Isosteviol ameliorates diabetic cardiomyopathy in rats by inhibiting ERK and NF-κB signaling pathways

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Abstract

Diabetes-induced injury of myocardium, defined as diabetic cardiomyopathy (DCM), accounts for significant mortality and morbidity in diabetic population. Alleviation of DCM by a potent drug remains considerable interests in experimental and clinical researches because hypoglycemic drugs cannot effectively control this condition. Here, we explored the beneficial effects of isosteviol sodium (STVNa) on type 1 diabetes-induced DCM and the potential mechanisms involved. Male Wistar rats were induced to diabetes by injection of streptozotocin (STZ). One week later, diabetic rats were randomly grouped to receive STVNa (STZ/STVNa) or its vehicle (STZ). After 11 weeks of treatment or 11 weeks treatment following 4 weeks of removal of the treatment, the cardiac function and structure were evaluated and related mechanisms were investigated. In diabetic rats, oxidative stress, inflammation, blood glucose and plasma advanced glycation end products (AGEs) were significantly increased, whereas superoxide dismutase 2 (SOD-2) expression and activity were decreased. STVNa treatment inhibited cardiac hypertrophy, fibrosis and inflammation, showed similar ratio of heart to body weight and antioxidant capacities almost similar to the normal controls, which can be sustained at least 4 weeks. Moreover, STVNa inhibited diabetes-induced stimulation of both extracellular signal-regulated kinase (ERK) and nuclear factor κB (NF-κB) signal pathways. However, blood glucose, plasma AGE and insulin levels were not altered by STVNa treatment. These results indicate that STVNa may be developed into a potent therapy for DCM. The mechanism underlying this therapeutic effect involves the suppression of oxidative stress and inflammation by inhibiting ERK and NF-κB without changing blood glucose or AGEs.

Introduction

Diabetic cardiomyopathy (DCM) induced by diabetes is increasingly recognized as the most important cause of elevated morbidity and mortality among diabetic patients (Chavali et al. 2013). DCM is characterized by abnormalities of structure and function of the left ventricle (LV), including hypertrophy and fibrosis, as well as diastolic and/or systolic contractile dysfunction. It is generally recognized that hyperglycemia-induced oxidative stress is a critical cause for this in diabetic complication. In fact, DCM is frequently accompanied...
with elevated formation of reactive oxygen species (ROS) (Nishikawa et al. 2000, Haidara et al. 2006) and reduced endogenous antioxidant capacity to clear the excessive ROS (Maritim et al. 2003). Increased ROS exacerbate cardiac inflammation and fibrotic remodeling (Rajesh et al. 2009, Huynh et al. 2013). Additionally, hyperglycemia-induced glycation of proteins, lipids and nucleic acids can generate advanced glycation end products (AGEs). AGEs interact with its receptors (RAGEs) to affect the intracellular signaling pathways, produce free radicals and release pro-inflammatory cytokines (Ma et al. 2009, Bodiga et al. 2014). It has been suggested that extracellular signal-regulated kinase (ERK) and nuclear factor κB (NF-κB) are the key mediators activated by AGEs–RAGEs interaction (Barlovic et al. 2011) to promote the pathophysiological process of DCM (Lorenzo et al. 2011, Xu et al. 2016).

Despite extensive studies in the past decade, the exact mechanisms of hyperglycemia causing DCM are not fully demonstrated (Dobrin & Lebeche 2010, Liu et al. 2014), and there are no effective therapies for this serious condition. Stevioside as well as the analog isosteviol, components of Stevia rebaudiana leaf, whose toxicology and safety were fully investigated, were used as safe conventional noncaloric sweeteners for hundreds of years (Brahmachari et al. 2011). Several researches have shown the anti-type 2 diabetic and cardioprotective effects of isosteviol and related compounds (Ma et al. 2007, Xu et al. 2007). Isosteviol sodium (STVNa) is an improved formulation with higher solubility and bioavailability (Lai et al. 2017), which has been shown by our laboratory to have the ability to alleviate cerebral ischemia injuries (Hu et al. 2016) and ischemia reperfusion-induced mitochondrial dysfunction in vitro (Sun et al. 2017). However, the effects of isosteviol and/or related compounds on DCM have not been investigated.

Since hyperglycemia and AGE-RAGE-stimulated ERK and NF-κB signal pathways play important roles in the formation of oxidative stress and development of DCM, and a previous study showed that isosteviol could improve glucose tolerance (Ma et al. 2007), we hypothesized that STVNa could depress blood glucose, AGEs and inhibit ERK and NF-κB, further attenuate diabetes-induced cardiac injuries and dysfunctions and activate endogenous antioxidant mechanisms. The present study evaluated: (1) whether STVNa could ameliorate or inhibit the development and progression of type 1 diabetes-induced cardiac dysfunction; (2) whether the potential effect of STVNa was related to anti-hyperglycemia and/or AGE-RAGE/ERK/NF-κB inhibiting effects; (3) was there any memory of cardioprotection after stopping the treatment.

Materials and methods

Animals and treatment

The study was performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 82-23, revised 1996) and was reviewed and approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Male Wistar (6- to 8-week-old) rats (N=80; body weight (BW): 190–220 g) were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China) and housed 3–4/cage under 12/12-h light/darkness cycles, kept at constant room temperature of 22±2°C with standard laboratory rat diet and water provided ad libitum. Streptozotocin (STZ; Sigma) was freshly prepared by dissolving it in 0.1 M citrate buffer (pH=4.5). The rats were fasted overnight after one week of acclimatization, diabetes were induced by single i.p. injection of STZ (55 mg/kg BW). Control animals received an equivalent volume of citrate buffer. One week later, the blood glucose concentrations were measured using a glucometer (AccuSoft) and glucose test strips (Accu-Chek Advantage; Roche Diagnostics), the rats with hyperglycemia (≥11.1 mmol/L) were considered as diabetic and allowed to be assigned to diabetic with or without treatment groups.

After one week of citrate buffer (Normal control group, N=10) or STZ injection (N=40), animals with diabetes (N=32) successfully induced were randomly assigned into solvent treatment (STZ, N=16) and STVNa treatment (STZ/STVNa, N=16). All rats except normal group started treatment accordingly for 11 weeks (total for 12 weeks), cardiac function was measured for 25 rats (N=5, 10, 10 for each group) and then killed, 17 rats (N=5, 6, 6 for each group) were further fed without any treatment for another 4 weeks (total for 16 weeks), and cardiac function was measured and killed. The oral dose of STVNa (dissolved in normal saline solvent) was 8 mg/kg/day (twice daily), which optimized by our researches about STVNa on pressure overload induced cardiac hypertrophy (unpublished) and doses previously shown to be neuroprotective in rats (Hu et al. 2016). Additionally, 22 rats were divided into Normal and STZ groups (N=4–6/group) and sustained for 4 or 8 weeks to study the progress of the DCM. The above research design diagram is shown in Fig. 1. In addition, ten rats were divided into normal group (N=5) and normal rats treated with STVNa group (N=5) and sustained for 6 weeks to study the potential effects of STVNa on normal rats.
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Briefly, rats were sedated and mechanically ventilated, the hemodynamic function was recorded by inserting a SPR-838 micro tip catheter (Millar Instruments, Inc., Houston, TX, USA) equipped with MPCU-400 PV signal conditioning hardware into LV via the right carotid artery. LV diastolic and systolic functions were evaluated by assessment maximal rate of pressure rise and pressure fall (\(dp/dt_{\text{max}}\) and \(dp/dt_{\text{min}}\)), LV end diastolic pressure (LVEDP), LV end systolic pressure (LVESP), heart rate (HR) and mean arterial pressure (MAP) in each study group. The data were analyzed by LabChart software (Millar Instruments, Inc.; version 7.0).

**Hemodynamic measurements**

Cardiac function was measured in rats anesthetized with 3% pentobarbital sodium (40–50mg/kg) as previously described (Pacher et al. 2008). Briefly, rats were sedated and mechanically ventilated, the hemodynamic function was recorded by inserting a SPR-838 micro tip catheter (Millar Instruments, Inc., Houston, TX, USA) equipped with MPCU-400 PV signal conditioning hardware into LV via the right carotid artery. LV diastolic and systolic functions were evaluated by assessment maximal rate of pressure rise and pressure fall (\(dp/dt_{\text{max}}\) and \(dp/dt_{\text{min}}\)). LV diastolic and systolic functions were evaluated by assessment maximal rate of pressure rise and pressure fall (\(dp/dt_{\text{max}}\) and \(dp/dt_{\text{min}}\)). LV end diastolic pressure (LVEDP), LV end systolic pressure (LVESP), heart rate (HR) and mean arterial pressure (MAP) in each study group. The data were analyzed by LabChart software (Millar Instruments, Inc.; version 7.0).

**Tissue collection and plasma analysis**

After the hemodynamic analysis was performed, hearts were excised surgically and blood was collected and centrifuged to obtain plasma. Hearts were washed totally with ice-cold PBS to clear the blood. HW and BW were weighted and the ratios of HW to BW were calculated. Then, the ventricular portions of the heart were cut into 2 cm sections and fixed or snap frozen in liquid nitrogen for further experiments. Following excision of the heart, plasma was separated from blood collected from the chest cavity and stored at ~80°C until use. Rat circulation tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-6 (IL-6), insulin and AGEs levels were measured in plasma from every sample by commercially available RATTNF-\(\alpha\), IL-6 and INSULIN ELISA kits (JissKang Biotech, Qingdao, China) and RAT Advanced Glycation End Products ELISA kit (SenBeijia Biological, Nanjing, China) respectively following the manufacturer’s instructions. As an auxiliary method, fluorescence measurements of AGEs were monitored using a luminescence spectrometer Berthold LB943 at excitation (ex) and emission (em) wavelengths of 370/440 nm for the total AGEs level, as previously described (Piwowar et al. 2008).

**Histological and immunohistochemical evaluation**

The ventricles were fixed in 10% formalin for at least 24 h, and then paraffin-embedded and were sectioned at 3\(\mu\)m for routine staining with hematoxylin and eosin (H&E) to determine cardiomyocyte sizes, as well as 0.1% Sirius Red to measure LV fibrosis, as previously described (Ritchie et al. 2012). All histological analyses were performed by two investigators who had no knowledge of identities of the slides.

Cardiomyocyte cross-sectional areas were assessed by H&E staining of LVs. At least five fields were randomly selected at magnification of 400× containing the capillary profiles and nuclei of myocardium. Images of the heart sections were obtained by an Axioplus image-capturing system (Zeiss) and analyzed by Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). At least 50 cells per heart were chosen for analysis. For cardiac tissue fibrosis evaluation, cardiac sections were stained with Sirius Red (Huamaike Biotech, Beijing, China). Images were captured by the image-capturing system (Zeiss) at magnification of 200× and analyzed by Image-Pro Plus. The content of tissue fibrosis (expressed as the fibrotic area to the entire cross area) was averaged over all fields.

Immunohistochemical (IHC) analysis of 8-hydroxy-2′-deoxyguanosine (8-OHdG) or CD68 was performed with mouse monoclonal antibody against 8-OHdG (Santa Cruz; #sc-393871, 1:100) or CD68 (Santa Cruz; #sc-20060, 1:100) and a biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) as described previously (Yamaleyeva et al. 2012) with some modifications. Briefly, the sections of each heart were dewaxed, hydrated with graded series of ethanol, and then treated with 0.3% H\(_2\)O\(_2\) for 30 min. After washing with Tris buffered solution, the sections were blocked with goat serum to reduce nonspecific reactions and incubated with mouse monoclonal antibody against 8-OHdG or CD68 at 4°C, followed by incubation with biotinylated anti-mouse immunoglobulin for 30 min. The horseradish peroxidase-labeled streptavidin solution was added for 30 min. The peroxidase-binding sites and nuclei were visualized with DAB (Sigma-Aldrich) and hematoxylin, respectively. The density of positive staining was analyzed using Image-Pro Plus.
Semi-quantitative PCR and real-time PCR for gene expression analysis

Cardiac tissues were frozen and stored at −80°C until use. Total RNA was extracted using TRIzol reagent (Generay Biotech, Shanghai, China). The concentrations of total RNA samples were determined by NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Inc.), and agarose gel electrophoresis was used to confirm the purities. First-strand cDNAs were synthesized with 1 μg total RNA in a 20 μL reaction system using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol.

The levels of gene were determined by semi-quantitative PCR first. The primers, whose sequences were listed in Supplementary Table 1 (see section on supplementary data given at the end of this article), were synthesized by Generay Biotech. The PCR reactions were performed with a 20 μL solution containing 10 μL MasterMix (universal PCR mix solution, Generay), 1 μL cDNA, 2 μL primer (1 μL for forward and reverse, respectively) and 7 μL water, using the ABI 2720 Thermocycler (Life Technologies Corp.). In order to ensure the amplification was within the logarithmic phase, the number of PCR cycles of every gene was optimized. PCR products were resolved on a 1.2–1.5% agarose gel. The intensity of the target gene was calculated using Image Pro Plus (Media Cybernetics) and expressed as fold changes normalized by internal gene control beta actin (Actb). Each experiment was performed in duplicate or triplicate.

The expression of Nppb (BNP), Tgfb1 (TGF-β1), p47 phox, Sod2, interleukin 1-beta (IL-1β) and advanced glycosylation end product-specific receptor (Ager) were confirmed by real-time PCR using SYBR Green PCR Premix HS Taq (Genview, Beijing, China) in the Applied Biosystems Prism 7500 Fast real-time PCR system. The cycling conditions were: 1 cycle of 30 s at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Each RNA sample was analyzed in duplicate. The relative amounts of mRNA were determined by ΔΔCt method with normalization to Actb.

Measurements of total anti-oxidative capacity, SOD activity, total GSH content and malondialdehyde content

Proteins of the heart were collected according to the guidelines of manufacturer (Nanjing Jiancheng Chemical Co, Nanjing, China) and the concentrations were determined by BCA assay (Pierce). Total antioxidant capacity (T-AOC) and SOD activity from tissue were
determined by T-AOC (according to the redox potential of Fe$^{3+}$-TPTZ) detection kit and SOD activity (WST-1 method) assay kit (Nanjing Jiancheng Chemical Co), respectively according to the manufacturer's instructions. Enzyme activities were normalized to protein concentration in samples (U/mg protein). Intracellular total glutathione (GSH) was measured using a Total Glutathione Assay Kit (Beyotime, Nantong, China) following the manufacturer’s protocol. Malondialdehyde (MDA), a lipid peroxidation product, was measured using the Malondialdehyde Assay Kit (Beyotime) by thiobarbituric acid assay, which is based on thiobarbituric acid (TBA) reacted with MDA to produce thiobarbituric acid reactive substances. The content of GSH and MDA was normalized to protein concentration and expressed as $\mu$mol/mg protein and nmol/mg protein, respectively.

**SDS-PAGE/Western blotting**

The cardiac tissues were lysed in ice-cold RIPA buffer (Genview) containing 1 mM PMSF and 1x protease inhibitor cocktail (Roche) by 100 mg tissue/1 mL buffer. The supernatant after centrifugation was collected and the protein concentration was determined by BCA method (Pierce). After separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to PVDF membrane (Millipore). The membrane was then blocked by 5% (w/v) nonfat milk in Tris-buffered saline with 0.1% Tween 20 at room temperature for 1 h. The primary antibodies, anti-collagen 1 (Abcam #ab7778, 1:1000), anti-collagen 3 (Abcam #ab34710, 1:1000), anti-RAGE (Santa Cruz #sc-365154, 1:1000), anti-phospho-ERK1/2 (CST #4370, 1:2000), anti-ERK1/2 (Abcam #196883, 1:2000), anti-Nuclear factor kappa B subunit p65 (NF-κB p65) anti-NF-κB p65 (ProteinTech #10745-1-AP, 1:1000) and anti-β-actin (CST #3700, 1:1000) were added to react with the proteins on the membrane overnight at 4°C. The membrane was then incubated with secondary HRP-conjugated antibody (Jackson, 1:3000) against primary antibodies at room temperature for 45 min. The bands were detected by ECL chemiluminescence kit (Pierce) and analyzed by ImageLab software (Bio-Rad).
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Statistical analysis
All data were presented as the mean±s.d. P<0.05 was considered as significant. Statistical significance was assessed firstly with one-way ANOVAs for comparisons among multiple groups. When significance existed, two-tailed Student’s t-test between two groups was performed. All statistical analyses were performed using Prism 6.0 GraphPad software (GraphPad).

Results
STZ-induced progressively development of DCM in rats
In order to demonstrate the dynamical process of DCM in rats, the changes of the morphology and cardiac function in rats following induction of diabetes were monitored at several time points. Compared with normal controls, diabetic rats showed cardiac dysfunction and myocardium hypertrophy from 8 weeks after STZ injection, with significant declines in LV diastolic function, increases in the HW/BW ratio (Table 1) and cardiomyocyte cell sizes (Fig. 2A and B). Meanwhile, diabetes significantly induced LV systolic dysfunction from 12 weeks after induction of diabetes (Table 1). These results were consistent with the previous research showed that DCM characterized by diastolic dysfunction of LV followed by systolic dysfunctions later (Huynh et al. 2014). Furthermore, diabetic animals also had increased fibrosis after 8 weeks of STZ injection (Fig. 2C and D), which further aggravated at 12 and 16 weeks.

Cardiac dysfunction was reversed with STVNa treatment
At the end of animal study, cardiac functions were measured by hemodynamic methods. The results indicated that diabetes induced significant LV diastolic and systolic dysfunction after 12 weeks or 16 weeks of STZ injection. As shown in Table 1, the diabetic rats (STZ) showed an elevated LV end diastolic pressure (LVEDP) and weakened LV −dP/dt_min (indicators of impaired LV diastolic function) compared with normal rats. STVNa administration (STZ/STVNa) for 11 weeks inhibited impairments of LVEDP and −dP/dt_min induced by diabetes. Analysis of end point systolic function showed significantly decreased LVESP and dP/dt_max (indicators of impaired LV contractility) in diabetic rats, and STVNa treatment could attenuate these impairments. Meanwhile, STZ-injected...
diabetic rats showed decreased MAP and STVNa can reverse this decrease, the heart rates were not changed by diabetes or drug treatment. More importantly, these cardioprotection trends of STVNa lasted at least 4 weeks even after stopping the treatment from the 12th week (16 weeks).

**Cardiac hypertrophy was inhibited with STVNa treatment**

After 12 weeks or 16 weeks of induction of diabetes, HW/BW ratios were increased in diabetes than normal control groups (Table 1). As shown in the H&E-stained hearts (Fig. 3A and B), cardiomyocyte cross-sectional areas (CSAs) were significantly increased in diabetes than normal groups. Meanwhile, the hypertrophic biomarkers, Nppa (ANP) and Nppb (BNP) mRNA expression were greatly enhanced in diabetic rats (Fig. 3C and D). STVNa treatments for 11 weeks or 11 weeks following 4 weeks without treatment could reverse the effects induced by DCM, including alleviated cardiac hypertrophy and decreased expressions of Nppa and Nppb mRNAs.

**Cardiac fibrotic remodeling was limited by STVNa administration**

Diabetes induced significantly increasing in LV interstitial collagen deposition compared to normal rats (Fig. 4A and B). STVNa treatment ameliorated LV fibrosis significantly compared to diabetic rats. Gene expression of Tgfβ1 and the downstream signal molecule Smad-3 were similarly elevated in diabetic rats and attenuated with STVNa treatment (Fig. 4C and D). Meanwhile, mRNA levels of collagen 1 (Col1a1) and collagen 3 (Col3a1), the fibrosis and extracellular matrix markers, were significantly increased in diabetic rats (Fig. 4C and D). As shown in Fig. 4E, both collagen 1 and 3 proteins showed trends of increasing in diabetic rats of 12 weeks (although without significance for collagen I, \( P=0.058 \)), which to some extent were attenuated by STVNa administration (\( P=0.059 \) for...
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Collagen I). STVNa administration significantly attenuated the diabetes-induced elevation in these markers of cardiac fibrosis, which could last at least 4 weeks.

**STVNa-attenuated type 1 diabetes-induced cardiac tissue oxidative stress**

The total antioxidant capacity and total of glutathione (GSH) of cardiac tissues from diabetic rats of 12 weeks decreased significantly, which were reversed by STVNa treatment (Fig. 5A and B). The activity of SOD showed the same trends (P>0.05, Fig. 5C). Meanwhile, type 1 diabetes markedly promoted LV MDA concentration (Fig. 5D) and NADPH oxidase expression, including increased NADPH oxidase 1 (Nox2) and p47 phox subunits mRNA content and declined expression of superoxide dismutase 2 (Sod2) (Fig. 5E and F), compared to nondiabetic normal rats. Moreover, IHC examinations confirmed the cardiac tissues of 12-week diabetic rats had significantly elevated 8-OHdG (Fig. 5G and H), another marker of oxidative stress. All these molecular markers of oxidative stress were significantly inhibited by STVNa treatment.

**STVNa ameliorated LV inflammation in type 1 diabetes**

The expression of Il1b, a pro-inflammatory cytokine (Fig. 6A and B), was significantly upregulated after 12 or 16 weeks of STZ injection. The pro-inflammatory chemokines, including Ccl2 (MCP1), Vcam1, Vcam2 and Icam1 were elevated significantly in diabetic rats (Fig. 6A and B). Meanwhile, the concentration of TNF-α and IL-6 in plasma or cardiac tissues of 12-week-old diabetic rats were increased significantly (Fig. 6C and D). IHC staining showed that CD68, a macrophage cell marker, was notably increased in diabetic rats (Fig. 6E and F). All these inflammation biomarkers were significantly alleviated by treatment with STVNa (including suspending the treatment for 4 weeks) in diabetic rats.
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The alleviation of DCM by STVNa is not related to anti-hyperglycemia, insulin restoration or AGE-RAGE inhibition

Diabetic rats showed elevation of blood glucose levels (Fig. 7A) along with decrease in the plasma insulin levels (Fig. 7B) after STZ injection (12 weeks). STVNa did not lower blood glucose or promote serum insulin levels. Moreover, ELISA and fluorescence methods showed that STZ-induced diabetes caused significant increase in plasma AGE levels compared to normal groups (Fig. 7C and D). However, STVNa did not decrease plasma AGE levels. Meanwhile, diabetes induced increases of the mRNA and protein levels of Ager (RAGE) after 12 weeks of STZ injection (Fig. 7E and F), which cannot be repressed by STVNa administration.

STVNa inhibited ERK1/2 phosphorylation and NF-κB stimulated by DCM

In order to demonstrate the mechanism of STVNa for its anti-inflammation and inhibition of oxidative stress, the related signal pathways were studied by Western blot.

As shown in Fig. 8, STZ-induced diabetic rats (12 weeks) showed significantly increased ERK1/2 phosphorylation (Thr 202/Tyr 204) correlated with elevated NF-κB p65 expression. STVNa-treated diabetic rats had significantly depressed the ERK1/2 and NF-κB pathways.

Effects of STVNa administration on normal rats

To investigate if there were any harmful effects of STVNa treatment on normal rats, we examined the HW/BW, BW increasing and marker genes expression of hypertrophy, fibrosis, oxidative stress and inflammation in cardiac tissues. As summarized in Fig. 9, after 6 weeks of administration of STVNa to normal rats, there were no significant differences between normal rats and rat administrated with STVNa.

Discussion

The present study investigated the effects of STVNa on DCM with an STZ-induced type-1 diabetic rats, a widely accepted animal model for the assessment of new drugs.
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Figure 7

Effects of STVNa on circulating levels of glucose, insulin and AGEs and RAGE expression in heart. (A) Diabetes-induced hyperglycemia and STVNa showed no any lowering effects on blood glucose level. (B) The plasma insulin levels of each group after 12 weeks of STZ injection. Insulin decreased significantly in diabetic group, STVNa had no effects on insulin concentration. ELISA (C) and fluorescence methods (D) showed that diabetes induced significant increase of advanced glycation end products in plasma after 12 weeks of STZ injection. STVNa did not show any effects on AGEs. (E) The mRNA expression and protein expression (F) of RAGE measured by semi-quantitative PCR was elevated in diabetic rats of 12 weeks, which cannot be reversed by STVNa administration. *, **, ***P<0.05, P<0.01 and P<0.001 vs normal group. AGE, advanced glycation end product; RAGE, AGEs interact with its receptors; STVNa, isosteviol sodium; STZ, streptozotocin.

Many diabetes patients develop cardiomyopathy or cardiac implications because of poor control of hyperglycemia. Once DCM is established, it cannot be easily treated with hypoglycemic drugs (Falcão-Pires & Leite-Moreira 2012). Isosteviol, derived from Stevia by hydrolysis of the β-glycoside, is a known compound but STVNa is an innovative pharmaceutical formulation suitable for injection, which greatly increased the oral bioavailability of isosteviol (Lai et al. 2017). In the present study, for the first time, we demonstrated that application of STVNa (11 weeks) prevented a multitude of cardiac complications of DCM including diastolic and systolic dysfunction, hypertrophy and fibrosis, accompanied with decreased inflammation and oxidative stress in STZ induced diabetic rats. Meanwhile, we discovered that the anti-cardiomyopathy effect of isosteviol was independent of AGE-RAGE levels, glucose lowering or increasing insulin content. More importantly, since ERK 1/2 and NF-κB signals were activated and played critical roles in the pathological processes of DCM, including stimulating hypertrophy, fibrosis and cell apoptosis (Rajesh et al. 2010, Singh et al. 2017), the compounds inactivating ERK1/2 and/or NF-κB would be very effective approaches, which were confirmed by a few studies (Thomas et al. 2014, Chen et al. 2016, Dong et al. 2016). The present study firstly showed that the beneficial effects of STVNa on DCM were attributed to inhibition of ERK 1/2 and NF-κB signaling pathways, which would be of great clinic significance.

We set up two time points to evaluate the protective effects of STVNa on DCM rats, in which some rats were treated with STVNa for 11 weeks followed by non-treatment for 4 weeks. The diabetes-induced cardiac injuries were significantly attenuated for the 11 weeks by treatment with STVNa, which sustained for additional 4 weeks after the 11-week treatment. This memory of cardioprotection indicated a great therapeutic potential of our preparation of STVNa for cardiomyopathy in diabetes. More importantly, STVNa treatment did not alter relative HW and cardiac genes expression of hypertrophy, fibrosis, oxidative and inflammation in nondiabetic normal rats, suggesting these beneficial effects of STVNa were functional only under high fibrosis, oxidative stress and inflammation in the diabetes hearts.

DCM is a complex multifactorial pathological process characterized by early impairments in cardiac diastolic followed by systolic dysfunction (Boudina & Abel 2010), as well as notably structural abnormalities including
cardiomyocytes as well as upregulation of Nppa/Nppb expression (Huynh et al. 2014, Mátyás et al. 2015). Our data demonstrated that STVNa had anti-hypertrophic and anti-fibrotic effects by histological methods (decreased cardiomyocyte size, cardiac fibrosis area) and molecular analysis (significantly reduced hypertrophic and fibrotic genes expression). Endothelial activation and increased monocyte adhesion molecules including Ccl2, Vcam1 and Vcam2, along with macrophage infiltration, represent the key early events that trigger vascular inflammation as a part of the pathophysiology of DCM (Potenza et al. 2009). Meanwhile, various studies showed that high glucose promoted NADPH oxidase subunits such as Nox2 and p47 phox expression, which increased or prolonged ROS production in response (Thallas-Bonke et al. 2008). Our data confirmed superoxide formation and significant inflammation responses in the cardiac tissue of diabetic rats. Moreover, increasing evidence indicated the enhanced ERK and NF-κB signal pathways during DCM development with the promotion of much oxidative stress, inflammatory, hypertrophy and fibrosis factors (Rajesh et al. 2010, Gao et al. 2016, Singh et al. 2017). Our results showed that administration of STVNa did not depress AGEs, RAGEs and glucose, but efficiently inhibited DM-induced phosphorylation of ERK and NF-κB p65 expression. Therefore, STVNa treatment could ameliorate cardiac dysfunction in type 1 diabetes, attenuate cardiac hypertrophy and fibrosis, possibly by inhibiting the oxidative stress and inflammation-related ERK and NF-κB signal pathways rather than anti-hyperglycemic effects. However, besides glucose, the disturbance of free fatty acids (FFAs) metabolism plays a critical role in the development of DCM (Ritchie et al. 2017), and the expression of some genes related FFAs metabolism were altered in DCM (Inoue T et al. 2015). We will investigate the effects of STVNa on FFAs metabolism and related pathways in our future study.

In a previous study using Zucker diabetic fatty (ZDF) rats, administration of isosteviol at 5 and 10 mg/kg could significantly decrease the AUC of glucose during the intravenous glucose tolerance test (IVGTT), without any effect on plasma insulin concentration (Ma et al. 2007). Another team demonstrated in vivo acute anti-hyperglycemic effects of a series of novel isosteviol and derivatives (Chen et al. 2010). However, our study showed that STVNa treatment did not show any depression of hyperglycemia or AGEs in plasma. These differences may be probably due to different animal models. Insulin-dependent STZ-induced type 1 diabetes was used in our study while the type 2 diabetic Zucker rats are insulin dependent.

**Figure 8**
STVNa inhibited STZ-induced activation of ERK1/2 and NF-κB signaling pathways. (A) Western blot to detect the phosphorylated and total ERK 1/2 as well as NF-κB p65 expression. The phosphorylated ERK 1/2 to total ERK1/2 (B) and the expression of NF-κB p65 (C) were increased significantly in diabetic rats, which were reversed by STVNa treatment. ***P<0.001 vs normal group; **P<0.01 vs STZ group. ERK, extracellular signal-regulated kinase; NF, nuclear factor; STVNa, isosteviol sodium; STZ, streptozotocin.
independent. Our study showed that STVNa could not promote insulin level of plasma, which is consistent with the previous study (Ma et al. 2007).

In addition, many previous studies of STZ-induced diabetic rats used various time spans from 2 to 24 weeks and showed different and inconsistent findings (Becher et al. 2013, Gupta et al. 2015, Harasiuk et al. 2016); therefore, the precise time course of DCM development is vital for elaborating and explaining varieties between findings. In the present study, we described the time course of DCM development, including the impairment in systolic and diastolic LV performance as well as cardiac hypertrophy and fibrosis over a long period of 4–16 weeks in diabetic Wistar rats, indicated by a progressive decrease in LV relaxation (from 8th week), contractility (from 12th week) and an increase in cardiac hypertrophy (from 8th week) and fibrosis (from 8th week).

In summary, the results presented here suggested that STVNa treatment was able to improve impaired cardiac function by attenuating hypertrophy and fibrosis as well as decreasing the oxidative stress, and inhibiting inflammatory responses, and the cardioprotective effects lasted at least 4 weeks after suspending of treatment. Meanwhile, the diabetes stimulated ERK and NF-κB signal pathways were inhibited by STVNa treatment. Therefore, STVNa, whose effects were not related to glucose lowering or AGE-RAGE inhibition, could be pursued as a novel therapeutic approach specific to DCM. However, additional in vitro researches will be required to definitively identify molecular mechanisms of STVNa.

Conclusions

This study shows the efficacies of STVNa on DCM by prevention of cardiac dysfunction, restriction on cardiac hypertrophy and fibrosis, as well as inhibition of oxidative stress and inflammation. Moreover, our study shows that the cardioprotective mechanism of STVNa in STZ-induced type 1 diabetes is not dependent on alleviating hyperglycemia and inhibiting AGEs formation, but through inhibition of ERK and NF-κB signal pathways. To our knowledge, this is the first study demonstrating the anti-hypertrophic and anti-fibrotic effects of pharmacological STVNa on DCM type-1 diabetes.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-17-0681.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Author contribution statement
Prof. Tan and Ye conceived and directed the project. Sheng-Gao Tang and Xiao-Yu Liu directed rats diabetic cardiomyopathy and tissue analysis related studies. Sheng-Gao Tang designed the experiments of molecular biology. Sheng-Gao Tang and Ying-Ying Yang carried out biochemical and molecular biological experiments. Ting-Ting Hu helped analyze some data of the experiments. Ting Han helped animal care and treatments.

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