEMPO (0.7 mg/kg/day, i.p., Enzo Life Sciences, Inc., the product number: ALX-430-150) [28] or vehicle for 30 days.

Type-2 diabetic db/db mice were produced by breeding db+/- mice. Male db/db mice and their littermate db+/- mice received daily injection of mito-TEMPO (0.7 mg/kg/day, i.p.) starting at age of 2.5 months for 30 days.

2.3. Echocardiography

Animals were lightly anaesthetized with inhalant isoflurane (1%) and imaged using a 40-MHz linear array transducer attached to a preclinical ultrasound system (Vevo 2100, FIIJIFILM VisualSonics, Canada) with nominal in-plane spatial resolution of 40 (μm (axial) $\times$ 80 (μm (lateral)). M-mode and 2-D parasternal short-axis scans (133 frames/s) at the level of the papillary muscles were used to assess changes in left ventricle (LV) end-systolic inner diameter, LV end-diastolic inner diameter, LV posterior wall thickness in end-diastole and end-systole, fractional shortening (FS%) and ejection fraction (EF%).

An apical four chamber view of the left ventricle was obtained and the pulsed wave Doppler measurements were performed in the apical view with a cursor at mitral valve inflow: maximal early (E) and late (A) transmitral velocities in diastole. The diastolic function was determined by the ratio of E to A peak.

2.4. Histological analyses

For cardiomyocyte cross-sectional area, several sections of the heart (5 μm thick) were prepared and stained for membranes with fluorescein isothiocyanate-conjugated wheat germ agglutinin (Invitrogen) and for nuclei with Hoechst. A single cardiomyocyte was measured by using an image quantitative digital analysis system (NIH Image version 1.6) as described [11]. The outline of at least 200 cardiomyocytes was traced in each section.

2.5. Measurement of ROS generation in isolated mitochondria

Interfibrillar mitochondria were isolated from freshly harvested hearts as described previously [29]. The isolated mitochondria were further purified using Percoll density gradient centrifugation. The freshly isolated mitochondria (10 μg) was incubated with pyruvate/malate (5/5 mmol/l) in a reaction buffer containing Amplex Red (0.05 mmol/l, Life Technologies Inc. Burlington, Ontario, Canada) and horseradish peroxidase (0.1 units/ml) at 37 °C. The fluorescent signals were monitored by spectrofluorometer at 520/580 nm for every 10 min.

2.6. Determination of oxidative stress levels in diabetic hearts

The oxidative stress levels in heart tissue lysates were measured by using 2,7-dichlorodihydro-fluorescein diacetate (DCF-DA, Invitrogen, USA) as an indicator [11]. Briefly, fresh heart tissues were homogenized in an assay buffer. The homogenates (50 μg protein) were incubated with DCF-DA at 37 °C for 3 h. The fluorescent product formed was quantified by spectrofluorometer at 485/525 nm. Changes in fluorescence were expressed as arbitrary unit.

The protein oxidation in heart tissue lysates was assessed by measuring protein carbonyl content using a commercial assay kit (Cayman Chemical, USA) following manufacturer's instructions.

2.7. Measurement of mitochondrial superoxide generation in cardiomyocytes

Superoxide flashes in single mitochondrion were measured to determine mitochondrial superoxide generation in living cardiomyocytes as described [30]. Briefly, cardiomyocytes

were infected with an adenoviral vector expressing mt-cpYFP (Ad-mt-cpYFP, kindly provided by Dr. Wang Wang from the University of Washington, Seattle, USA). Ad-mt-cpYFP expresses a circularly permuted yellow fluorescent protein (cpYFP) in the mitochondrial matrix of cells using the cytochrome C oxidase subunit IV targeting sequence (mt-cpYFP). Twenty-four hours after infection, confocal imaging was recorded using the Olympus FV 1000 laser-scanning microscope equipped with a 63 ×, 1.3NA oil immersion objective and a sampling rate of 0.7 s/frame. At least 20 cardiomyocytes per culture in each group were analyzed.

Mitochondrial superoxide generation was also assessed in living cardiomyocytes from Tg-mt-cpYFP mice by using the MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes) and oxidant levels measured by using Dihydroethidium (DHE, Molecular Probes). The cpYFP signals were used to identify mitochondrial MitoSOX™ Red and DHE staining in cardiomyocytes.

2.8. Determination of apoptotic cell death

Caspase-3 activity in myocardial tissues and cardiomyocytes was measured by using a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories).

Cell death was also determined in cardiomyocytes by annexin V/Hoechst staining as described [31].

2.9. Real-time RT-PCR

Total RNA was extracted from heart tissues using the Trizol Reagent (Life Technologies Inc., Burlington, Ontario, Canada). Realtime RT-PCR was performed to analyze mRNA expression for β -myosin heavy chain (β -MHC), atrial natriuretic peptide (ANP), gp91^{phox}, p47^{phox} and GAPDH as previously described [11].

2.10. Western Blot analysis

The protein levels of Bcl-2, phosphorylated and total extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun NH2-terminal kinase-1/2 (JNK1/2), p38 kinase, and GAPDH were determined by western blot analysis using their specific antibodies (Cell Signaling, Danvers, MA).

2.11. Statistical analysis

All data were given as means $\pm$ SD. Differences between two groups were compared by unpaired Student's *t*-test. For multi-group comparisons, ANOVA followed by Newman-Keuls test was performed. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. Mito-TEMPO inhibited high glucose-induced mitochondrial superoxide generation and cell death in cardiomyocytes

To determine the effects of mito-TEMPO on mitochondrial superoxide generation and cell death, we incubated adult cardiomyocytes with normal glucose (5 mmol/l) or high glucose

(30 mmol/l) in the presence of mito-TEMPO (25 nmol/l) or vehicle for 24 h. This dose of mito-TEMPO was chosen because it has been shown to increase mitochondrial superoxide dismutation by 3-fold while not affecting cytoplasmic dismutation in cultured cells [20]. As shown in Fig. 1A, high glucose increased mitochondrial flashes in cardiomyocytes, which were abrogated by mito-TEMPO, indicating an increase in mitochondrial superoxide generation. This was further confirmed by the MitoSOX™ Red or DHE staining in living cardiomyocytes during high glucose stimulation (Fig. 1B and C).

High glucose induced cardiomyocyte death as determined by annexin-V/Hoechst staining. Co-incubation with mito-TEMPO prevented high glucose-induced cell death in cardiomyocytes (Fig. 1D and E). These results demonstrate that inhibition of mitochondrial superoxide generation prevents high glucose-induced cardiomyocyte death.

3.2. Administration of mito-TEMPO abolished mitochondrial ROS generation and oxidative stress in hearts of diabetic mice

Injection of mito-TEMPO did not affect the blood glucose levels, heart weight and body weight in both sham and diabetic mice (Supplemental Tables 1 and 2). Neither abnormal behaviors or health problems including myocardial function and death nor changed intake of food and water was observed due to the use of mito-TMEPO in sham mice (Supplemental Table 3), suggesting there might be no obvious toxic side-effects of mito-TEMPO.

Mitochondrial ROS generation was significantly increased in both db/db and STZ-induced mouse hearts. Administration of mito-TEMPO abolished mitochondrial ROS generation in diabetic mouse hearts (Fig. 2A and B).

To assess oxidative stress in diabetic hearts, we first measured oxidative stress levels in heart tissue lysates. The oxidative stress levels were increased in db/db and STZ-induced mouse hearts, which was inhibited by mito-TEMPO (Fig. 2C and D). The total antioxidant capacity (including small molecule and protein antioxidants) was also increased in diabetic hearts (Supplemental Fig. 1). We then determined the oxidative damage in diabetic mice hearts by measuring the protein carbonyl content. The protein carbonyl content was elevated in db/db and STZ-induced mouse hearts. However, injection with mito-TEMPO abrogated diabetes-induced protein carbonyl content (Fig. 2E and F). These results suggest that mito-TEMPO effectively blocks mitochondrial ROS production and prevents oxidative stress in diabetic mouse hearts.

Since NADPH oxidase is another important source of ROS in the heart, we measured NADPH oxidase expression. Real-time PCR revealed that the mRNA levels of gp91^{phox} and p47^{phox} were significantly increased in STZ-induced type-1 diabetic hearts but not in db/db mouse hearts. Administration of mito-TEMPO decreased the mRNA levels of gp91^{phox} and p47^{phox} in STZ-induced mouse hearts whereas it did not have significant impact on their expression in db/db mouse hearts (Fig. 3A–D).

To examine whether mitochondrial superoxide interacts with gp91^{phox}-containing NADPH oxidase (Nox2) activation, we used gp91ds-tat peptide to inhibit Nox2 activation in cardiomyocytes [32]. Cardiomyocytes were incubated with normal or high glucose in the

presence of gp91ds-tat (10 μ mol/l, AnaSpec, Inc., Fremont, CA, USA), mito-TEMPO (25 nmol/l) and vehicle, either alone or in combination for 24 h. Gp91ds-tat and mito-TEMPO alone or in combination prevented mitochondrial superoxide production and oxidant levels induced by high glucose as determined by the MitoSOX Red and DHE staining, respectively (Fig. 3E and F). Similarly, high glucose-induced cell death was inhibited by gp91ds-tat or mito-TEMPO; however, a combination of gp91ds-tat and mito-TEMPO did not provide further protection in cardiomyocytes (Fig. 4), suggesting a potential interaction between mitochondrial superoxide and Nox2 activation.

3.3. Mito-TEMPO reduced cardiomyopathic changes in type-2 diabetic db/db mice

Histological analysis of cardiomyocyte cross-sectional areas showed an increase in cardiomyocyte size from db/db mouse hearts. However, administration of mito-TEMPO did not affect cardiomyocyte size in sham animals but reduced cardiomyocyte size in db/db mice hearts (Fig. 5A). Similarly, the mRNA levels of ANP and β -MHC were elevated in diabetic db/db mouse hearts and significantly reduced in db/db mice receiving mito-TEMPO (Fig. 5B and C). These results demonstrate that mito-TEMPO prevents myocardial hypertrophy in type-2 diabetic db/db mice.

Apoptosis plays an important role in development of diabetic cardiomyopathy. We examined the effects of mito-TEMPO on apoptosis in db/db mouse hearts. Diabetes increased caspase-3 activity, an indicator of apoptosis, and decreased the protein levels of BCL-2, an important anti-apoptotic factor in db/db mouse hearts. Administration of mito-TEMPO prevented caspase-3 activity and increased BCL-2 protein expression in db/db mouse hearts (Fig. 5D and E). These results suggest that inhibition of mitochondrial ROS protects the heart against apoptosis in diabetic mice.

Echocardiographic analysis revealed a decline of E/A ratio in db/db mice (Fig. 5F), indicating myocardial diastolic dysfunction, while myocardial systolic function was preserved in db/db mice as determined by FS% and EF% (Fig. 5G and H, and Supplemental Table 4). Injection of mito-TEMPO significantly increased the E/A ratio in db/db mice (Fig. 5F). Taken together, administration of mito-TEMPO reduces cardiomyopathy in type-2 diabetic db/db mice.

3.4. Mito-TEMPO mitigated diabetic cardiomyopathy in STZ-induced mice

We also examined the effects of mito-TEMPO on diabetic cardiomyopathy in STZ-induced mice. Consistently, cardiomyocyte cross-sectional areas and gene expression of ANP and β -MHC were significantly increased in STZ-induced type-1 diabetic hearts (Fig. 6A–C). These hypertrophic changes were attenuated by mito-TEMPO (Fig. 6A–C). Injection of STZ increased caspase-3 activity (Fig. 6D) and reduced anti-apoptotic protein BCL-2 in the heart (Fig. 6E), which were prevented by mito-TEMPO (Fig. 6D and E). Finally, myocardial diastolic and systolic functions were decreased in STZ-induced mice as determined by the E/A ratio (Fig. 6F), FS% and EF% (Fig. 6G and H, and Supplemental Table 5), respectively. However, administration of mito-TEMPO restored both myocardial diastolic and systolic functions in STZ-induced type-1 diabetic mice (Fig. 6F–H and Supplemental Table 4).

3.5. Mito-TEMPO attenuated ERK1/2 activation in diabetic mouse hearts and high glucose-stimulated cardiomyocytes

Since activation of mitogen-activated protein kinase has been implicated in diabetic cardiomyopathy and they are sensitive to oxidative stress, we therefore examined the phosphorylation of its three family members: ERK1/2, JNK1/2 and p38. Western blot analysis showed that phosphorylated ERK1/2 was increased in both diabetic mouse hearts (Fig. 7A and B) while no change in phosphorylated JNK1/2 and p38 was observed (data not shown). Mito-TEMPO significantly reduced ERK1/2 phosphorylation in db/db and STZ-induced mouse hearts (Fig. 7A and B).

To further address the involvement of ERK1/2, we incubated adult cardiomyocytes with normal (5 mmol/l) or high glucose (30 mmol/l) in the presence of mito-TEMPO (25 nmol/l) or vehicle for 24 h. High glucose increased phosphorylated ERK1/2, which was abolished by mito-TEMPO (Fig. 7C). In addition, co-incubation with ERK1/2 inhibitor PD98059 (10 μ mol/l) prevented high glucose-induced cell death (Fig. 7D).

4. Discussion

The present study provides the first evidence that therapeutic inhibition of mitochondrial ROS by mito-TEMPO reduces cardio-myopathic changes and improves myocardial function in type-1 and type-2 diabetic mice. Our study also provides direct evidence that high glucose induces mitochondrial superoxide generation, which is prevented by mito-TEMPO. We further suggest that ROS amplified in mitochondria subsequently activates downstream ROS-sensitive signaling pathways (e.g. ERK1/2) implicated in pathological cardiac changes in diabetes (Supplemental Fig. 2).

This study demonstrates that both type-1 and type-2 diabetes promoted mitochondrial ROS generation, increased intracellular oxidative stress levels and induced oxidative damage in the heart, which are consistent with previous reports [13–17]. The total antioxidant capacity was also increased in diabetic hearts as well. Furthermore, our in vitro study provides direct evidence that high glucose induced mitochondrial superoxide generation in cultured cardiomyocytes. Thus, the elevation of oxidative stress levels and oxidative damage may be caused by increased ROS generation rather than decreased antioxidant capacity. Whereas we used cpYFP as a probe to measure mitochondrial superoxide flashes in cardiomyocytes, we are aware there has been a controversy concerning the use of the cpYFP as a superoxide probe [33,34]. To validate mitochondrial superoxide generation in cardiomyocytes, we also assessed mitochondrial superoxide production by using the MitoSOX™ Red mitochondrial superoxide indicator. In addition, DHE was employed to measure oxidant levels in cardiomyocytes. Both MitoSOX™ Red and DHE staining showed an increase in mitochondrial oxidant levels during high glucose stimulation, which was inhibited by mitochondrial-targeted antioxidant mito-TEMPO, similar to the changes in mitochondrial flashes.

It is well known that excessive mitochondrial ROS causes mitochondrial dysfunction in cardiomyocytes [35], compromising ATP production and inducing cell death [36], both of which directly contribute to myocardial dysfunction [37]. In fact, ATP product is reduced

and apoptosis is induced in diabetic hearts [38,39]. Mitochondrial ROS over-production also promotes adverse myocardial hypertrophy, an important cellular hallmark of diabetic cardiomyopathy [40,41]. Thus, targeted inhibition of mitochondrial ROS by transgenic over-expression of SOD2 and mitochondrial catalase prevents cardiac adverse changes and dysfunction in a mouse model of type-1 diabetes and in insulin-resistant and obese Ay mice, respectively [18,19]. The present study extends these previous findings to investigate the therapeutic potentials of targeted inhibition of mitochondrial ROS in both type-1 and type-2 diabetic mice. We show that incubation with mito-TEMPO efficiently inhibited mitochondrial superoxide generation in high glucose-stimulated cardiomyocytes and treatment of mito-TEMPO after diabetes onset abolished diabetes-induced mitochondrial ROS production and oxidative damage in hearts. Consequently, administration of mito-TEMPO prevented hypertrophy and attenuated myocardial dysfunction in both type-1 and type-2 diabetic mice. Thus, antioxidant strategies specifically targeting mitochondria may have therapeutic benefit in diabetic cardiac complications.

Gp91^{phox}-containing NADPH oxidase is another important source of ROS in cardiomyocytes [42]. It consists of cytosolic subunits (p67^{phox}, p47^{phox}, p40^{phox} and Rac1) and membrane subunits (gp91^{phox} and p22^{phox}). We have recently demonstrated that gp91^{phox} containing NADPH oxidase significantly contributes to diabetic cardiomyopathy [11,31]. The present study shows that gp91^{phox} and p47^{phox} were up-regulated in STZ-induced mouse hearts, which is consistent with our recent report [11]. In contrast, NADPH oxidase expression was not altered in type-2 diabetic db/db mice, suggesting a differential expression of NADPH oxidase in type-1 and type-2 diabetes. Inhibition of mitochondrial ROS by mito-TEMPO prevented up-regulation of NADPH oxidase expression in type-1 diabetic hearts. This suggests that mitochondrial ROS signaling may promote NADPH oxidase expression in type-1 diabetic hearts. In fact, this has been recognized that mitochondrial ROS positively regulates NADPH oxidase subunits expression and activation under pathological conditions including diabetes [43]. On the other hand, we have recently reported that deletion of Rac1 or pharmacological inhibition of NADPH oxidase activation reduces mitochondrial ROS generation in diabetic hearts, suggesting that NADPH oxidase promotes mitochondrial ROS generation [31]. Thus, it is most likely that cross-talks between mitochondria and NADPH oxidase form a positive feedback loop in favor of ROS production and oxidative damage in type-1 diabetic hearts, and disruption of this feedback loop by inhibiting either of them provides beneficial effects in diabetes. This is indeed supported by our findings that inhibition of either mitochondrial superoxide or gp91^{phox}-containing NADPH oxidase prevents mitochondrial superoxide production and attenuates apoptosis in high glucose-stimulated cardiomyocytes.

Apoptosis has been implicated in the pathogenesis of diabetic cardiomyopathy [4,39,44]. Cardiomyocyte apoptosis has been reported to occur in diabetic animal models and patients [44]. Apoptotic cell death in the heart causes a loss of contractile tissue which initiates cardiac remodeling. The loss of cardiomyocytes and hypertrophy of the remaining viable cardiomyocytes characterize the diabetic cardiomyopathy. As such, inhibition of cardiomyocyte apoptosis has been shown to prevent the development of diabetic heart diseases [45]. In the present study, administration of mito-TEMPO prevented caspase-3 activation in type-1 and type-2 diabetic mouse hearts. This effect of mito-TEMPO was

associated with up-regulation of BCL-2 protein in diabetes. In cultured cardiomyocytes, we provide direct evidence in support of our hypothesis that inhibition of mitochondrial superoxide generation protects cardiomyocytes under diabetic conditions. Thus, inhibition of apoptotic cell death may be one of important mechanisms by which mito-TEMPO reduces diabetic cardiomyopathy.

ROS production and subsequent oxidative damage have been demonstrated to activate a variety of signaling pathways, among which ERK1/2, p38 and JNK1/2 have been implicated in apoptosis and hypertrophy in the heart [46,47]. Studies have shown that ERK1/2 is activated in diabetic hearts whereas p38 and JNK1/2 activities are either increased or decreased in diabetes [48,49]. Activation of ERK1/2 and p38 contributes to apoptosis and hypertrophy in cardiomyocytes under diabetic conditions [50,51]. The present study shows that the levels of phosphorylated ERK1/2 were increased in type-1 and type-2 diabetic hearts; however, phosphorylation of p38 and JNK1/2 remained unaltered. Importantly, administration of mito-TEMPO prevented ERK1/2 activation in diabetic hearts and cardiomyocytes under diabetic conditions, and inhibition of ERK1/2 prevented cell death in high glucose-stimulated cardiomyocytes. Thus, blocking ERK1/2 signaling may represent a potential mechanism underlying the cardiac protection of mito-TEMPO in diabetes.

Although mito-TEMPO is a mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties [20], it is currently unknown whether there are any off-target effects of mito-TEMPO when it is accumulated in mitochondria, and it is also unclear how much of mito-TEMPO has actually gone to the heart. The present study shows that administration of mito-TEMPO did not display any effects on blood glucose, body weight, activity and dietary ingestion in both sham and diabetic mice, suggesting no significant side-effects of this reagent. In addition, systemic administration of mito-TEMPO may provide protective effects on other organs in diabetes, which may benefit the heart.

5. Conclusions

Administration of mitochondria-targeted antioxidants may be an effective therapy for diabetic cardiac complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ERK1/2	extracellular signal-regulated kinase-1/2
Mito-TEMPO	(2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride, monohydrate
ROS	reactive oxygen species
STZ	streptozotocin

Fig. 1.

Effects of mito-TEMPO (M-TEMPO) on mitochondrial superoxide generation and cell death in cardiomyocytes. Adult mouse cardiomyocytes were incubated with normal glucose (NG) or high glucose (HG) for 24 h in the presence of M-TEMPO or vehicle. (A) Single mitochondrial superoxide flashes were determined. (B) Representative microphotographs from 5 different cultures for the MitoSOX staining, cpYFP signals and nuclei (Hoechst staining) show overlap of MitoSOX staining and cpYFP signals in cardiomyocytes. (C) Representative microphotographs from 5 different cultures for the DHE staining, cpYFP signals and nuclei (Hoechst staining) show overlap of DHE staining and cpYFP signals in cardiomyocytes. (D) A representative staining for annexin V (green color) and nuclei (blue color) and (E) quantification of death cells (%). Data are mean $\pm$ SD from 5 different cell cultures. * $P < 0.05$ versus vehicle +NG and † $P < 0.05$ versus vehicle +HG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Effects of mito-TEMPO (M-TEMPO) on mitochondrial ROS generation and oxidative stress. Mitochondrial oxidant levels were assessed using Amplex Red in db+/- and db/db hearts (A) or sham and STZ-treated hearts (B). Oxidative stress levels were determined using DCF-DA in db+/- and db/db hearts (C) or sham and STZ-treated hearts (D). Protein carbonyl content was measured in db+/- and db/db hearts (E) or sham and STZ-treated hearts (F). Data are mean $\pm$ SD; $n=6-8$. * $P < 0.05$ versus vehicle in db+/- or sham and † $P < 0.05$ versus vehicle in db/db or STZ.

Fig. 3.

(A–D) Effects of mito-TEMPO (M-TEMPO) on NADPH oxidase expression in diabetic mice. The mRNA levels of gp91^{phox} and p47^{phox} mRNA in db+/- and db/db mouse hearts (A and B) or sham and STZ-treated mouse hearts (C and D) were determined by real-time RT-PCR. Data are mean $\pm$ SD; $n=6-8$. * $P < 0.05$ versus sham and † $P < 0.05$ versus vehicle +STZ. (E and F) Effects of gp91ds-tat and mito-TEMPO (M-TEMPO) on mitochondrial superoxide production in cardiomyocytes. Cultured cardiomyocytes isolated from Tg-mtcpYFP mice were incubated with normal (NG) or high glucose (HG) in the presence of gp91ds-tat, M-TEMPO or vehicle, either alone or in combination for 24 h. Mitochondrial superoxide production was then determined. (E) Representative microphotographs for the MitoSOX staining, cpYFP signals and nuclei (Hoechst staining) show overlap of MitoSOX staining and cpYFP signals in cardiomyocytes. (F) Representative microphotographs for the DHE staining, cpYFP signals and nuclei (Hoechst staining) show overlap of DHE staining and cpYFP signals in cardiomyocytes. Data are from 5 different cultures.

Fig. 4. Effects of gp91ds-tat and mito-TEMPO (M-TEMPO) on cell death. Cultured cardiomyocytes isolated from Tg-mtcpYFP mice were incubated with normal (NG) or high glucose (HG) in the presence of gp91ds-tat, M-TEMPO or vehicle, either alone or in combination for 24 h. Cell death was determined by annexin V staining. (A) Representative staining for annexin V (green color) and nuclei (blue color). (B) Quantification of death cells (%). Data are mean $\pm$ SD from 5 different cultures. * $P < 0.05$ versus vehicle+control+NG and † $P < 0.05$ versus vehicle+control+HG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Effects of mito-TEMPO (M-TEMPO) on cardiomyopathic changes in db/db mice. (A) Cardiomyocyte cross-sectional areas. (B and C) The mRNA levels of ANP and β -MHC in db/db mouse hearts. (D) Caspase-3 activity in db/db hearts. (E) The upper panel is a representative western blot for BCL-2 in myocardial tissues, and the lower panel is the quantitative data from western blot analysis for BCL-2/GAPDH. (F) Myocardial diastolic function (E/A). Data are mean $\pm$ SD; $n=6-8$. * $P < 0.05$ versus vehicle in db+/- and † $P < 0.05$ versus vehicle in db/db.

Fig. 6. Effects of mito-TEMPO (M-TEMPO) on cardiomyopathic changes in STZ-injected mice: (A–D) Cardiomyocyte cross-sectional areas (A), the mRNA levels of ANP (B) and β -MHC (C) and caspase-3 activity (D) were determined in STZ-treated hearts. (E) The upper panel is a representative western blot for BCL-2, and the lower panel is the quantification of BCL-2 protein levels relative to GAPDH. (F) Echocardiographic analysis (E/A). Data are mean $\pm$ SD; $n=6-8$. * $P < 0.05$ versus sham and † $P < 0.05$ versus vehicle+STZ.

Fig. 7.

Effects of mito-TEMPO(M-TEMPO) on ERK1/2 phosphorylation in hearts and cardiomyocytes. Upper panels are representative western blots for phosphorylated ERK1/2 (p-ERK) and total ERK1/2 (T-ERK), and lower panels are quantitative data of p-ERK/T-ERK ratio in db+/- and db/db hearts (A), sham and STZ-treated hearts (B) or high glucose-stimulated cardiomyocytes (C). (D) Cell death was determined by annexin V staining. Upper panel is a representative staining for annexin V (green color) and nuclei (blue color) and lower panels are the quantification of death cells (%). Data are mean $\pm$ SD; $n=6-8$ or 5 different cultures. * $P < 0.05$ versus vehicle in db+/-, sham or normal glucose and † $P < 0.05$ versus vehicle in db/db, STZ or high glucose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)