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MALONDIALDEHYDE AND 4-HYDROXYNONENAL ADDUCTS ARE NOT FORMED ON CARDIAC RYANODINE RECEPTOR (RyR2) AND SARCO(ENDO)PLASMIC RETICULUM Ca^{2+} ATPase (SERCA2) IN DIABETES

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Abstract

Recently, we reported an elevated level of glucose-derived carbonyl adducts on cardiac ryanodine receptor (RyR2) and sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2) in hearts of streptozotocin(STZ)-induced diabetic rats. We also showed these adduct impaired RyR2 and SERCA2 activities, and altered evoked Ca^{2+} transients. What is less clear is if lipid-derived malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) also chemically react with and impair RyR2 and SERCA2 activities in diabetes. This study used Western blot assays with adduct-specific antibodies and confocal microscopy to assess levels of MDA, 4-HNE, N^{ϵ} -carboxy(methyl)lysine (CML), pentosidine and pyrraline adducts on RyR2 and SERCA2 and evoked intracellular transient Ca^{2+} kinetics in myocytes from control, diabetic and treated-diabetic rats. MDA and 4-HNE adducts were not detected on RyR2 and SERCA2 from control or 8 weeks diabetic rats with altered evoked Ca^{2+} transients. However, CML, pentosidine, and pyrraline adducts were elevated 3–5 fold ($p < 0.05$). Treating diabetic rats with pyridoxamine (a scavenger of reactive carbonyl species, RCS) or aminoguanidine (a mixed reactive oxygen species-reactive carbonyl species scavenger) reduced CML, pentosidine and pyrraline adducts on RyR2 and SERCA2 and blunted SR Ca^{2+} cycling changes. Treating diabetic rats with the superoxide

dismutase mimetic tempol had no impact on MDA and 4-HNE adducts on RyR2 and SERCA2, and on SR Ca^{2+} cycling. From these data we conclude that lipid-derived MDA and 4-HNE adducts are not formed on RyR2 and SERCA2 in this model of diabetes, and are therefore unlikely to be directly contributing to the SR Ca^{2+} dysregulation.

Keywords

Diabetes mellitus; rats; malondialdehyde; 4-hydroxynonenal; post-translational modifications; type 2 ryanodine receptor; sarco(endo)plasmic reticulum Ca^{2+} -ATPase

Introduction

World governments currently spend in excess of \$500 billion to diagnose, treat and care for the >350 million individuals with diabetes mellitus (DM) [1, 2]. This syndrome, which is characterized by an elevation in blood glucose, alters the responsiveness of small blood vessels to physiological stimuli, precipitating hypertension and other microvascular-related diseases including stroke, retinopathy, and nephropathy [3]. Approximately, 70% of individuals with DM will also develop a unique type of heart failure referred to as a diabetic cardiomyopathy, for which the etiology remains poorly understood [4]. What we know to date is that alterations in cellular metabolism, perturbation in sarcoplasmic reticulum Ca^{2+} cycling, and stiffening of the extracellular matrix are characteristic features of the diabetic heart [5–8]. Of interest in our laboratory are the underlying causes for dysregulation in the flow of Ca^{2+} from and into the sarcoplasmic reticulum (SR), an established cause for the reduction in contractility and increased incidence of ventricular arrhythmias in individuals with diabetes mellitus.

The release of Ca^{2+} from the SR of cardiac ventricular myocytes occurs via type 2 ryanodine receptors (RyR2) and its reuptake into the SR via the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2). Using a rat model of type 1 diabetes, we recently discovered increased carbonylation by the glucose-derived reactive carbonyl species (RCS), methylglyoxal, 3-deoxyglucosone, N^{ϵ} -carboxy(methyl)lysine and pyrraline is a major cause for RyR2 and SERCA2 dysregulation and the perturbation in myocyte SR Ca^{2+} cycling [9, 10]. The heart is unique amongst organs in that only 30% of the ATP needed for functioning is generated from glucose (pyruvate and lactate), with the remaining 70% of the ATP generated from beta-oxidation of free fatty acids (FFAs) [11, 12]. During DM, expression of the fatty acid transport proteins FATP and CD36 are up-regulated and ATP production via oxidation of FFAs is potentiated [12–14]. More FFAs than that which is required for ATP generation are also taken up, resulting in accumulation of FFAs, triglyceride, phospholipids, and other lipid subspecies inside myocytes [12]. At the same time, ATP production via pyruvate and lactate is down regulated [6, 15].

In diabetic myocytes, production of reactive oxygen and nitrogen species (ROS/RNS) is also upregulated from increased activation of NADPH oxidase, xanthine oxidases and nitric oxide synthases, and uncoupling of the mitochondrial electron transport chain [16, 17]. This excess of ROS/RNS react with the FFAs on myocyte membranes, within mitochondria, and in the cytoplasm of myocytes to increase production of the lipid-derived RCS,

malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) [18–20]. MDA and 4-HNE production are also upregulated from increased prostaglandin, thromboxane and arachadonic acid biosynthesis in the diabetic heart [21, 22]. Like glucose-derived RCS, MDA and 4-HNE are capable of reacting with nucleophilic groups on proteins (basic and cysteine residues) to form adducts [23, 24]. By acting as a nucleophile, MDA can also react with electrophilic moieties on proteins [24].

To the best of our knowledge, no data are available to assess if formation of MDA and 4-HNE adducts are also contributing to RyR2 and SERCA2 dysregulation in diabetes. Thus, the objectives of the present study were (i) to determine relative levels of MDA and 4-HNE adducts on RyR2 and SERCA2 from hearts of control and streptozotocin (STZ)-induced diabetic rats with cardiomyopathy and to compare with CML, pentosidine and pyrraline levels, and (ii) to assess efficacies of tempol (superoxide dismutase mimetic), aminoguanidine (mixed ROS-RCS scavenger) and pyridoxamine (RCS scavenger) treatments to blunt formation of carbonyl adduct formation on RyR2 and SERCA2 and perturbation in myocyte SR Ca^{2+} cycling.

Materials and Methods

Antibodies and Reagents

RyR2 and SERCA2 antibodies were obtained from Thermo Fisher Scientific (Waltham, MA). Antibodies to malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4HNE) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA) and EMD Millipore (Billerica, MA) and used at 1:500 and 1:1000 dilutions. Antibodies to N^{ϵ} -carboxy(methyl)lysine, 3-deoxyglycosone/imidazolone, pentosidine and pyrraline were provided by Dr. Royji Nagai (Department of Bioscience, Tokai University, Tokyo, Japan) and used at 1:1000 dilutions. [^3H]Ryanodine and $^{45}\text{Ca}^{2+}$ were purchased from GE-Healthcare (Pittsburg, PA). Pyridoxamine, aminoguanidine and tempol were from Sigma-Aldrich Chemical (Saint Louis, MO). Fluo-3AM was from Invitrogen (Carlsbad, CA). All other reagents used were of the highest grade commercially available.

Induction and verification of type 1 DM

Male Sprague Dawley rats (200 ± 10 g, Harland Laboratories, Indianapolis, IN) were used in this study and animal procedures used were approved by the Institutional Animal Care and Use Committee, University of Nebraska Medical Center, and adhered to the Guide for the Care and Use of Laboratory Animals [9]. The induction of diabetes with streptozotocin (STZ) and the care for rats with DM are described in details elsewhere [10, 25].

Treatment of rats with DM

Two weeks after injection of STZ (45 mg/kg in 0.1 M citrate buffer, pH 4.5), diabetic rats were randomly divided into four groups. The first group was administered tempol (T-D, 0.17 g/L) via drinking water for 5–6 weeks [26, 27], the second group was given aminoguanidine (Ag-D, 0.14 g/L) [28], the third group was given pyridoxamine (Py-D, 0.17 g/L) [9] and a fourth group remained untreated (D). Two weeks after injection with citrate buffer, control animals were also divided into four groups: one group was administered

pyridoxamine for 5–6 weeks (Py-C, 0.05 g/L, as they drank one third less water [29]), a second group with aminoguanidine (Ag-C, 0.05 g/L), the third group with tempol (TC, 0.05 g/L) and a fourth group remained untreated (C). Water bottles containing tempol, aminoguanidine and pyridoxamine were wrapped around with aluminum foil to protect them from light and changed daily.

Establishing diabetic cardiomyopathy *in vivo*

- i. *M-mode echocardiography* was performed at the end of the 8-week protocol in anaesthetized animals (100 mg/kg ketamine/2.5 mg/kg acepromazine, i.p.) to establish reduced left ventricular fraction shortening and ejection fraction [25]. Rats were lightly anesthetized with ketamine-acepromazine (91 mg/kg i.p. ketamine and 1.8 mg/kg i.p. acepromazine), and left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV) were assessed using a Vevo 770™ High Resolution In Vivo Micro Imaging System (Visual Sonics Inc, Toronto, Canada). Percent fractional shortening (FS) was calculated using the equation $FS = [(LVEDD - LVESD)/LVEDD] \times 100$. Percent ejection fraction (EF) was calculated using the equation $EF = [(LVEDV - LVESV)/LVEDV] \times 100$.
- ii. *In vivo hemodynamics*: Basal and isoproterenol-induced changes in rates of left ventricular pressure rise and decline ($-dP/dt$), peak left ventricular pressure (LVP) and left ventricular end diastolic volume (LVEDV) were evaluated as described earlier [30]. Briefly, animals were anesthetized with Inactin (thiobutabarbital, sodium salt, 20 mg/kg given i.p) and their left carotid arteries were isolated and cannulated with Millar catheters containing a pressure transducer. Each Millar catheter was fed into the artery until it reaches inside the left ventricle for assessment of pressure changes during contraction/relaxation cycle. Right hind leg femoral vein was also cannulated and used for injection of isoproterenol. A Powerlab, a computer-based data acquisition system (ADInstruments) was used for obtaining basal heart rate, left ventricular pressure, $\pm dP/dT$, and arterial blood pressure.

Blood parameters

Blood samples were collected via left renal arteries after deep anesthesia and assayed for glucose, insulin, thiobarbituric acid-reactive substances (predominantly malondialdehyde), % glycosylated hemoglobin, and semicarbazide-sensitive amine oxidase as described previously [10, 28].

Assessment of evoked Ca^{2+} transients

Ventricular myocytes were isolated through retrograde collagenase perfusion and evoked Ca^{2+} transients were assessed as described previously [25, 28]. For the latter, cells on laminin-coated coverslips in Dulbecco's Modified Eagle's Medium (DMEM) containing F12 were loaded with Fluo-3 (5 μ M) for 30 min at 37°C. At the end of the incubation, cells were washed and placed in a chamber on the stage of the confocal microscope. Cells were

then field stimulated at 0.5 Hz (10 V for 10 ms), and rate of rise of Ca^{2+} transient, Ca^{2+} transient amplitude and Ca^{2+} transient decay time were measured. Fluo-3 was excited by light at 488 nm, and fluorescence was measured at wavelengths of >515 nm. LSM 5 Meta, Prism 4.0, and Microsoft Excel were used for data analysis.

Assessment of myocyte contractile kinetics

Contraction kinetics of myocytes were assessed using high-speed video-based edge detection system (IonOptix Corporation, Milton, MA) described previously [30]. For this, myocytes in DMEM were placed in a Warner chamber mounted on the stage of an inverted microscope (Zeiss X-40, Thornwood, NJ) at room temperature (22°C – 24°C). Cells were then field stimulated at 0.5 Hz ((10 V for 10 msec) using a pair of platinum wires placed on opposite sides of the chamber and movements were recorded using a proprietary data acquisition program (IonOptix Corporation, Milton, MA). Rates of myocyte shortening and relengthening and extent of cell shortening were then calculated using IonWizard version 5.0.

RyR2 activity

The ability of RyR2 to bind the Ca^{2+} -dependent binding [^3H]ryanodine was used as an index of its activity. For this, crude SR membranes (0.1 mg/ml) prepared as described earlier [9] were incubated in binding buffer (500 mM KCl, 20 mM Tris-HCl, 0.3 mM Ca^{2+} , 2 mM reduced glutathione, and 100 μM EGTA, 6.7 nM [^3H]ryanodine, pH 7.4) for 2 hr at 37°C . After incubation, membranes were filtered and washed, and the amount of [^3H]ryanodine bound was determined using liquid scintillation counting. Non-specific binding was determined simultaneously by incubating vesicles with 1 μM unlabeled ryanodine [30].

SERCA2 activity

The ability of SERCA2 to transport Ca^{2+} (steps 1 through 6 of the Post-Elbers cycle) was used as an index of its activity. Crude SR membrane vesicles were resuspended in 1 ml buffer (30 mM Tris-HCl, pH 7.0, 100 mM KCl, 5 mM NaN_3 , 50 μM ryanodine, 5 mM MgCl_2 , 0.15 mM EGTA, 0.12 mM CaCl_2 , 1 μCi $^{45}\text{Ca}^{2+}$ and 10 mM potassium oxalate) and were divided into 2×500 μl aliquots. One aliquot was incubated with a SERCA2a inhibitor cocktail (10 μM thapsigargin, 100 μM ammonium molybdate, 0.05 μg bafilomycin) and the other aliquot remained untreated. Ca^{2+} uptake was initiated by the addition of ATP (Na_2ATP) and after 20 min SR vesicles were cold-filtered and washed as described earlier [10]. The amount of $^{45}\text{Ca}^{2+}$ remaining on the filter paper was used as a measure of SERCA2 activity.

Determination of carbonyl adducts

Ventricular homogenates (60 g) and crude SR membranes (30 g) prepared from hearts of control, diabetic and were solubilized in gel dissociation medium and electrophoresed on Criterion 4–15% gradient Tris-glycine polyacrylamide gels (BioRad Technologies Inc Burlingame CA) for 210 min at 150V. For experiments involving lipid-derived adducts, Criterion TGX gels were used (BioRad Inc, Burlingame CA). At the end the electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (PVDF, Millipore

Corporation, Bedford, MA, 100 mA, 16 hr at 4°C) using a semidry electroblotter (Panther; Owl Scientific Inc., Woburn, MA) with CAPS buffer (10 mM cyclohexylamino-1-propanesulfonic acid/0.01% SDS in 10% methanol, pH 9.0) and Western blots were performed with adduct-specific antibodies to determine MDA, 4HNE, N^ε-carboxy(methyl)lysine, pentosidine and pyrraline adducts. Primary antibodies against MDA and 4HNE adducts were used at 1:1000 and 1:500 dilutions, antibodies against N^ε-carboxy(methyl)lysine, pentosidine and pyrraline adducts were used at 1:1000 dilutions, and secondary antibodies were used at 1:5000 dilutions. All gels were stained with Coomassie Blue dye after semi-dry transfer to assess sample loading [9, 10].

Determination of protein identity

After semi-dry transfer, gels were stained with Coomassie Blue dye and destained with 25% methanol. Protein bands corresponding to those with carbonyl adduct (RyR2, SERCA2, NCX, myosin heavy chain) were excised, digested with trypsin, desalted with ZipTips® (Millipore, Billerica, MA) and subjected to matrix-assisted laser desorption ionization time-of-flight and Tandem mass spectrometry as described earlier [9, 10]. Peptide sequences were submitted to Mascot Wizard Search Engine to confirm identity (Matrix Science, Boston, MA).

Statistical Analysis

Differences among RyR2 and SERCA2 values from each of the groups (C, D, Py-C, Py-D, Ag-C, Ag-D, T-C and T-D) were evaluated using analysis of variance (ANOVA) employing GraphPad Prism. Data are presented as means ± S.E.M. The results were considered significantly different if $p < 0.05$ (95% confidence interval).

Results

Table 1 shows the general characteristics of animals employed in this study. Diabetes increased % glycosylated hemoglobin, serum thiobarbituric acid-reactive substances (TBARS) and semicarbazide-sensitive amine oxidase (SSAO) activity. Pyridoxamine (Py), aminoguanidine (Ag) or tempol (T) treatments did not significantly differently alter body weight, blood glucose, % glycosylated hemoglobin, or serum insulin. However, pyridoxamine treatment significantly lowered SSAO activity ($P < 0.05$) while aminoguanidine and tempol treatments lowered serum TBARS ($P < 0.05$).

Establishing diabetic cardiomyopathy

- i. *M-Mode echocardiography*: After 7–8 weeks of diabetes, ejection fraction, % fractional shortening and heart rate were significantly ($p < 0.05$) reduced in diabetic rats compared with controls, indicative of a cardiomyopathy (Figure 1(i), Table 2, left lanes).
- ii. *In vivo hemodynamics*: Basal and isoproterenol-induced heart rates, rates of left ventricular pressure increase and decline ($-dP/dt$) and peak left ventricular pressure (LVP) were also significantly ($p < 0.05$) lower in diabetic rats compared to controls (Figure 1(ii), Figure 1(iii) and Table 2, left lanes), consistent with diabetic

cardiomyopathy. Left ventricular end diastolic pressure (LVEDP) was significantly ($p<0.05$) elevated in diabetic rats compared to controls.

Evoked Ca^{2+} transient kinetics

After Ca^{2+} reconstitution, $68 \pm 6\%$ of myocytes from control rats were rod-shaped and viable, $53 \pm 5\%$ were rod shaped and viable from diabetic rats, $65 \pm 6\%$ were rod shaped and viable from pyridoxamine-treated diabetic rats, $63 \pm 6\%$ were rod shaped and viable from aminoguanidine-treated diabetic rats, and $56 \pm 2\%$ were rod shaped and viable from tempol-treated diabetic rats. More than 80% of viable cells were quiescent and responded to field stimulated. Rate of rise of evoked Ca^{2+} transient, rate of decay of evoked Ca^{2+} transient and Ca^{2+} transient amplitude was significantly ($p<0.05$) reduced in diabetic myocytes compared with control myocytes (Figure 1 (v), Table 3, right lanes), consistent with altered SR Ca^{2+} cycling. About 35% of diabetic myocytes also exhibited diastolic Ca^{2+} release in between stimulation.

Confirming reduced RyR2 and SERCA2 activities

- i. *RyR2*- Steady-state levels of RyR2 protein did not change after 7–8 weeks of diabetes (Figure. 1 (vi), upper autoradiogram) [9, 30]. At equivalent amounts of protein, RyR2 from diabetic rat hearts bound significantly ($p<0.05$) less [^3H]ryanodine than RyR2 from control hearts, consistent with a net reduction in activity (Figure 1(vi), graph).
- ii. *SERCA2*- Steady state SERCA2, monomeric and pentameric PLN proteins also remained unchanged after 7–8 weeks of diabetes (Figure 1(vii)) [10]. However, SERCA2 from diabetic rat was less effective in transporting Ca^{2+} .

Carbonyl adducts on RyR2 and SERCA2

Malondialdehyde and 4-HNE adducts were not detected on RyR2 or SERCA2 proteins from hearts of control and diabetic rats using 30 μg of SR membranes and 1:1000 dilution of primary antibodies (Figure 2, left lanes, and Figure 3, left lanes). Higher amounts of SR membrane proteins (75 μg) and increased concentration of antibodies (1:500) also did not revealed MDA and 4-HNE adduct on RyR2 and SERCA2 (data not shown). MDA adducts were detected on other proteins in the SR preparations (M_w ~13 kDa, ~40 kDa, ~45 kDa, ~56 kDa, ~70 kDa, ~85 kDa and ~200 kDa, Figure 2-left lanes, indicating that the antibodies were detecting immuno-reactive MDA adducts. With an exception of the protein band at ~50 kDa, the amount of MDA adducts did not change with DM. 4-HNE adducts were also detected on proteins of M_w ~4 kDa, ~10 kDa, ~44 kDa, ~48kDa, and ~66kDa (Figure 3), indicating that the antibodies were detecting immuno-reactive 4-HNE adducts

In agreement with recent reports [9, 10], in this study elevated levels of immuno-reactive N^ϵ -carboxy(methyl)lysine, pentosidine, and pyrroline adducts were formed on RyR2 and SERCA2 in diabetes (Figure 4, Figure 5 and Figure 6, left lanes, $p<0.05$). Higher levels of pentosidine (80%), and pyrroline (250%) adducts were also found on the sodium-calcium exchanger (NCX) from diabetic rat (Figure 4 and Figure 5). N^ϵ -carboxy(methyl)lysine and

pentosidine adducts were also significantly ($p<0.05$) elevated (2–3 fold) on several yet to be identified proteins in diabetic rat hearts (Figure 3 and Figure 4, black arrows).

Comparison between aminoguanidine, pyridoxamine and tempol treatments

- (i) *M-mode echocardiography*: Treating diabetic rats with pyridoxamine and aminoguanidine for 6 weeks, starting 2 weeks after the onset of DM significantly ($p<0.05$) showed higher ejection fraction and percent fractional shortening (Table 4). Tempol treatment did not attenuate the loss in cardiac ejection fraction and percent fractional shortening (Table 4). Pyridoxamine, aminoguanidine and tempol treatments did not blunt the slowing in heart rate.
- (ii) *In vivo hemodynamics*: Treating diabetic rats with pyridoxamine, aminoguanidine or tempol for 6 weeks, starting 2 weeks after the onset of DM significantly ($p<0.05$) blunted the reduction in rate of left ventricular pressure development (+dP/dt) and enhanced the responsiveness of diabetic hearts to isoproterenol stimulation (Table 4). Pyridoxamine and aminoguanidine also ($p<0.05$) increased basal peak LVP and rate of left ventricular pressure decline (–dP/dt) and lowered left ventricular end diastolic pressure (LVEDP) (Table 4).
- (ii) *Myocyte contractile kinetics*: Treating diabetic rats with pyridoxamine, aminoguanidine or tempol for 6 weeks, starting 2 weeks after the onset of DM significantly ($p<0.05$) blunted the reduction in myocyte contraction velocity induced by DM (Table 5). Only pyridoxamine and aminoguanidine blunted the reduction in extent in cell shortening. Pyridoxamine, aminoguanidine and tempol treatments also significantly ($p<0.05$) enhanced myocyte relaxation rate (Table 5).
- (iv) *Evoked Ca^{2+} transient kinetics*: Pyridoxamine and aminoguanidine treatments significantly ($p<0.05$) enhanced the rate of evoked Ca^{2+} rise in ventricular myocytes from diabetic rats, but not tempol treatment (Figure 7, Table 5). Although there were substantial increases in the Ca^{2+} transient amplitude in myocytes from pyridoxamine-treated, aminoguanidine-treated and tempol-treated diabetic myocytes, these increases did not attain statistical significance ($p=0.07$). Pyridoxamine, aminoguanidine and tempol shortened evoked Ca^{2+} transient decay time (Table 5) and reduced diastolic Ca^{2+} release in between pulses (Figure 7).
- (v) *Carbonyl adducts on RyR2 and SERCA2*
MDA and 4HNE adducts were not detected on RyR2 and SERCA2 proteins from pyridoxamine-, aminoguanidine-, and tempol-treated diabetic rat hearts (Figure 2 and Figure 3). Tempol treatment, did lower the amount of MDA adduct on a protein of $M_w \sim 50$ kDa (Figure 2) and a protein with 4-HNE adduct with $M_w \sim 42$ kDa (Figure 3). Treating diabetic animals with either pyridoxamine or aminoguanidine blunted the increase in N^ϵ -carboxy(methyl)lysine, pentosidine, and pyrroline on RyR2 and SERCA2, but not on all SR proteins (Figure 4, Figure 5 and Figure 6, respectively). Tempol treatment did not blunt formation of immuno-reactive N^ϵ -

carboxy(methyl)lysine, pentosidine, and pyrraline on RyR2 and SERCA2 (data not shown).

- (vi) *RyR2 and SERCA2 activities:* Pyridoxamine, aminoguanidine and tempol treatments did not alter expressions of RyR2 (Figure 8) and SERCA2 (Figure 9). Interestingly, only pyridoxamine and aminoguanidine treatments significantly ($p < 0.05$) blunted the loss in RyR2 activity (as assessed using [^3H]ryanodine binding to RyR2, and ability of SERCA2 to transport Ca^{2+} induced by DM (Figure 8, graph below, and Figure 9, graph below).

Discussion

Individuals with chronic and/or long-standing diabetes mellitus (DM) have reduced cardiac contractile function and are more likely to develop stress-induced arrhythmias, compared with age-matched individual [31]. These defects are attributed in part to perturbation in the flow of Ca^{2+} out of and into the SR arising from dysregulation of RyR2 and SERCA2, respectively [32–35]. However, the molecular mechanisms underlying RyR2 and SERCA2 dysregulation during diabetes remains incompletely defined. We recently showed that these slowly-turned over Ca^{2+} cycling proteins ($t_{1/2} \sim 8$ days for RyR2, and $t_{1/2} \sim 14$ days for SERCA2, [35]) become post-translationally modified by glucose-derived reactive carbonyl species (RCS) during DM, and that incorporation of these adducts are responsible in part for their dysregulation. We also showed that oxidation of cysteine residues also contributes to RyR2 dysregulation during DM, but the identity of these cysteine residues remains undefined [36]. What is not clear is if lipid-derived RCS like MDA and 4HNE also post-translationally modify and alter the function of RyR2 and SERCA2 in DM.

The principal finding of the present study is that RyR2 and SERCA2 from hearts of control animals and STZ-induced diabetic rats with established diabetic cardiomyopathy do not contain either malondialdehyde or 4-hydroxy-2-nonenal adducts. This conclusion is based on extensive Western blotting assays using ventricular homogenates and SR membranes enriched in RyR2 and SERCA2, and several highly selective commercially available antibodies that immuno-reacts specifically with malondialdehyde adducts (N^ϵ - β -lysyl-aminoacrolein and N^δ -(2-pyrimidyl)-L-orithine) and 4-hydroxy-2-nonenal adducts. MDA and 4-HNE antibodies were used at 2 times the recommended manufactures' dilutions (1:500) and with up to 75 μg SR membrane proteins/lane in SDS-PAGE gels. Although RyR2 and SERCA2 were not chemically modified by MDA and 4HNE, several proteins in our SR membrane preparations were, indicating that these antibodies were detecting immuno-reactive MDA and 4-HNE adducts. These data are consistent with studies reported by Shah et al., [37] and Minamiyama et al., [38]. Why some proteins in our preparations (control) are post-translationally modified by MDA and 4HNE while others are not, remains undefined. What we suspect is that our SR preparations contain proteins from other intracellular organelles including mitochondria, Golgi, cytoplasm, and cell membranes that reside in proximity to where MDA and 4-HNE are synthesized.

Although MDA is up-regulated in DM, its ability to rearrange at neutral and physiological pHs to the less reactive enol form [23] could account for the absence of adducts on RyR2

and SERCA2. Another reason could be the reduction in expression of the malondialdehyde degrading enzyme, aldehyde dehydrogenase (ADLH2) [39]. Conversion of cytoplasmic malondialdehyde to methylglyoxal [40] and degradation of methylglyoxal to D-lactate by glyoxalase I and II and reduced glutathione [41] may also contribute. In diabetes, increased expression and activity of aldose reductase, the major enzyme that degrades 4-HNE, may also help explain the reduction in 4-HNE adducts on proteins [42]. Increased expression of glutathione S-transferases (GSTA4-4 and GST5.8) may also contribute [43].

In this study, glucose-derived *N*^ε-carboxy(methyl)lysine, pentosidine and pyrraline adducts were significantly ($p < 0.05$) elevated on RyR2, SERCA2 and NCX isolated from diabetic rat hearts (Figure 4, Figure 5 and Figure 6). What was also apparent from the autoradiograms was that *N*^ε-carboxy(methyl)lysine, pentosidine and pyrraline adducts were more likely to be formed on proteins $M_w > 200$ kDa and < 30 kDa (Figure 4, 5 and 6), while MDA and 4HNE adducts were more likely to be formed on proteins of M_w between 30 kDa and 80 kDa (Figures 2 and 3). The reason(s) for the selectivity in proteins targeted for MDA and 4HNE, and by *N*^ε-carboxy(methyl)lysine, pentosidine and pyrraline remains incompletely understood.

Another major finding of the present study is that treating diabetic rats with tempol was less effective than treatment with aminoguanidine and pyridoxamine in blunting cardiac function loss. Pyridoxamine and aminoguanidine treatments increased percent cardiac fractional shortening, ejection fraction, peak $\pm dP/dt$, basal LVP and reduced LVEDP, while tempol treatment restored $+dP/dt$. Pyridoxamine and aminoguanidine treatments also significantly improved myocyte contraction and relaxations rates, extent of cell shortening and rates of Ca^{2+} release and uptake from the SR. Hearts from pyridoxamine- and aminoguanidine-animals were also more responsive to isoproterenol-induced contraction/relaxation compared with hearts from tempol-treated animals (Table 4). These data are not in agreement with those of Shi et al., [44], who found that oral administration of tempol (2 mmol/L) for six weeks after coronary artery ligation prevented the signs of cardiac dysfunction and failure manifest as an increase in left ventricular end diastolic pressure and volume, reduced ejection fraction and enhanced renal sympathetic nerve activity and plasma norepinephrine. The reason(s) for the discrepancy in our data is not clear at this time. One reason could be differences in the models. The second may be the amount of tempol administered may be toxic. Diabetic rats typically drink $> 3X$ more water than control rats (300 ± 40 mL compared with 80 ± 10 mL per day for controls), resulting in nearly 1.2 $\mu\text{mol/kg/day}$ tempol being consumed [45]. Since tempol reduces ROS production/generation through its superoxide dismutase mimetic action, scavenges $O_2^{\bullet-}$, exhibits catalase-like action, and reduces NADPH-dependent $O_2^{\bullet-}$ generation [27], we expected tempol treatment to reduce ventricular stiffening and increase cardiac distensibility by reducing activation of metalloproteases [27, 46, 47]. In this study, tempol treatment also did not improve RyR2 or SERCA2 activities, suggesting that ROS/superoxide is unlikely to be the primary cause for their dysregulation in diabetes.

The third major finding of the present study is that pyridoxamine is more efficacious than aminoguanidine in reducing formation of glucose-derived *N*^ε-carboxy(methyl)lysine, pentosidine and pyrraline adducts on RyR2 and SERCA2 in diabetic rats. This conclusion is

in agreement with earlier studies by Voziyan and Hudson [48] who indicated that pyridoxamine, but not aminoguanidine, strongly inhibited the formation of N^{ϵ} -carboxy(methyl)lysine by directly scavenging RCS. At this time, it is not clear if the higher hydrophilic properties of aminoguanidine prevent it from entering the cell and attenuating carbonylation of RyR2 and SERCA2 [49]. From its chemical structure, pyridoxamine is less hydrophilic and therefore more likely to enter cells and scavenge RCS. In addition to its ability to scavenge RCS, aminoguanidine also inhibits nitric oxide synthases and semicarbazide-sensitive amine oxidase [49] which may dilute its RCS-scavenging actions *in vivo*.

In conclusion, this study shows for the first time that lipid-derived MDA and 4HNE adducts are not formed on RyR2 or SERCA2 in either control or diabetic rat hearts. The reason for this unexpected finding is not clear at this time. What we do know is that MDA rearrange at physiological pH to form the less reactive enol derivative, and expression of enzymes that degrades it, aldehyde dehydrogenase and glyoxalase 1 are upregulated in the heart in diabetes. Expression and activity of aldose reductase, the enzyme that metabolizes 4-HNE is also upregulated in the heart during diabetes. In a recent study Sansbury et al., [50] indicated that in this study treating diabetic animals with the RCS scavengers, pyridoxamine and aminoguanidine were more efficacious in blunting the development of a diabetic cardiomyopathy than treatment with the ROS/superoxide scavenger, tempol, and that this beneficial effect is due in part to reducing formation of glucose-derived RCS adducts on the principal intracellular Ca^{2+} cycling proteins RyR2 and SERCA2.

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References

1. American Diabetes A. Economic costs of diabetes in the U.S. In 2007. *Diabetes Care*. 2008; 31:596–615. [PubMed: 18308683]
2. Giorda CB, Manicardi V, Diago Cabezudo J. The impact of diabetes mellitus on healthcare costs in Italy. *Expert Rev Pharmacoecon Outcomes Res*. 2011; 11:709–719. [PubMed: 22098288]
3. Nosadini R, Tonolo G. Cardiovascular and renal protection in type 2 diabetes mellitus: the role of calcium channel blockers. *J Am Soc Nephrol*. 2002; 13(Suppl 3):S216–S223. [PubMed: 12466317]
4. Battiprolu PK, Gillette TG, Wang ZV, Lavandero S, Hill JA. Diabetic Cardiomyopathy: Mechanisms and Therapeutic Targets. *Drug Discov Today Dis Mech*. 2010; 7:e135–e143. [PubMed: 21274425]
5. Thandavarayan RA, Giridharan VV, Watanabe K, Konishi T. Diabetic Cardiomyopathy and Oxidative Stress: Role of Antioxidants. *Cardiovasc Hematol Agents Med Chem*. 2011 Sep 9. [Epub ahead of print].
6. Boudina S, Abel ED. Diabetic cardiomyopathy, causes and effects. *Rev Endocr Metab Disord*. 2010; 11:31–39. [PubMed: 20180026]
7. Duncan JG. Mitochondrial dysfunction in diabetic cardiomyopathy. *Biochim Biophys Acta*. 2011; 1813:1351–1319. [PubMed: 21256163]

8. D'Souza A, Howarth FC, Yanni J, Dobrynski H, Boyett MR, Adeghate E, Bidasee KR, Singh J. Left ventricle structural remodelling in the prediabetic Goto-Kakizaki rat. *Exp Physiol.* 2011; 96:875–888. [PubMed: 21622965]
9. Shao C, Tian C, Ouyang S, Moore C, Alomar F, Nemet I, D'Souza A, Nagai R, Kutty S, Rozanski GJ, Ramanadham S, Singh J, Bidasee K. Carbonylation Induces Heterogeneity in Cardiac Ryanodine Receptors (RyR2) Function During Diabetes. *Mol Pharmacol.* 2012; 82(3):383–399. [PubMed: 22648972]
10. Shao CH, Capek HL, Patel KP, Wang M, Tang K, DeSouza C, Nagai R, Mayhan W, Periasamy M, Bidasee KR. Carbonylation contributes to SERCA2a activity loss and diastolic dysfunction in a rat model of type 1 diabetes. *Diabetes.* 2011; 60:947–959. [PubMed: 21300842]
11. Taha M, Lopaschuk GD. Alterations in energy metabolism in cardiomyopathies. *Ann Med.* 2007; 39:594–607. [PubMed: 17934906]
12. Boudina S, Abel ED. Diabetic cardiomyopathy revisited. *Circulation.* 2007; 115:3213–3223. [PubMed: 17592090]
13. Stanley WC, Lopaschuk GD, McCormack JG. Regulation of energy substrate metabolism in the diabetic heart. *Cardiovasc Res.* 1997; 34:25–33. [PubMed: 9217869]
14. Kota SK, Kota SK, Jammula S, Panda S, Modi KD. Effect of diabetes on alteration of metabolism in cardiac myocytes: therapeutic implications. *Diabetes Technol Ther.* 2011; 13:1155–1160. [PubMed: 21751873]
15. Wright JJ, Kim J, Buchanan J, Boudina S, Sena S, Bakirtzi K, Ilkun O, Theobald HA, Cooksey RC, Kandror KV, Abel ED. Mechanisms for increased myocardial fatty acid utilization following short-term high-fat feeding. *Cardiovasc Res.* 2009; 82:351–360. [PubMed: 19147655]
16. Munzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J.* 2010; 31:2741–2748. [PubMed: 20974801]
17. Tousoulis D, Briasoulis A, Papageorgiou N, Tsioufis C, Tsiamis E, Toutouzas K, Stefanadis C. Oxidative stress and endothelial function: therapeutic interventions. *Recent Pat Cardiovasc Drug Discov.* 2011; 6:103–114. [PubMed: 21513492]
18. Marjani A. Lipid peroxidation alterations in type 2 diabetic patients. *Pak J Biol Sci.* 2010; 13:723–730. [PubMed: 21850933]
19. Piconi L, Quagliaro L, Ceriello A. Oxidative stress in diabetes. *Clin Chem Lab Med.* 2003; 41:1144–1149. [PubMed: 14598863]
20. Uchida K. Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol Med.* 2000; 28:1685–1696. [PubMed: 10946210]
21. Basu S. Isoprostanes: novel bioactive products of lipid peroxidation. *Free Radic Res.* 2004; 38:105–122. [PubMed: 15104204]
22. Dirx E, Schwenk RW, Glatz JF, Luiken JJ, van Eys GJ. High fat diet induced diabetic cardiomyopathy. *Prostaglandins Leukot Essent Fatty Acids.* 2011; 85:219–225. [PubMed: 21571515]
23. Slatter DA, Bolton CH, Bailey AJ. The importance of lipid-derived malondialdehyde in diabetes mellitus. *Diabetologia.* 2000; 43:550–557. [PubMed: 10855528]
24. Voulgaridou GP, Anestopoulos I, Franco R, Panayiotidis MI, Pappa A. DNA damage induced by endogenous aldehydes: current state of knowledge. *Mutat Res.* 2011; 711:13–27. [PubMed: 21419140]
25. Shao CH, Rozanski GJ, Patel KP, Bidasee KR. Dyssynchronous (non-uniform) Ca²⁺ release in myocytes from streptozotocin-induced diabetic rats. *J Mol Cell Cardiol.* 2007; 42:234–246. [PubMed: 17027851]
26. Troncoso Brindeiro CM, Lane PH, Carmines PK. Tempol prevents altered K(+) channel regulation of afferent arteriolar tone in diabetic rat kidney. *Hypertension.* 2012; 59:657–664. [PubMed: 22252401]
27. Wilcox CS. Effects of tempol and redox-cycling nitroxides in models of oxidative stress. *Pharmacol Ther.* 2010; 126:119–145. [PubMed: 20153367]
28. Shao CH, Rozanski GJ, Nagai R, Stockdale FE, Patel KP, Wang M, Singh J, Mayhan WG, Bidasee KR. Carbonylation of myosin heavy chains in rat heart during diabetes. *Biochem Pharmacol.* 2010; 80:205–217. [PubMed: 20359464]

29. Dai S, Fraser H, Yuen VG, McNeill JH. Improvement in cardiac function in streptozotocin-diabetic rats by salt loading. *Can J Physiol Pharmacol.* 1994; 72:1288–1293. [PubMed: 7767868]
30. Shao CH, Wehrens XH, Wyatt TA, Parbhu S, Rozanski GJ, Patel KP, Bidasee KR. Exercise training during diabetes attenuates cardiac ryanodine receptor dysregulation. *J Appl Physiol.* 2009; 106:1280–1292. [PubMed: 19131475]
31. Bertoni AG, Hundley WG, Massing MW, Bonds DE, Burke GL, Goff DC Jr. Heart failure prevalence, incidence, and mortality in the elderly with diabetes. *Diabetes Care.* 2004; 27:699–703. [PubMed: 14988288]
32. Choi KM, Zhong Y, Hoit BD, Grupp IL, Hahn H, Dilly KW, Guatimosim S, Lederer WJ, Matlib MA. Defective intracellular Ca(2+) signaling contributes to cardiomyopathy in Type 1 diabetic rats. *Am J Physiol Heart Circ Physiol.* 2002; 283:H1398–H1408. [PubMed: 12234790]
33. Belke DD, Dillmann WH. Altered cardiac calcium handling in diabetes. *Curr Hypertens Rep.* 2004; 6:424–429. [PubMed: 15527685]
34. Bidasee KR, Dincer UD, Besch HR Jr. Ryanodine receptor dysfunction in hearts of streptozotocin-induced diabetic rats. *Mol Pharmacol.* 2001; 60:1356–1364. [PubMed: 11723243]
35. Ferrington DA, Krainev AG, Bigelow DJ. Altered turnover of calcium regulatory proteins of the sarcoplasmic reticulum in aged skeletal muscle. *J Biol Chem.* 1998; 273:5885–5891. [PubMed: 9488726]
36. Bidasee KR, Nallani K, Besch HR Jr, Dincer UD. Streptozotocin-induced diabetes increases disulfide bond formation on cardiac ryanodine receptor (RyR2). *J Pharmacol Exp Ther.* 2003; 305:989–998. [PubMed: 12606683]
37. Shah G, Pinnas JL, Lung CC, Mahmoud S, Mooradian AD. Tissue-specific distribution of malondialdehyde modified proteins in diabetes mellitus. *Life Sci.* 1994; 55:1343–1349. [PubMed: 7934642]
38. Minamiyama Y, Bito Y, Takemura S, Takahashi Y, Kodai S, Mizuguchi S, Nishikawa Y, Suehiro S, Okada S. Calorie restriction improves cardiovascular risk factors via reduction of mitochondrial reactive oxygen species in type II diabetic rats. *J Pharmacol Exp Ther.* 2007; 320:535–543. [PubMed: 17068205]
39. Zhang Y, Babcock SA, Hu N, Maris JR, Wang H, Ren J. Mitochondrial aldehyde dehydrogenase (ALDH2) protects against streptozotocin-induced diabetic cardiomyopathy: role of GSK3beta and mitochondrial function. *BMC Med.* 2012; 10:40. [PubMed: 22524197]
40. Agadjanyan ZS, Dmitriev LF, Dugin SF. A new role of phosphoglucose isomerase. Involvement of the glycolytic enzyme in aldehyde metabolism. *Biochemistry (Mosc).* 2005; 70:1251–1255. [PubMed: 16336185]
41. Thornalley PJ. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem J.* 1990; 269:1–11. [PubMed: 2198020]
42. Iwata K, Nishinaka T, Matsuno K, Kakehi T, Katsuyama M, Ibi M, Yabe-Nishimura C. The activity of aldose reductase is elevated in diabetic mouse heart. *J Pharmacol Sci.* 2007; 103:408–416. [PubMed: 17384488]
43. Choudhary S, Xiao T, Srivastava S, Zhang W, Chan LL, Vergara LA, Van Kuijk FJ, Ansari NH. Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells. *Toxicol Appl Pharmacol.* 2005; 204:122–134. [PubMed: 15808518]
44. Shi Z, Chen AD, Xu Y, Chen Q, Gao XY, Wang W, Zhu GQ. Long-term administration of tempol attenuates postinfarct ventricular dysfunction and sympathetic activity in rats. *Pflugers Arch.* 2009; 458:247–257. [PubMed: 19132383]
45. Matsumoto K, Krishna MC, Mitchell JB. Novel pharmacokinetic measurement using electron paramagnetic resonance spectroscopy and simulation of in vivo decay of various nitroxyl spin probes in mouse blood. *J Pharmacol Exp Ther.* 2004; 310:1076–1083. [PubMed: 15105413]
46. Grote K, Flach I, Luchtefeld M, Akin E, Holland SM, Drexler H, Schieffer B. Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxygen species. *Circ Res.* 2003; 92:e80–e86. [PubMed: 12750313]

47. Zhao W, Zhao T, Chen Y, Ahokas RA, Sun Y. Oxidative stress mediates cardiac fibrosis by enhancing transforming growth factor-beta1 in hypertensive rats. *Mol Cell Biochem.* 2008; 317:43–50. [PubMed: 18581202]
48. Voziyan PA, Hudson BG. Pyridoxamine: the many virtues of a maillard reaction inhibitor. *Ann N Y Acad Sci.* 2005; 1043:807–816. [PubMed: 16037308]
49. Thornalley PJ. Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Arch Biochem Biophys.* 2003; 419:31–40. [PubMed: 14568006]
50. Sansbury BE, Riggs DW, Brainard RE, Salabei JK, Jones SP, Hill BG. Responses of hypertrophied myocytes to reactive species: implications for glycolysis and electrophile metabolism. *Biochem J.* 2011; 435:519–528. [PubMed: 21275902]

Figure 1. Establishing a diabetic cardiomyopathy

Panel (i) shows representative M-mode echocardiograms of control (C) and diabetic (D) rat hearts. Panel (ii) shows basal and isoproterenol-induced peak +dP/dt for hearts from control (C) and diabetic (D) rats. Values shown are mean $\pm$ S.E.M from $n = 12$ animals per group. Panel (iii) shows basal and isoproterenol-induced peak -dP/dt for hearts from control (C) and diabetic (D) rats. Values shown are mean $\pm$ S.E.M from $n = 12$ animals per group. Panel (iv) shows representative evoked contractile kinetics of ventricular myocytes isolated from hearts of control (C) and diabetic (D) rats. Panel (v) shows representative evoked Ca^{2+}

transients in ventricular myocytes isolated from hearts of control (C) and diabetic (D) rats. Panel (vi) upper shows representative autoradiogram of steady state level of RyR2 protein in hearts from control (C) and diabetic (D) rats. β -actin served as the internal control for protein load. Graph below shows the ability of equivalents of RyR2 protein from control (C) and diabetic (D) rat hearts to bind the ligand [^3H]ryanodine. Values shown are mean $\pm$ S.E.M for four separate experiments. Panel (vii) upper show representative autoradiograms of steady state level of SERCA2 and PLN proteins in hearts from control (C) and diabetic (D) rats. β -actin served as the internal control for protein load. Graph below shows the ability of equivalents of SERCA2 protein from control (C) and diabetic (D) rat hearts to transport Ca^{2+} . Values shown are mean $\pm$ S.E.M for four separate experiments.

Figure 2. MDA adducts on sarcoplasmic reticulum membrane proteins

Representative autoradiogram (left panel) showing MDA adduct in SR membranes isolated from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control, (Ag-C) aminoguanidine-treated diabetic (Ag-D), tempol-treated control (T-C) and tempol-treated diabetic (T-D) rats. Graph, right side shows quantitative data for relative levels of MDA adducts on two protein bands present in SR membranes, Mw ~ 50 kDa and 40 kDa. Values shown were from three separate experiments. * significantly different from control (higher, $p < 0.05$), ** significant different from diabetic ($p < 0.05$), # significantly different from control (lower, $p < 0.05$), ## significantly different from T-C ($p < 0.05$).

Figure 3. 4-HNE adducts on sarcoplasmic reticulum membrane proteins

Representative autoradiogram (left panel) for 4-hydroxy-2-nonenal adduct in SR membranes isolated from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control, (Ag-C) aminoguanidine-treated diabetic (Ag-D), tempol-treated control (T-C) and tempol-treated diabetic (T-D) rats. Graph on right panel shows quantitative data for relative levels of 4-HNE adduct on two protein bands present in SR membranes, $M_w \sim 66$ kDa and 42 kDa. Values shown were from three separate experiments. * significantly different from control (higher, $p < 0.05$), ** significant different from diabetic ($p < 0.05$), # significantly different from control (lower, $p < 0.05$), ## significantly different from T-C ($p < 0.05$).

Figure 4. Effects of pyridoxamine and aminoguanidine treatments on formation of N^{ϵ} -carboxy(methyl)lysine adduct on RyR2 and SERCA2

Representative autoradiogram (left panel) for N^{ϵ} -carboxy(methyl)lysine adducts in SR membranes isolated from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control, and (Ag-C) aminoguanidine-treated diabetic (Ag-D) rats. Graphs on right side show quantitative data for relative levels of N^{ϵ} -carboxy(methyl)lysine adduct on RyR2 (upper) and SERCA2 (lower). Values shown are mean $\pm$ S.E.M for five separate experiments. *significantly different from control ($p < 0.05$).

Figure 5. Effects of pyridoxamine and aminoguanidine treatments on formation of pentosidine adduct on RyR2 and SERCA2

Representative autoradiogram for pentosidine adduct in SR membranes isolated from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control, and (Ag-C) aminoguanidine-treated diabetic (Ag-D) rats. Graphs on right show quantitative data for relative levels of pentosidine adduct on RyR2 (upper) and SERCA2 (lower). Values shown are mean $\pm$ S.E.M for five separate experiments. * significantly different from control ($p < 0.05$), ** significantly different from diabetic ($p < 0.05$)

Figure 6. Effects of pyridoxamine and aminoguanidine treatments on formation of pyrraline adduct on RyR2 and SERCA2

Representative autoradiogram for pyrraline adduct in SR membranes isolated from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control, and (Ag-C) aminoguanidine-treated diabetic (Ag-D) rats. Panel right side (upper) shows quantitative data for relative levels of pyrraline adduct on RyR2 and SERCA2 (lower). Values shown are mean $\pm$ S.E.M for five separate experiments. * significantly different from control ($p < 0.05$).

Figure 7. Effects of pyridoxamine, aminoguanidine and tempol treatments on evoked Ca^{2+} transients

Upper panels show representative evoked Ca^{2+} transient in myocytes isolated from hearts of diabetic (D), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated diabetic (Ag-D) and tempol-treated diabetic (T-D) rats. White arrows points to diastolic Ca^{2+} release in between stimulation in myocytes. Graph below shows the percent of myocytes isolated from four animals in each exhibiting diastolic Ca^{2+} release. Data are mean $\pm$ S.E.M; n = 60 cells or more cells from 6–7 hearts. *p<0.05 for diabetic treated compared to diabetic untreated hearts.

Figure 8. Steady state levels and activity of RyR2

Representative autoradiograms showing relative levels of RyR2 proteins in hearts from control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control (Ag-C), aminoguanidine-treated diabetic (Ag-D), tempol-treated control (T-C) and tempol-treated diabetic rats. β -actin served as the internal control to correct for protein amounts. Graph below shows [³H]ryanodine bound to equivalent amounts of RyR2 protein from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control (Ag-C), aminoguanidine-treated diabetic (Ag-D), tempol-treated control (T-C) and tempol-treated diabetic rats. Data shown are means $\pm$ S.E.M for four separate experiments.

Figure 9. Steady state levels and activity of SERCA2 and phospholamban (PLN) proteins
 Representative autoradiograms showing relative levels of SERCA2 and PLN proteins in hearts from control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control (Ag-C), aminoguanidine-treated diabetic (Ag-D), tempol-treated control (T-C) and tempol-treated diabetic rats. β -actin served as the internal control to correct for protein amounts. Graph below shows ability of equivalent amount of SERCA2 protein from hearts of control (C), diabetic (D), pyridoxamine-treated control (Py-C), pyridoxamine-treated diabetic (Py-D), aminoguanidine-treated control (Ag-C), aminoguanidine-treated diabetic (Ag-D), tempol-treated control (T-C) and tempol-treated diabetic rats to transport Ca^{2+} . Data shown are means $\pm$ S.E.M for four separate experiments; *significantly different from control ($p < 0.05$). ** significant different from diabetic ($p < 0.05$).

Table 1

General characteristics of animals used in the study

PARAMETER	Control (C) n = 12	Diabetic (D) n = 12	Pyridoxamine- treated control (Py-C) n = 12	Pyridoxamine- treated diabetic (Py-D) n = 12	Aminoguanidine- treated control (Ag-C) n = 12	Aminoguanidine- treated diabetic (Ag-D) n = 12	Tempol- treated control (T-C) n = 12	Tempol- treated diabetic (T-D) n = 12
Body mass (g)	393.0 ± 13.3	289.1 ± 14.3 [*]	394.8 ± 10.9	284.5 ± 13.6	380.9 ± 8.1	282.2 ± 10.8	383.3 ± 3.3	268.6 ± 12.1
Blood glucose (mmol) %	5.2 ± 0.5	22.6 ± 1.4 [*]	4.9 ± 1.1	20.1 ± 1.6	7.9 ± 0.3	23.4 ± 2.1	5.2 ± 0.7	19.3 ± 3.1
Glycosylated hemoglobin	4.3 ± 0.1	8.9 ± 0.1 [*]	4.2 ± 0.2	8.2 ± 0.3	4.6 ± 0.2	8.2 ± 0.2	4.2 ± 0.2	8.1 ± 0.3
Serum insulin (ng/ml)	1.12 ± 0.21	0.3 ± 0.0 [*]	1.1 ± 0.1	0.3 ± 0.0	0.7 ± 0.07	0.3 ± 0.0	0.7 ± 0.1	0.3 ± 0.0
Serum TBARS (nmol/ml)	2.8 ± 1.3	9.8 ± 1.5 [*]	1.1 ± 0.2	8.0 ± 1.3	3.2 ± 0.9	6.7 ± 0.7 ^{***}	1.7 ± 0.9	6.9 ± 0.7 ^{***}
Serum SSAO activity (units/ml/min)	0.3 ± 0.0	0.5 ± 0.0 [*]	0.2 ± 0.0	0.4 ± 0.0 ^{***}	0.4 ± 0.0 [*]	0.4 ± 0.1 ^{**}	0.3 ± 0.0	0.5 ± 0.0

^{*} significantly different from control ($p < 0.05$),^{***} significantly different from diabetic ($p < 0.05$)

Table 2Establishing diabetic cardiomyopathy *in vivo*

PARAMETER	Heart rate (beat per min) n = 12	% Cardiac fractional shortening n = 12	Cardiac ejection fraction n = 12	Peak +dP/dt (mm Hg/sec) n = 12	Peak left ventricular pressure (mm Hg) n = 12	Peak -dP/dt (mm Hg/sec) n = 12	Left ventricular end diastolic pressure (mm Hg) n = 12
Control (C)	382 ± 12	61 ± 3	85 ± 2	9753 ± 456	130 ± 4	9088 ± 935	0.6 ± 0.3
Diabetic (D)	291 ± 18 *	51 ± 2 *	70 ± 2 *	5176 ± 433 *	104 ± 7 *	4723 ± 606 *	4.8 ± 0.9 *

* significantly different from control (p < 0.05)

Table 3

Establishing reductions in myocyte contraction and alterations in evoked intracellular Ca^{2+} transient kinetics

PARAMETER	Rate of evoked cytoplasmic Ca^{2+} rise in myocytes at 0.5 Hz (f.a.u/msec) n = 75	Evoked Ca^{2+} transient amplitude at 0.5 Hz (f.a.u) n = 75	Rate of decay of evoked Ca^{2+} transient in myocytes at 0.5 Hz (f.a.u/msec) n = 75	Myocyte contraction velocity at 0.5 Hz ($\mu\text{m}/\text{sec}$) n = 90	% Myocyte shortening at 0.5 Hz n = 90	Myocyte relaxation velocity at 0.5 Hz ($\mu\text{m}/\text{sec}$) n = 90
Control (C)	90.7 $\pm$ 4.3	6.1 $\pm$ 0.3	280.5 $\pm$ 15.6	228.6 $\pm$ 18.5	14.3 $\pm$ 0.9	186.5 $\pm$ 17.6
Diabetic (D)	70.7 $\pm$ 4.3 *	3.8 $\pm$ 0.6 *	710 $\pm$ 22.3 *	89.9 $\pm$ 10.4 *	9.6 $\pm$ 0.9 *	77.1 $\pm$ 12.0 *

* significantly different from control (p < 0.05)

Effect of pyridoxamine, aminoguanidine and tempol treatments on *in vivo* cardiac parameters

Table 4

PARAMETER	Heart rate (beat per min) n = 12	% cardiac fractional shortening n = 12	cardiac ejection fraction n = 12	Amount of ISO injected	Peak +dP/dt (mm Hg/sec) n = 12	Peak left ventricular pressure (mm Hg) n = 12	Peak -dP/dt (mm Hg/sec) n = 12	Left ventricular end diastolic pressure (mm Hg) n = 12
Control (C)	382 ± 12	61 ± 3	84 ± 2	0.0 µg/kg 0.1 µg/kg	9753 ± 456 14971 ± 1536	130 ± 4 130 ± 3	9087 ± 934 10089 ± 556	0.6 ± 0.3 1.5 ± 0.6
Diabetic (D)	291 ± 18*	51 ± 2*	70 ± 2*	0.0 µg/kg 0.1 µg/kg	5176 ± 433* 11167 ± 579	104 ± 7* 108 ± 6	4721 ± 606* 7060 ± 1111	4.8 ± 0.9* 1.5 ± 0.7
Pyridoxamine-treated control (Py-C)	362 ± 20	58 ± 3	80 ± 4	0.0 µg/kg 0.1 µg/kg	9339 ± 506 13451 ± 578	123 ± 5 117 ± 3	8553 ± 846 10067 ± 1158	1.7 ± 0.6 1.1 ± 0.1
Pyridoxamine-treated diabetic (Py-D)	318 ± 19	57 ± 3**	82 ± 3**	0.0 µg/kg 0.1 µg/kg	8702 ± 278** 13451 ± 498**	120 ± 5** 122 ± 7**	6351 ± 623** 10104 ± 1472**	2.1 ± 0.5** 0.25 ± 0.4**
Aminoguanidine-treated control (Ag-C)	402 ± 17	59 ± 2	80 ± 5	0.0 µg/kg 0.1 µg/kg	10318 ± 415 14355 ± 915	135 ± 9 129 ± 7	8404 ± 612 11386 ± 1595	0.9 ± 1.0 0.4 ± 0.2
Aminoguanidine-treated diabetic (Ag-D)	285 ± 20	58 ± 3**	82 ± 3**	0.0 µg/kg 0.1 µg/kg	8786 ± 800** 13438 ± 915**	117 ± 7** 115 ± 8	6149 ± 839** 7891 ± 1254	2.5 ± 1.0** 0.9 ± 0.2
Tempol-treated control (T-C)	346 ± 20	60 ± 1	84 ± 1	0.0 µg/kg 0.1 µg/kg	11285 ± 591.8 14222 ± 840	132 ± 11 124 ± 8	8337 ± 620 12122 ± 914	1.0 ± 0.6 0.5 ± 1.8
Tempol-treated diabetic (T-D)	315 ± 14	50 ± 2	75 ± 3	0.0 µg/kg 0.1 µg/kg	8820 ± 465 12566 ± 982**	112 ± 6 118 ± 5	5381 ± 511 7938 ± 540	3.1 ± 2.2 3.0 ± 0.4**

¹ Isoproterenol,

* significantly different from control ($p < 0.05$),

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significantly different from diabetic ($p < 0.05$)
**

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Table 5Effect of pyridoxamine, aminoguanidine and tempol treatments on myocyte contractility and evoked Ca^{2+} transient kinetics

PARAMETER	Rate of evoked cytoplasmic Ca^{2+} rise in myocytes at 0.5 Hz (f.a.u./msec) n = 75	Evoked Ca^{2+} transient amplitude at 0.5 Hz (f.a.u) n = 70	Rate of decay of evoked Ca^{2+} transient in myocytes at 0.5Hz (f.a.u./msec) n = 75	Myocyte contraction velocity at 0.5Hz (μm/sec) n = 80	% myocyte shortening at 0.5Hz n = 85	Myocyte relaxation velocity at 0.5 Hz (μm/sec) n = 96
Control (C)	90.7 ± 4.3	6.1 ± 0.3	280.5 ± 15.6	228.6 ± 18.5	14.3 ± 0.9	186.5 ± 17.6
Diabetic (D)	70.7 ± 4.3*	3.8 ± 0.6*	710 ± 22.3*	89.9 ± 10.4*	9.6 ± 0.5*	77.1 ± 12.0*
Pyridoxamine-treated Control (Py-C)	87.4 ± 4.3	5.7 ± 0.4	301.5 ± 15.3	219.3 ± 12.3	13.4 ± 0.4	162.4 ± 7.5
Pyridoxamine-treated Diabetic (Py-D)	82.6 ± 6.7**	4.4 ± 0.4	480.3 ± 28.3**	164.5 ± 8.2**	11.3 ± 0.4**	131.6 ± 3.6**
Aminoguanidine-treated Control (Ag-C)	85.6 ± 7.3	5.4 ± 0.8	310.5 ± 21.8	193.2 ± 0.3	13.6 ± 1.2	172.1 ± 7.4
Aminoguanidine-treated Diabetic (Ag-D)	79.6 ± 4.3**	4.1 ± 0.6	510.3 ± 26.4**	159.2 ± 8.9**	11.2 ± 0.5**	122.6 ± 5.8**
Tempol-treated Control (T-C)	82.1 ± 6.3	5.4 ± 0.5	320.6 ± 12.4	215.7 ± 9.1	14.7 ± 0.5	187.4 ± 9.5
Tempol-treated Diabetic (T-D)	71.2 ± 4.1	3.9 ± 0.5	600 ± 32.3**	125.3 ± 6.1**	10.7 ± 0.5	111.3 ± 9.3**

* significantly different from control ($p < 0.05$),** significantly different from diabetic ($p < 0.05$)