Supporting Information

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SI Results

Echocardiography. Echocardiographic parameters were measured from 8-wk-old and 43-wk-old wild-type (*wt*) mice and 8-wk-old and 43-wk-old Duchenne muscular dystrophy (*mdx*) mice under light methoxyflurane anesthesia (Table S5) as described in *SI Materials and Methods*.

Mass Spectrometry Analysis of Immunoprecipitated $I_{Ca\text{-}L}$ Protein. Immunoprecipitated $I_{Ca\text{-}L}$ proteins from *wt* heart and *mdx* heart were analyzed by mass spectrometry on a Velos Orbitrap (Tables S1–S4) as described in *SI Materials and Methods*.

SI Materials and Methods

Isolation of Ventricular Myocytes. Myocytes were isolated from 8-wk-old and 43-wk-old male C57BL/10ScSn-Dmdmdx/Arc (*mdx*), C57BL/10ScSnArc *wt* mice, and *mdx mice* treated with phosphorodiamidate morpholino oligomer (PMO) at 10 mg/kg per week by i.p. injection for 24 wk from 7 d of age. Animals were anesthetized with i.p. injection of pentobarbitone sodium (240 mg/kg) before excision of the heart as approved by The Animal Ethics Committee of The University of Western Australia in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (Eighth Edition, 2013).

Data Acquisition for Patch-Clamp Studies. The whole-cell configuration of the patch-clamp technique was used to measure changes in $I_{Ca\text{-}L}$ currents in intact ventricular myocytes (1, 2). Microelectrodes with tip diameters of 3–5 μm and resistances of 0.5–1.5 MΩ contained the following: 115 mM CsCl, 10 mM Hepes, 10 mM EGTA, 20 mM tetraethylammonium chloride, 5 mM MgATP, 0.1 mM Tris-GTP, 10 mM phosphocreatine, and 1 mM $CaCl_2$ (pH adjusted to 7.05 at 37 °C with CsOH). Currents were measured in extracellular modified Tyrode's solution containing the following: 140 mM NaCl, 5.4 mM CsCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM Hepes, and 11 mM glucose (pH adjusted to 7.4 with NaOH). All experiments were performed at 37 °C. Macroscopic currents were recorded by using an Axopatch 200B voltage-clamp amplifier (Molecular Devices) and an IBM compatible computer with a Digidata 1322A interface and pClamp9 software (Molecular Devices). An Ag/AgCl electrode was used to ground the bath. Once the whole-cell configuration was achieved, the holding potential was set at –80 mV. Na^+ channels and T-type Ca^{2+} channels were inactivated by applying a 50-ms prepulse to –30 mV immediately before each test pulse. The time course of changes in Ca^{2+} conductance were monitored by applying a 100-ms test pulse to −10 mV once every 10 s.

Measurement of Mitochondrial Membrane Potential. The fluorescent indicator 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide [JC-1, 200 nM, excitation (ex) 480 nm, emission (em) 580/535 nm; Molecular Probes] was used to measure mitochondrial membrane potential (Ψ_m) at 37 °C as described (2). Individual 580-nm and 535-nm signals were assessed in each experiment to confirm that the fluorescent indicator was accurately measuring Ψ_m. Fluorescent signal was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Fluorescent images were taken at 2-min intervals with 50-ms exposure. Metamorph 6.3 was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used as background and was subtracted. The 580/535 nm ratiometric fluorescent values recorded over 6 min before and 4 min after the addition of drugs were averaged, and alterations in fluorescent ratios were reported as a percentage increase from the basal average. To confirm that the JC-1 signal was indicative of Ψ_m, 4 mM potassium cyanide (KCN) was added at the end of each experiment to collapse Ψ_m. In addition, individual 580-nm and 535-nm signals were assessed in each experiment to confirm that the fluorescent indicator was accurately measuring Ψ_m.

Measurement of Mitochondrial Flavoprotein Oxidation. Flavoprotein autofluorescence was used to measure flavoprotein oxidation in intact mouse cardiac myocytes based on described methods (3). Fluorescence at ex 480 nm and em 535 nm was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Fluorescent images were taken at 1-min intervals with 1.5-s exposure. Metamorph 6.3 was used to quantify the signal by manually tracing myocytes. An equivalent region not containing cells was used as background and was subtracted. Fluorescent values recorded over 5 min before and 10 min following the addition of drugs were averaged and alterations in fluorescent ratios were reported as the percentage increase from the basal average. Ten micomolar carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) was added at the end of each experiment to achieve a maximum fluorescence value indicative of maximum flavoprotein oxidation.

RNA Preparation and RT-PCR Analysis. Total RNA was extracted from 2 to 3 mg of sections, cut from frozen tissue blocks, using TRIzol (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed on 100 ng of total RNA for 40 cycles of amplification across exons 22–24, using 1 U SuperScript III (Invitrogen) in a 12.5-μL reaction. Primers were used at 94 °C for 30 s, 55 °C annealing for 1 min, 72 °C extension for 2 min. A 2-μL sample from this reaction was then used as the template for 35 cycles of secondary PCR amplification by using 0.5 U AmpliTaq Gold (Applied Biosystems) under cycling conditions described above. Primers are listed in Table S6. Products were then electrophoresed on a 2% (vol/vol) Tris, acetic acid, and EDTA agarose gel.

Immunostaining. Heart and diaphragm tissue from untreated *mdx* mice, C57BL/10ScSnArc *wt*, and four *mdx mice* treated with PMO at 10 mg/kg per week by i.p. injection for 24 wk from 7 d of age were embedded in OCT compound (Tissue-Tek; Sakura Finechemicals) before being snap frozen in liquid nitrogen-cooled isopentane. Immunohistochemistry was performed on serial 10-mm frozen sections adhered to silanated slides by using the Mouse-on-Mouse kit with Texas Red substrate according to the manufacturer's instructions (Vector Laboratories) with the monoclonal antibody DYS2 (NCL-DYS2; Novocastra) and Zenon Alexafluor 647 antibody (Z-25002; Molecular Probes). Fluorescence was visualized with a Nikon A1 confocal microscope at 20× magnification and shown in green. All images were captured and processed using identical conditions (4, 5).

MTT Assay. Reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) depends on the presence of reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), and intact mitochondrial electron transport (6). Reduction of MTT occurs predominantly at the mitochondria in cardiomyocytes (7). The basis for this assay is the cleavage of the yellow tetrazolium salt (MTT) to purple formazan crystals by the electron transport chain within the mitochondria (8). An increase in absorbance represents

an increase in formazan production and, therefore, an increase in metabolic activity. Myocytes in suspension in 96-well plates were treated with relevant agonists/antagonists. MTT was then added to each well (final concentration 0.5 mg/mL). The rate of increase in absorbance was immediately measured by using a spectrophotometer (PowerWave XS) at 570 nm with a reference wavelength of 620 nm at 37 °C. The rate of increase in absorbance of treated myocytes was expressed as a percentage of the rate of increase in absorbance of untreated myocytes.

Extraction of $I_{Ca\text{-}L}$ Protein from Membrane Fractions. Membrane fractions were prepared from crude heart homogenates. Ventricular tissue was homogenized 3 × 5 s at 22,000 × *g* on ice in a buffer containing 300 mM sucrose, 20 mM imidazole-HCl at pH 7.0, 1 mM EDTA, and an EDTA-free protease inhibitor tablet. Homogenates were centrifuged for 15 min at 3,800 × *g* at 4 °C, and the supernatant was ultracentrifuged for 2 h at 100,000 × *g* at 4 °C. The pellet was resuspended on ice in 4.0 mL of buffer containing the following: 600 mM KCl, 300 mM sucrose, 20 mM imidazole-HCl at pH 7.0, 1 mM EDTA, 0.025 mM leupeptin, and 0.25 mM Pefabloc. The resuspended pellet was incubated on ice for 1 h, then placed in the ultracentrifuge at 100,000 × *g* at 4 °C. The membrane fraction was obtained by resuspension of the pellet in 0.5 mL of homogenization buffer.

Immunoprecipitation of $I_{Ca\text{-}L}$ Protein and VDAC Protein. One milligram of protein was diluted into 500 μL of immunoprecipitation (IP) buffer containing the following 10 mM Na_2HPO_4, 0.2 mM H_2O, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 5 mM NaF, 0.1% mM Triton X-100, and a EDTA-free mini protease inhibitor tablet (added just before use). Anti-Ca_V pan α_1 subunit antibody (1.2 μg of ACC-004; Alomone Labs) or 4 μg of Porin monoclonal antibody (MSA03; MitoSciences) was added to the protein/IP buffer mix and incubated overnight at 4 °C. For negative controls, 2 μg of rabbit polyclonal antibody raised in house against a gold *N*-heterocyclic carbene compound (α-GOLD) or 20 μg of serum from pooled BALB/c and C57BL/6 mice (MS) was added to the protein/IP buffer mix and incubated overnight at 4 °C. Following overnight incubation, 100 μL of PureProteome Protein G Magnetic Beads were washed as per manufacturer's instruction (Millipore), added to the protein/IP buffer mix and incubated for 10 min at room temperature with continuous mixing to capture the protein/antibody complex. The beads were then allowed to migrate toward a magnetic stand, upon which supernatant was removed, and beads were washed three times with 500 μL of PBS containing 0.1% Tween 20. Following the last wash, 60 μL of 0.2 M Glycine (pH 2.5) (Elution Buffer) was added to the beads and incubated for 2 min at room temperature. The beads were then allowed to migrate toward a magnetic stand, upon which supernatant was removed and neutralized by adding 5 μL of 1 M Tris at pH 8.5.

Immunoblot of Immunoprecipitated $I_{Ca\text{-}L}$ and VDAC Protein. Immunoprecipitated proteins were treated with sample buffer (50 mM Tris·Cl at pH 6.8, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) for 4 min at 95–100 °C. Sample proteins were separated on a 12.5% SDS-polyacrylamide gel (SDS/PAGE) at pH 8.8, at 80 V for 30 min until the dye front passed through the stacking gel, then at 120 V until the dye front ran off the bottom the gel. Proteins were electrophoretically transferred from the SDS/PAGE to a PVDF membrane in transfer buffer (40 mM Tris·Cl, 20 mM sodium acetate, 2 mM EDTA, 0.05% SDS, 20% methanol at pH 7.4) for 1 h, 100 V, 2 A, 200 W at 4 °C. Following transfer, the membrane was incubated in blocking buffer (5% BSA and 0.05% Tween-20 in PBS, pH 7.5) for 1 h at room temperature (RT). The membrane was then rinsed and incubated with primary antibody for overnight at 4 °C. Primary antibodies used were either Anti-Ca_V pan α_1 subunit antibody (ACC-004, 1:400 dilution; Alomone Labs) or Porin monoclonal antibody (MSA03, 1:2,000 dilution; MitoSciences). Following incubation with primary antibodies, membranes were then rinsed three times for 10 min then incubated with secondary antibody for 1 h at RT. Secondary antibodies used were either Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (172-1019, 1:10,000 dilution; Bio-Rad) or Goat Anti-Mouse IgG (H+L)-HRP Conjugate (172-1011, 1:10,000 dilution; Bio-Rad). Signal was detected by using ECL detection reagent (GE Healthcare) and exposed to film (GE Healthcare).

Mass Spectrometry Analysis of Immunoprecipitated $I_{Ca\text{-}L}$ Protein. Ten micrograms of immunoprecipitated $I_{Ca\text{-}L}$ protein from three *wt* hearts and 10 μg of immunoprecipitated $I_{Ca\text{-}L}$ protein from three *mdx* hearts were analyzed. Digest peptides were separated by nano-LC by using an Ultimate 3000 HPLC and autosampler system (Dionex) at The Bioanalytical Mass Spectrometry Facility, University of New South Wales, Sydney. Samples (2.5 μL) of protein were concentrated and desalted onto a micro C18 precolumn (500 μm × 2 mm; Michrom Bioresources) with H_2O:CH_3CN (98:2, 0.05% TFA) at 15 μL/min. After a 4-min wash, the precolumn was switched (Valco 10 port valve; Dionex) into line with a fritless nano column (75 μm × ~10 cm) containing C18 media (5 μm, 200 Å Magic; Michrom). Peptides were eluted by using a linear gradient of H_2O:CH_3CN (98:2, 0.1% formic acid) to H_2O:CH_3CN (64:36, 0.1% formic acid) at 250 nL/min over 30 min. High voltage (2,000 V) was applied to low volume tee (Upchurch Scientific), and the column tip was positioned ~0.5 cm from the heated capillary (T = 280 °C) of an Orbitrap Velos (Thermo Electron) mass spectrometer. Positive ions were generated by electrospray and the Orbitrap operated in data-dependent acquisition mode (DDA).

A survey scan *m/z* 350–1,750 was acquired in the Orbitrap (resolution = 30,000 at *m/z* 400, with an accumulation target value of 1 million ions) with lockmass enabled. Up to the 10 most abundant ions (>5,000 counts) with charge states > +2 were sequentially isolated and fragmented within the linear ion trap by using collisionally induced dissociation with an activation $q = 0.25$ and activation time of 30 ms at a target value of 30,000 ions. *M/z* ratios selected for MS/MS were dynamically excluded for 30 s. Peak lists were generated by using Mascot Daemon/extract_msn (Matrix Science, Thermo) by using the default parameters, and submitted to the database search program Mascot (version 2.1, Matrix Science). Search parameters were as follows: Precursor tolerance 4 ppm and product ion tolerances ± 0.4 Da; Met(O) specified as variable modification, enzyme specificity was trypsin, one missed cleavage was possible, and the nonredundant National Center for Biotechnology Information (NCBInr) protein database containing a total number of 14,987,464 peptides was searched across species. To estimate the false discovery rate (FDR), a search against a decoy NCBInr database in which the sequences were reversed or randomized was performed. The FDR was calculated as follows: (number of false positives/(number of false positives + number of true positives) × 100. The FDR was estimated to be 3.3% and 4.9% for *wt* and *mdx* immunoprecipitated samples, respectively.

Mitochondrial Respiration Studies. Mitochondria were isolated from three pooled mouse hearts (9), and ~50–100 μg was resuspended in 0.25 mL of mitomedium B (0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 1 $g{\cdot}L^{-1}$ fatty acid-free BSA, 60 mM lactobionate, 110 mM mannitol, and 0.3 mM DTT at pH 7.1 with KOH) and added to 0.5 mL of mitomedium B in a 1-mL OROBOROS high-resolution respirometer thermostatically maintained at 37 °C. The system was left to equilibrate for 5 min, before adding digitonin (50 $\mu g{\cdot}mL^{-1}$) and waiting for 5 min for the oxygen consumption to decline. Respiration on 0.5 mM

tetramethyl-p-phenylenediamine and 2 mM ascorbate was measured in the permeabilized membranes.

Echocardiography. Echocardiographic studies to measure left ventricular function were performed on mice under light methoxyflurane anesthesia with the use of an i13L probe on a Vivid 7 Dimension (GE Healthcare). Echocardiographic measurements were taken on M mode in triplicate from separate mice. The quantitative measurements represent the average of 8-wk-old *wt* ($n = 3$) and 43-wk-old *wt* ($n = 5$) and 8-wk-old *mdx* ($n = 3$) and 43-wk-old *mdx* ($n = 6$) mice. M-mode recordings were made at a sweep speed of 200 mm/s. Measurements of left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), fractional shortening (FS), left ventricular posterior wall in diastole, left ventricular posterior wall in systole, intraventricular septum in diastole, end diastolic diameter (EDD), and intraventricular septum in systole were made. FS was calculated by the formula [(LVEDD−LVESD)/EDD] × 100.

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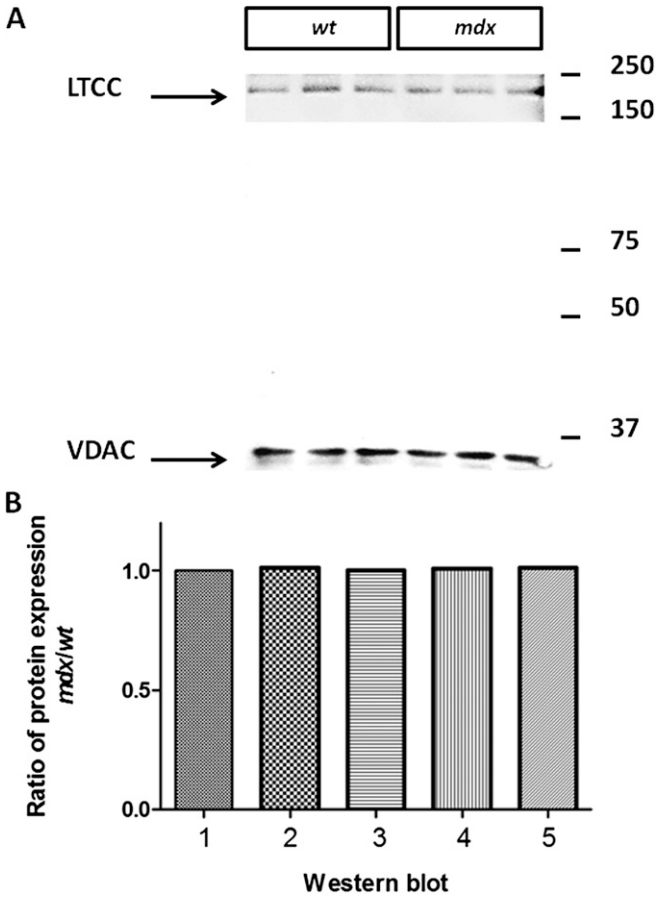

Fig. S1. Immunoblot analysis of L-type calcium channel (LTCC) protein expression in three hearts from 8-wk-old *wt* mice and three hearts from 8-wk *mdx* mice. (*A*) Representative immunoblots probed with Anti-Ca_V pan α_1 subunit antibody (LTCC) or Porin monoclonal antibody (voltage-dependent anion channel, VDAC) for $I_{Ca\text{-}L}$ protein extracted from membrane fractions from *wt* hearts and *mdx* hearts as shown. For further detail, see *Materials and Methods*. (*B*) LTCC protein expression was normalized to VDAC protein and shown as a ratio of expression of protein from *mdx* hearts and from *wt* hearts after analysis on densitometry for each of five immunoblots performed.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)
Table S4 (DOCX)
Table S5 (DOC)
Table S6 (DOC)