Online supplement

Interleukin-1β, oxidative stress, and abnormal calcium handling mediate diabetic arrhythmic risk

Hong Liu, MD, PhD[a], Yang Zhao, MD[b], An Xie, PhD[a], Tae-Yun Kim, PhD[c], Radmila Terentyeva, BS[c,d], Man Liu, PhD[a], Guangbin Shi, MD[c], Feng Feng, BS[a], Bum-Rak Choi, PhD[c], Dmitry Terentyev, PhD[c,d], Shanna Hamilton, PhD[d], Samuel C. Dudley Jr, MD, PhD[a]*

a. Division of Cardiology, Department of Medicine, the Lillehei Heart Institute, University of Minnesota at Twin Cities, Minneapolis, Minnesota.
b. Division of Cardiology, Lanzhou University Second Hospital, Lanzhou University, Lanzhou, Gansu, China
c. Division of Cardiology, Cardiovascular Research Center, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, Rhode Island
d. Department of Physiology and Cell Biology, Ohio State University, Columbus, Ohio

Supplemental methods

High fat diet (HFD)-induced diabetic mouse model

As a well-established model of metabolic syndrome and type 2 DM (1-3), HFD-fed male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 24 weeks of age, when they have established DM (4). These mice were started on the HFD (60 kcal% fat, Research Diet, New Brunswick, NJ) at 6 weeks old and were continued on the same type of HFD throughout the experiments. Female mice were excluded because they are less susceptible to the development of DM under a high fat diet (5). Age- and gender-matched C57BL/6J mice fed normal chow (Harlan, Indianapolis, IN) were used as controls. The diabetic mice were randomly assigned to three different treatments: 1) MitoTEMPO, (2-(2,2,6,6-tetramethyl-piperidin-1-oxyl-4-ylamino)-2-oxoethyl-triphenylphosphonium chloride) (Enzo Life Sciences, Farmingdale, NY), daily intraperitoneal injection of 1 mg/kg for 2 weeks; 2) Interleukin-1 receptor antagonist (IL-1RA,

Biolegend, San Diego, CA), daily intraperitoneal injection at 3 mg/kg for 2 weeks; 3) S107 (Millipore, Burlington, MA), subcutaneous injection of 30 mg/kg for 1 week. S107 is known to inhibit resting Ca^{2+} leak through sarcoplasmic reticulum (SR) Ca^{2+} release channels, otherwise known as the ryanodine receptor (RyR2) channels (6). At the end of each treatment, mice underwent echocardiography, surface ECG recording, and in vivo electrophysiology testing before euthanasia for tissue collection.

Animal care were provided in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals, and all animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Non-invasive echocardiographic assessment

Echocardiography was performed under isoflurane anesthesia using the Vevo 2100 (VisualSonics, Toronto, Canada) ultrasound system as in previous studies (7). Body temperature and heart rate were maintained at 37-38 °C and above 400 bpm, respectively. B-mode and M-mode images were obtained along the left ventricular parasternal long axis and at the mid-papillary level of the short-axis views, respectively. Measurements were averaged from five consecutive beats during expiration. Percent left ventricular ejection fraction (EF) was calculated as: $100\times[(7/2.4+LVEDd)\times LEDd3] - [(7/2.4+LVESd)\times LVESd3] / [(7/2.4+LVEDd)\times LEDd3]$ (8).

Surface ECG recording, telemetry, and programmed ventricular stimulation

Under isoflurane anesthesia, a standard limb ECG was recorded at sampling rate of 4000 Hz using 25-gauge needle electrodes connected to an amplifier (ADinstruments, Colorado Spring, CO). One minute of ECG signals from limb lead II was averaged for QT interval measurement. Corrected QT interval (QTc) was calculated using Mitchell's formula (8). Under anesthesia, mice were implanted with ETA-F10 telemetry monitors (Data Sciences International, St. Paul,

Minnesota). Ventricular arrhythmia inducibility was determined by programmed ventricular stimulation where a 1.1F Millar electrophysiology catheter (Millar Instruments, Houston, TX) was advanced through the right jugular vein into the right ventricle. A STG2004 stimulator (ADinstruments, Colorado Spring, CO) was used along with MCStimulus software (Multichannel System, Baden-Württemberg, Germany) to pace the heart. Two programmed stimulation protocols were employed: 1) burst pacing at cycle lengths of 55 ms down to 30 ms for 1, 3, 5, 10 seconds; 2) a train of twenty beats (S1) at 80 ms interval followed by 3-11 extra stimuli (S2) with incrementally deceasing cycle lengths (by 2 seconds) between 30-55 ms. Inducible ventricular tachycardia (VT) was defined as reproducible episodes of ≥ 5 successive ventricular complexes. The ECG signals were analyzed using the LabChart 7.1 (AD Instrument) software.

Optical mapping for APD and arrhythmias

As described previously (9), mice were anaesthetized with isoflurane and injected with heparin before euthanasia. Hearts were rapidly excised and washed in oxygenated (95%/5% O_2/CO_2) modified Tyrode solution (in mM: 128.2 NaCl, 4.7 KCl, 1.19 NaH_2PO_4, 1.05 $MgCl_2$, 1.3 $CaCl_2$, 20.0 $NaHCO_3$, and 11.1 glucose, pH 7.35 ± 0.05), placed in a custom-made optical mapping chamber, attached to a pacing electrode and oriented so that the optical mapping field contained the anterior and posterior free walls (1x1 cm^2 field of view). Hearts were retrograde-perfused (3 mL/min at 37° C) with control Krebs-Henseleit buffer (in mM: 119 NaCl, 25 $NaHCO_3$, 4 KCl, 1.2 KH_2PO_4, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose and 2 sodium pyruvate, pH 7.4) and with the excitation-contraction uncoupler blebbistatin (10 μM, Abcam) to prevent motion artifacts. After 10 min of stabilization, hearts were perfused with fluorescence probes for membrane potential V_m (Di-4 ANEPPS or RH237). With a light source at an excitation wavelength of 530 nm, optical signals were recorded using a MiCAM Ultima-L CMOS camera (SciMedia) with high spatial (1x1 cm^2

field of view, 100×100 pixels) and temporal (2,000 frames/s) resolution (emission filter >650 nm). Optical action potentials were recorded during sinus rhythm and during ventricular pacing (S1S2 or rapid pacing protocol). IL-1β and mitoTEMPO were delivered by a microsyringe pump (World Precision Instruments). The average action potential duration at 90% repolarization (APD_{90}) was measured.

Isolation of ventricular cardiomyocytes

Adult mice ventricular cardiomyocytes were isolated as described before (10,11). Hearts were excised under isoflurane and perfused with buffer (in mM: 113 NaCl, 4.7 KCl, 0.6 Na_2HPO_4, 0.6 KH_2PO_4, 1.2 $MgSO_4$, 0.032 Phenol Red, 12 $NaHCO_3$, 10 $KHCO_3$, 10 HEPES, 30 Taurine, 10 2-3-butanedione monoxime) for 7 min at 3 mL/min flow rate using a temperature controlled Langendorff perfusion system. Hearts were then perfused with collagenase II (0.8 mg/mL, Worthington Biochemical Co. Lakewood, NJ) for 10 min at 37°C. Following suspension with serially increasing Ca^{2+} concentrations (0.2, 0.5, and 1 mM), cardiomyocytes were placed in a minimal Eagle's medium (MEM) containing 5% fetal bovine serum, 1% penicillin/streptomycin, 1 mM pyruvate, 5.5 mM glucose, and 1% insulin-transferrin-selenium (ITS).

Mitochondrial reactive oxygen species (mitoROS) measurement

MitoROS was measured by flow cytometry of ventricular cardiomyocytes (LSR, BD Biosciences, San Jose, CA) as described before (12,13). Isolated cardiomyocytes were incubated with MitoSOX Red (5 μM; Thermo Fisher Scientific, Waltham, MA) in MEM medium for 10 min at 37°C in a 95%/5% O_2/CO_2 incubator and were washed three times with phosphate buffered saline before flow cytometry. Five thousand single myocytes were selected by appropriate forward and side scatter gating. MitoSOX Red was detected at excitation/emission of 510/580 nm. The mean of the fluorescence intensity was obtained from the MitoSOX histogram. To test the direct

effect of IL-1β on mitoROS, cardiomyocytes isolated from the same control heart were incubated in the MEM medium with 0, 10, 100, 1000 pg/mL IL-1β, respectively, in separate culture dishes at 37°C for 4h before stained with mitoSOX for flow cytometry.

Cellular electrophysiology

Cardiac action potentials were recorded in isolated myocytes by whole cell current-clamp at room temperature as described previously (11). The voltage pulses were 3 ms in duration, 1.2-fold above the threshold intensity, and at a stimulation rate of 0.5 Hz. Series resistance was partially compensated by feedback circuitry. Current-voltage curves was evaluated. Action potential duration at 90% repolarization (APD90) was measured. All records were filtered with cutoff frequencies designed to avoid aliasing and was digitized at speeds at least two or three times the filter cutoff frequency, generally 2 kHz and 10 kHz, respectively.

For K+ current recording, Tyrode's solution was used as a bath solution (in mM: NaCl 140, KCl 5.4, MgCl2 1, HEPES 10, CaCl2 1.8, and glucose 5.5, pH 7.4). The pipette solution was composed of the following (in mM: K+-aspartate 110, KCl 20, NaCl 8, MgC12 1, CaC12 1, 1,2-bis(o-aminophenoxy)ethane-N,N,N0,N0-tetraacetic acid 10, K2ATP 4, and HEPES 10, pH=7.2). Series resistance in the whole-cell mode was in the range of 2 to 4 MΩ; 80% to 90% series resistance compensation was always used. Voltage-clamp currents were lowpass filtered at 1 to 3 kHz and digitized at 4 to 10 kHz. Currents were recorded with a series of 5 s pulses from a holding potential of -80mV. Test potentials of each pulse were increased by +10 mV from -110 mV to +50mV. The interval between two pulse stimulations was 10 s. Ito was obtained by subtracting the current recorded with and without the inactivating prepulse (-40 mV for 1 sec before test potentials). Current amplitudes were normalized to the cell capacitance and expressed as pA/pF (14).

Ca^{2+} sparks in ventricular myocytes

Ca^{2+} spark activity in isolated ventricular myocytes was be monitored as previously described by a Leica SP2 confocal laser scanning system equipped with a 60× 1.4 NA oil-immersion objective in line-scan mode using Ca^{2+}-sensitive indicators Fluo-4 pentapotassium salt (Invitrogen/Molecular Probes, Carlsbad, CA, USA) (15). Myocytes were permeabilized with saponin (0.01% for ~20 s) and exposed to the intracellular solution (in mM: 120 K^+ aspartate, 20 KCl, 0.81 $MgCl_2$, 1 KH_2PO_4, 0.5 EGTA, 3 MgATP, 10 phosphocreatine, 0.03 Fluo-4 pentapotassium salt, 20 HEPES (pH 7.2) and 5 U ml^{-1} creatine phosphokinase, 50 nM free Ca^{2+}). To assess the SR Ca^{2+} load, 20 mM caffeine was applied at the end of the experiments. The dye was excited with the 488 nm line of an argon laser. Emission was collected at 500–600 nm. Parameters of Ca^{2+} sparks was analyzed using SparkMaster plug-in for NIH ImageJ software.

Protein Western blots and RyR2 oxidation

Proteins were isolated from left ventricles and separated on a 4-20% SDS-PAGE gel and transferred onto 0.2 μm polyvinyl difluoride membranes. Following block in 5% BSA/PBS for 1 h, the membranes were incubated overnight with the primary antibody at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA). Vinculin (Cell Signaling Technology) was used as a loading control. Optical density of the bands was measured with ChemiDoc MP system (Bio-Rad, Hercules, CA) and analyzed with Image-Lab software (Bio-Rad).

Assessment of RyR2 oxidation was performed using a modified protocol from Roussel et al (16). Freshly collected left ventricular tissue was lysed. A two-hour immunoprecipitation of RyR2 with anti-RyR2 antibody (Thermo Scientific) was carried out using a Pierce Co-IP Kit (Thermo Scientific). An Oxidized Protein Western Blot Kit (Abcam, Cambridge, MA) was used

to detect oxidation of immunoprecipitated RyR2. The carbonyl groups were derivatized to 2,4 dinitrophenylhydrasone (DNP) by reaction with dinitrophenylhydrazine. Treated samples were subjected to SDS PAGE gels, transferred onto nitrocellulose membranes, and incubated with anti-DNP antibodies included in the kit (1:5000). RyR2 phosphorylation at Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) site Ser-2814 and protein kinase A (PKA) site Ser-2808 was assessed using custom-made antibodies (Phosphosolutions, Aurora, CO) (12,15).

Glucose and insulin measurement

Mice were fasted for six hours before blood was collected from the tail vein. Fasting glucose level was measured by a glucometer (ACCU-CHEK, Roche Applied Science, Indianapolis, IN). Fasting insulin level was measured using an ultra-sensitive mouse insulin ELISA kit (Crystal Chem, Elk Grove Village, IL). Insulin resistance was determined by the HOMA-IR index (homeostasis model assessment of insulin resistance) calculated as: fasting glucose x fasting insulin/22.5 (13).

1. Petro AE, Cotter J, Cooper DA, Peters JC, Surwit SJ, Surwit RS. Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse. Metabolism 2004;53:454-7.
2. Surwit RS, Feinglos M, Rodin J , et al. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. Metabolism 1995;44:645-51.
3. Rossmeisl M, Rim JS, Koza RA, Kozak LP. Variation in type 2 diabetes--related traits in mouse strains susceptible to diet-induced obesity. Diabetes 2003;52:1958-66.

4. Liu M, Jeong EM, Liu H, et al. Magnesium supplementation improves diabetic mitochondrial and cardiac diastolic function. JCI Insight 2019;4:123182.
5. Pettersson US, Waldén TB, Carlsson PO, Jansson L, Phillipson M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. PLoS One 2012;7:e46057.
6. Andersson DC, Marks AR. Fixing ryanodine receptor Ca leak - a novel therapeutic strategy for contractile failure in heart and skeletal muscle. Drug Discov Today Dis Mech 2010;7:e151-e157.
7. Jeong EM, Chung J, Liu H, et al. Role of mitochondrial oxidative stress in glucose tolerance, insulin resistance, and cardiac diastolic dysfunction. J Am Heart Assoc 2016;5:e003046.
8. Mitchell GF, Jeron A, Koren G. Measurement of heart rate and Q-T interval in the conscious mouse. Am J Physiol 1998;274:H747-51.
9. Lau E, Kossidas K, Kim TY, et al. Spatially discordant alternans and arrhythmias in tachypacing-induced cardiac myopathy in transgenic LQT1 rabbits: The importance of IKs and Ca^{2+} Cycling. PLoS One 2015;10:e0132198.
10. Jeong EM, Monasky MM, Gu L, et al. Tetrahydrobiopterin improves diastolic dysfunction by reversing changes in myofilament properties. J Mol Cell Cardiol 2013;56:44-54.
11. Liu M, Gu L, Sulkin MS, et al. Mitochondrial dysfunction causing cardiac sodium channel downregulation in cardiomyopathy. J Mol Cell Cardiol 2013;54:25-34.
12. Terentyev D, Rees CM, Li W, et al. Hyperphosphorylation of RyRs underlies triggered activity in transgenic rabbit model of LQT2 syndrome. Circ Res 2014;115:919-28.

13. Fraulob JC, Ogg-Diamantino R, Fernandes-Santos C, Aguila MB, Mandarim-de-Lacerda CA. A mouse model of metabolic syndrome: Insulin resistance, fatty liver and non-alcoholic fatty pancreas disease (NAFPD) in C57BL/6 mice fed a high fat diet. J Clin Biochem Nutr 2010;46:212-23.

14. El Gebeily G, Fiset C. 4-Hydroxytamoxifen inhibits K(+) currents in mouse ventricular myocytes. Eur J Pharmacol 2010;629:96-103.

15. Cooper LL, Li W, Lu Y, Centracchio J, Terentyeva R, Koren G, Terentyev D. Redox modification of ryanodine receptors by mitochondria-derived reactive oxygen species contributes to aberrant Ca^{2+} handling in ageing rabbit hearts. J Physiol 2013;591:5895-911.

16. Roussel J, Thireau J, Brenner C, et al. Palmitoyl-carnitine increases RyR2 oxidation and sarcoplasmic reticulum Ca^{2+} leak in cardiomyocytes: Role of adenine nucleotide translocase. Biochim Biophys Acta 2015;1852:749-58.

Supplemental Tables

Table S1. Additional ECG results from control and diabetic mice with treatments

	Ctrl	DM	DM+IL1RA	DM+MT	DM+S107	P value
HR (bpm)	495 ± 16	483 ± 8	498 ± 8	488 ± 10	525 ± 26	0.292
P dur (ms)	23.4 ± 1.7	24.1 ± 0.5	24.8 ± 0.6	22.7 ± 1.2	24.2 ± 0.9	0.548
PR (ms)	37.4 ± 0.9	38.5 ± 0.5	38.9 ± 0.6	39.1 ± 0.9	40.1 ± 1.2	0.332
QRS (ms)	10.7 ± 0.4	11.4 ± 0.3	12.1 ± 0.3	11.7 ± 0.5	10.6 ± 0.3	0.069
Amp P (μV)	95 ± 6	79 ± 3	79 ± 5	78 ± 6	86 ± 6	0.121
Amp R (μV)	647 ± 59	721 ± 37	694 ± 39	685 ± 46	765 ± 82	0.703
N	14	32	20	19	8	

Note: Data are means ± SEM. Amp, amplitude; bpm, beats per minute; dur, duration; Ctrl, control; DM, diabetes; HR, heart rate; IL1RA, IL-1 receptor antagonist; MT, mitoTEMPO.

Table S2. Echocardiographic characterization of control and diabetic mice

	Ctrl (N=6)	DM (N=6)	P value
LVAW;d (mm)	0.97 ± 0.03	0.90 ± 0.05	0.226
LVAW;s (mm)	1.24 ± 0.04	1.13 ± 0.05	0.110
LVPW;d (mm)	0.78 ± 0.03	0.79 ± 0.03	0.686
LVPW;s (mm)	1.05 ± 0.03	1.05 ± 0.02	0.947
LVEDV (μL)	74.6 ± 1.7	68.3 ± 2.8	0.074
LVESV (μL)	33.0 ± 0.9	30.9 ± 2.0	0.323
HR (bpm)	480 ± 15	452 ± 14	0.200

Note: ： Data are means ± SEM. bpm, beats per minute; Ctrl, control; DM, diabetes; HR, heart rate; LVAW;d, left ventricular end-diastolic anterior wall thickness; LVAW;s, left ventricular end-systolic anterior wall thickness; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVPW;d, left ventricular end-diastolic posterior wall thickness; LVPW;s, left ventricular end-systolic posterior wall thickness.