Supplemental Material

Reactive Oxygen Species Originating from Mitochondria Regulate the Cardiac Sodium Channel

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Methods

All chemicals were purchased from Sigma (St. Louis, MO) except: diazoxide and FGIN-1-27 (Enzo Life Sciences International, Inc., Plymouth Meeting, PA); chelerythrine and cyclosporin A (Alomone Labs, Jerusalem, Israel); and Hoechst 33342, MitoSOX^{TM} Red, and tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR). MitoTEMPO was a generous gift from Dr. Sergey Dikalov (Emory University, Atlanta, GA).

Cell Culture

We maintained a human embryonic kidney (HEK) cell line stably expressing the human cardiac $Na_v1.5$ channel (SCN5A cells). Expression of $Na_v1.5$ was linked to green fluorescent protein (GFP) expression by an internal ribosomal entry site (SCN5A-IRES-GFP). SCN5A cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 0.2 mg/mL geneticin (for antibiotic selection) and 1% penicillin/streptomycin in a 95% O_2/5% CO_2 incubator at 37ºC. Rat neonatal ventricular myocytes (NVM) were isolated from neonatal rat hearts by collagenase treatment (Worthington Biochemical Corporation, Lakewook, NJ).

Nearly undetectable levels of GPD1-L protein are expressed in HEK cells.[1] Therefore, for whole-cell patch clamping experiments to study GPD1-L effects on $Na_v1.5$, SCN5A cells were transiently transfected with WT or A280V GPD1-L (a generous gift from Dr. Barry London, University of Pittsburgh, PA) and an expression vector containing red fluorescent protein (RFP) as described previously.[2] In these experiments, cells expressing both GFP and RFP were studied.

Electrophysiology

Na^+ currents were measured using the whole-cell patch clamp technique in voltage-clamp mode at room temperature. Pipettes (1-2 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, and Na_2ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 130, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1.2, HEPES 10 and glucose 5 (adjusted to pH 7.4 with CsOH). A stepped voltage protocol from -100 to +60 mV with a holding potential of -100 mV was applied to establish the presence of voltage-gated $Na_v1.5$ channels. Peak currents obtained during steps to -20 or -30 mV were used for comparison in determining the relative reduction of I_{Na}. Steady state fast inactivation was assessed during voltage depolarization from a holding potential of –140 to -20 mV for 500 ms, and measuring current at -20 mV. In all recordings, 80% of the series resistance was compensated, yielding a maximum voltage error of ~1 mV. Data were sampled at 50 kHz and later low pass filtered at 10 kHz for analysis. Currents were recorded and analyzed with an Axopatch 200B amplifier, Axon Digidata 1320A A/D converter and pClamp software (Molecular Devices, Sunnyvale, CA). To minimize time-dependent drift in gating parameters, all protocols were initiated 2-5 min after whole-cell configuration was obtained. The currents were normalized with cell capacitance prior to deriving ratios.

Rat NVM action potentials were measured using the whole-cell patch clamp technique in current-clamp mode at room temperature. Pipettes (2-4 MΩ) were filled with a pipette solution containing (in mmol/L): NaCl 10, potassium glutamate 130, EGTA 1.0, $MgCl_2$ 0.5, KCl 9, HEPES 10, glucose 10, and MgATP 5 (adjusted to pH 7.4 with KOH). The bath solution consisted of (in mmol/L): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.0, HEPES 10 and glucose 10 (adjusted to pH 7.4 with NaOH). Action potentials were evoked by brief (4 ms) current injections applied at 0.8-1 Hz. Eighty percent of the series resistance was compensated, yielding a maximum voltage error of ~1 mV. Data were sampled at 50 kHz and later low pass filtered at 10 kHz for analysis. Action potentials were recorded and analyzed with an Axopatch 200B amplifier, Axon Digidata 1320A A/D converter and pClamp software.

The following specific inhibitors or activators were applied directly in the pipette solution, alone or together: NADH (100-500 μmol/L), N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1-20 mmol/L), allopurinol (200 μmol/L), mitoTEMPO (5-20 μmol/L), rotenone (1-5 μmol/L), antimycin A (20-40 μmol/L), azide (10 mmol/L), 5-hydroxydecanoate (5-HD, 300 μmol/L), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 500 μmol/L), cycloporine A (CsA, 0.5 μmol/L), PK11195 (50 μmol/L), 4'-chlorodiazepam (4'-CD, 40-100 μmol/L), and FGIN-1-27 (500 μmol/L). Apocynin (100-300 μmol/L),

forskolin (1-5 μmol/L), NAD^+ (500 μmol/L) and malonate (1 mmol/L) were applied to bath solution, respectively. Concentrations were determined in our laboratory or by using the similar values used in literatures.

Intracellular NADH Level

Intracellular NADH levels ($[NADH]_i$) were detected by using the EnzyChromTM NAD^+/NADH Assay Kit (BioAssay Systems, Hayward, CA) in SCN5A cells with or without treatment of 1 mmol/L pyruvate and 10 mmol/L lactate for 10 min at room temperature. The intensity difference of the reduced product color, measured at 565 nm at time zero and 15 min later, was proportional to the change in $[NADH]_i$.

Confocal Microscopy

To measure mitochondrial ROS, the fluorecent probe MitoSOX™ Red was used according to the manufacturer's protocol. Briefly, three groups of SCN5A cells or rat NVM were studied: untreated cells, the PL group (cells treated with 1 mmol/L pyruvate and 10 mmol/L lactate for 10 min at room temperature, under which condition intracellular NADH level was increased[3-5], see "Results"), and the NAD-PL group (cells incubated with NAD^+ for ~6 hours at 37 ºC and then treated with 1 mmol/L pyruvate and 10 mmol/L lactate for 10 min at room temperature). The three groups of cells were then incubated with 2.5 μM MitoSOX™ Red in Hank's balanced salt solution (HBSS) for 10 min at 37 ºC, followed by three times wash with warm HBSS. Before treatment with MitoSOX™ Red, cells were first stained with Hoechst 33342 (0.4 μg/ml working concentration) for 20 min at 37 ºC. Images were taken on a Zeiss LSM510 META confocal microscope (Carl Zeiss GmbH, Oberkochen, Germany) using an argon laser excitation (514 nm) with emisson collection at more than 560 nm (red). The cell area was calculated, and the whole cell fluorescence of MitoSOX™ Red was measured with ImageJ software. The number of pixels of cell fluorescence divided by the cell area was used to determine the mitochondrial ROS generation. For each of the groups, 9 to 16 cells were used. NADH in water has an emission peak at 460 nm and less than 20% of the maximum value above 560 nm[6]. Therefore, NADH is unlikely to interfere with the fluorescence of MitoSOX™ Red in our experiments.

To measure the effect of elevated intracellular NADH level on the mitochondrial membrane potential ($\Delta\Psi_m$), the fluorescent membrane-permeant cationic probe TMRM, which is readily sequestered by mitochondria, was applied. SCN5A cells or rat NVM were loaded with TMRM (100 nmol/L)[7] for 30 min at 37 ºC in the dark. Then, cells were washed gently twice and kept with the bath solution used in the patch experiments before being placed on the stage of a Zeiss LSM510 META confocal microscope (35°C). TMRM was excited at 543 nm with a helium neon laser (3%), and the emission was collected through a 560 nm longpass filter. Images were collected at 30 s intervals for 10 min and then 2 min interval for 30 min. For the PL group, pyruvate and lactate were applied after the first image was taken. The mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μmol/L) was incubated for 1 min at 35 ºC with cells, which is sufficient to completely depolarize $\Delta\Psi_m$.[8] Images were then collected every 2 min for 20 min. The resulting fluorescence images were processed using Zeiss LSM510 META software to obtain the time course of the TMRM fluorescence changes.

Statistical Evaluations

Data are shown as the mean ± SEM. Determinations of statistical significance were performed with ANOVA with Bonferroni correction for comparisons of multiple means. A value of $P<0.05$ was considered statistically significant.

ONLINE TABLE I

Online Table I. Parameters of voltage dependence of steady state activation and inactivation of all groups.

	Voltage dependence of activation			Voltage dependence of inactivation		
	$V_{1/2}$, mV	k, mV	n	$V_{1/2}$, mV	k, mV	n
SCN5A	-44.7 ± 0.2	5.7 ± 0.1	14	-72.9 ± 0.3	6.8 ± 0.2	15
+ 100 μM $[NADH]_i$	-44.5 ± 0.3	5.5 ± 0.2	16	-75.6 ± 0.3#	7.0 ± 0.3	21
+ 100 μM $[NADH]_i$ + 10 mM $[L\text{-}NAME]_i$	-45.9 ± 0.6	6.0 ± 0.5	8	-74.8 ± 0.6	9.3 ± 0.5	7
+ 100 μM $[NADH]_i$ + 200 μM $[apocynin]_o$	-43.2 ± 0.3	6.9 ± 0.2	14	-78.5 ± 0.6*	8.6 ± 0.5	14
+ 100 μM $[NADH]_i$ + 200 μM $[allopurinol]_i$	-44.1 ± 1.1	6.6 ± 0.8	13	-77.5 ± 0.6*	6.9 ± 0.3	11
+ 100 μM $[NADH]_i$ + 5 μM $[mitoTEMPO]_i$	-44.1 ± 0.2	5.9 ± 0.2	11	-77.4 ± 0.2	6.1 ± 0.1	9
+ 100 μM $[NADH]_i$ + 1 μM $[rotenone]_i$	-45.8 ± 0.4	5.2 ± 0.3	10	-78.1 ± 0.3*	7.6 ± 0.3	9
+ 20 μM $[antimycin\ A]_i$	-46.1 ± 0.4	5.1 ± 0.3	13	-69.3 ± 0.8#	6.3 ± 0.3	7
+ 100 μM $[NADH]_i$ + 1 mM $[malonate]_o$	-45.9 ± 0.4	5.9 ± 0.3	12	-75.9 ± 0.2	6.6 ± 0.2	10
+ 100 μM $[NADH]_i$ + 10 mM $[azide]_i$	-46.6 ± 0.6	5.5 ± 0.5	16	-79.3 ± 0.2*	5.0 ± 0.2	9
+ 100 μM $[NADH]_i$ + 300 μM $[5\text{-}HD]_i$	-39.9 ± 0.2*	6.7 ± 0.1	13	-78.8 ± 0.2*	5.9 ± 0.2	7
+ 200 μM $[diazoxide]_i$	-45.1 ± 0.5	7.1 ± 0.5	11	-71.0 ± 0.3	7.5 ± 0.3	5
+ 100 μM $[NADH]_i$ + 500 μM $[DIDS]_i$	-45.7 ± 0.3	5.1 ± 0.2	13	-79.2 ± 0.2*	6.2 ± 0.2	9
+ 100 μM $[NADH]_i$ + 0.5 μM $[CsA]_i$	-41.2 ± 1.0*	5.3 ± 0.8	13	-75.5 ± 0.5	7.0 ± 0.4	9
+ 100 μM $[NADH]_i$ + 50 μM $[PK11195]_i$	-41.3 ± 0.7*	5.9 ± 0.5	15	-79.0 ± 0.2*	6.0 ± 0.2	7
+ 100 μM $[NADH]_i$ + 40 μM $[4'\text{-}CD]_i$	-45.6 ± 0.5	6.0 ± 0.4	14	-78.6 ± 0.4*	7.5 ± 0.4	10
+ 500 μM $[FGIN\text{-}1\text{-}27]_i$	-46.7 ± 0.4	5.4 ± 0.3	9	-78.9 ± 0.5#	8.4 ± 0.5	7
+ 100 μM $[NADH]_i$ + 500 μM $[FGIN\text{-}1\text{-}27]_i$	-46.7 ± 0.4	5.4 ± 0.3	9	-83.3 ± 0.3*	7.0 ± 0.2	5

Data were shown as mean ± SEM, n is sample number. For $V_{1/2}$, # $P<0.05$ vs. SCN5A, and *$P<0.05$ vs. + 100 μM $[NADH]_i$.

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