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L-type Ca\(^{2+}\) channel contributes to alterations in mitochondrial calcium handling in the \textit{mdx} ventricular myocyte

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Viola HM, Davies SM, Filipovska A, Hool LC. The L-type \textit{Ca}\(^{2+}\) channel contributes to alterations in mitochondrial calcium handling in the \textit{mdx} ventricular myocyte. \textit{Am J Physiol Heart Circ Physiol} 304: H767–H775, 2013. First published January 18, 2013; doi:10.1152/ajpheart.00700.2012. — The L-type \textit{Ca}\(^{2+}\) channel is the main route for calcium entry into cardiac myocytes, and it is essential for contraction. Alterations in whole cell L-type Ca\(^{2+}\) channel current and Ca\(^{2+}\) homeostasis have been implicated in the development of cardiomyopathies. Cytoskeletal proteins can influence whole cell L-type Ca\(^{2+}\) current and mitochondrial function. Duchenne muscular dystrophy is a fatal X-linked disease that leads to progressive muscle weakness due to the absence of cytoskeletal protein dystrophin. This includes dilated cardiomyopathy, but the mechanisms are not well understood. We sought to identify the effect of alterations in whole cell L-type \textit{Ca}\(^{2+}\) channel current on mitochondrial function in the murine model of Duchenne muscular dystrophy (\textit{mdx}). Activation of the L-type \textit{Ca}\(^{2+}\) channel with the dihydropyridine agonist BayK(\textit{−}) caused a significantly larger increase in cytosolic Ca\(^{2+}\) in \textit{mdx} vs. wild-type (\textit{wt}) ventricular myocytes. Consistent with elevated cytosolic Ca\(^{2+}\), resting mitochondrial Ca\(^{2+}\), NADH, and mitochondrial superoxide were significantly greater in \textit{mdx} vs. \textit{wt} myocytes. Activation of the channel with BayK(\textit{−}) caused a further increase in mitochondrial Ca\(^{2+}\), NADH, and superoxide in \textit{mdx} myocytes. The ratios of the increases were similar to the ratios recorded in \textit{wt} myocytes. In mitochondria isolated from 8-wk-old \textit{mdx} hearts, respiration and mitochondrial electron transport chain complex activity were similar to mitochondria isolated from \textit{wt} hearts. We conclude that mitochondria function at a higher level of resting calcium in the intact \textit{mdx} myocyte and activation of the L-type \textit{Ca}\(^{2+}\) channel contributes to alterations in calcium handling by the mitochondria. This perturbation may contribute to the development of cardiomyopathy.

L-type \textit{Ca}\(^{2+}\) channel; mitochondria; calcium; muscular dystrophy; cardiomyopathy

THE PROGRESSION OF CARDIAC hypertrophy to failure and development of many cardiomyopathies involves alterations in Ca\(^{2+}\) homeostasis, cardiac myocyte remodeling, disorganization of cytoskeletal proteins, and reduced energy metabolism (25). However, the mechanisms that lead to mitochondrial dysfunction and compromised cardiac function responsible for the development of the myopathy are poorly understood.

Cytoskeletal proteins stabilize cell structure. In mature muscle, intermediate filaments form a three-dimensional scaffold that extend from the Z disks to the plasma membrane and traverse cellular organelles such as t-tubules, sarcoplasmic reticulum, and mitochondria (35). Intermediate filaments and microtubules interact directly with mitochondria by binding to outer mitochondrial membrane proteins. In addition to a physical association, cytoskeletal proteins also regulate the function of proteins in the plasma membrane and within the cell (34). The L-type \textit{Ca}\(^{2+}\) channel (\textit{I_{Ca-L}}) or dihydropyridine receptor is anchored to F-actin networks by subsarcolemmal stabilizing proteins such as AHNAK that also tightly regulate the function of the channel (13, 20, 28). Disruption of actin filaments significantly alters the L-type \textit{Ca}\(^{2+}\) current (20, 23, 28). Abnormal mitochondrial function is associated with the absence of desmin intermediate filament protein in the heart (6). The abnormalities are detected early before structural defects develop, suggesting that the cytoskeletal environment influences the progression of the myopathy.

Alterations in Ca\(^{2+}\) homeostasis are associated with the development of cardiac hypertrophy and cardiomyopathy (25). Overexpression of the \textit{α}_{1C} or the \textit{β}_{2} subunit of \textit{I_{Ca-L}} leads to hypertrophy and failure (22, 33). In addition, exposure of \textit{I_{Ca-L}} to hydrogen peroxide can increase peak inward current and intracellular Ca\(^{2+}\) (1, 15, 37). This is sufficient to alter protein synthesis and increase cell size consistent with the development of hypertrophy (19, 32). Ca\(^{2+}\) influx through \textit{I_{Ca-L}} is a requirement for excitation and contraction in the heart. However, \textit{I_{Ca-L}} can also regulate mitochondrial function. Activation of the channel with application of the dihydropyridine receptor agonist BayK(\textit{−}) or voltage clamp of the plasma membrane can influence mitochondrial superoxide production, NADH production, and metabolic activity (36).

We hypothesized that disruption of the cytoskeletal architecture will result in altered \textit{I_{Ca-L}} current and Ca\(^{2+}\) handling by the mitochondria. We based this hypothesis on previous findings that depolymerization of actin with latrunculin A in healthy myocytes alters mitochondrial function after application of BayK(\textit{−}) (36). We investigated the hypothesis in a murine model of Duchenne muscular dystrophy (\textit{mdx}) that lacks dystrophin and exhibits disruption of the cytoskeletal architecture that leads to cardiomyopathy (3, 27, 30). We examined the effect of activation of \textit{I_{Ca-L}} on cytosolic Ca\(^{2+}\) and mitochondrial Ca\(^{2+}\) uptake. We performed experiments in quiescent ventricular myocytes with consistent ATP utilization or where we held ATP concentration constant (in the patch pipette) since this allowed us to more readily explore the effects of channel activation on mitochondrial function. We find that cardiac myocytes isolated from 8-wk-old \textit{mdx} mice

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that exhibit disorganized cytoskeletal protein networks but not yet overt cardiomyopathy (26) show significantly greater Ca\(^{2+}\) influx through I_{Ca-L} under resting conditions compared with wt myocytes. We also find that resting mitochondrial Ca\(^{2+}\), NADH production, and superoxide generation are increased in mdx myocytes. Activation of I_{Ca-L} further increases cytosolic and mitochondrial Ca\(^{2+}\), NADH production, and superoxide generation, indicating that I_{Ca-L} contributes to alterations in mitochondrial Ca\(^{2+}\) handling in the mdx myocyte.

**MATERIALS AND METHODS**

Isolation of adult mouse cardiac myocytes. Myocytes were isolated from 8-wk-old male C57BL/10ScSn-DmdmdxArc (mdx) and C57BL/10ScSn wild-type (wt) mice. Animals were anesthetized with intraperitoneal 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 (NHMRC, 7th ed., 2004). Cells were isolated based on methods described by O’Connell et al. (24). Mouse hearts were excised and cannulated onto a Langendorff apparatus via the aorta and perfused with Krebs-Henseleit buffer (KHB) containing the following (in mM): 120 NaCl, 25 NaHCO\(_3\), 4.8 KCl, 2.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), and 11 glucose (pH 7.35 with Na\(_2\)CO\(_3\) at 37°C) for 4 min at 37°C. KHB was then supplemented with 2.4 mg/ml collagenase B, and hearts were perfused for 3 min, followed by a further 8-min perfusion in the presence of 40 μM calcium. Aorta and atria were removed before ventricles were gently teased apart and triturated to dissociate myocytes into suspension in KHB supplemented with 10% FCS. Myocyte suspension was then spun at 500 rpm for 3 min, supernatant was discarded, and myocytes were resuspended in calcium-free HEPES-buffered solution (HBS) containing the following (in mM): 5.3 KCl, 0.4 MgSO\(_4\), 7H\(_2\)O, 139 NaCl, 5.6 NaHPO\(_4\), 2H\(_2\)O, 5 glucose, 20 HEPES, and 2 glutamine (pH 7.4 at 37°C). Calcium was then titrated back to achieve a final extracellular concentration of 2.5 mM. All experiments were performed in freshly isolated myocytes at 37°C.

Reagents. Fluorescent dyes including fura-2 AM, Rhod-2 AM (Rhod-2), and dihydroethidium (DHE) were purchased from Molecular Probes. All salt solutions, (S)-(−)-Bay K8644 ([BayK(−)], (R)−(−)-Bay K8644 [BayK(+)], nisoldipine, myothiazol, dantrolene, thapsigargin, ionomycin, carbonyl cyanide p-(tri-fluoromethoxy)phenylhydrazone (FCCP), and oligomycin were purchased from Sigma. Rat360 was purchased from Merck, ryanodine was from Alomone Labs, KB-R7943 was from Torcisia Bioscience, and gp91ds-t was synthesized by AnaSpec.

Fluorescent studies. All fluorescent studies including measurement of intracellular calcium, mitochondrial calcium, mitochondrial NADH, and superoxide were performed using a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. 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.

**Measurement of intracellular Ca\(^{2+}\).** Intracellular calcium was monitored in intact mouse cardiac myocytes using the fluorescent indicator Rhod-2 (200 nM; 515- to 560-nm ex filter and 580 long pass em) at 37°C. Fluorescent images were taken at 2-min intervals with 200-ms exposure. Four micromoles of ionomycin and 40 μM oligomycin and then 50 μM FCCP were added at the end of each experiment to achieve maximum (F_{max}) and minimum (F_{min}) fluorescent values, respectively. Fluorescent values recorded over 3 min before and 3 min following addition of drugs were averaged to give basal and treatment fluorescent values (F) and expressed as a percentage of full size changes in fluorescence according to the following equation:

\[
\%Rhod-2 = \left[ \frac{(F - F_{min})}{(F_{max} - F_{min})} \right] \times 100
\]

where F_{min} = 0.82 ± 0.09 (wt, n = 4) and 0.84 ± 0.05 (mdx, n = 7); F_{max} = 2.97 ± 0.76 (wt, n = 4) and 1.58 ± 0.14 (mdx, n = 7).

**Measurement of mitochondrial NADH.** Autofluorescence of NADH was monitored in intact mouse cardiac myocytes at 37°C as previously described (ex 365 nm and em 535 nm) (32). Fluorescent images were taken at 1-min intervals with 1.5-s exposure. Forty μM oligomycin and then 50 μM FCCP were added at the end of each experiment to achieve maximum (F_{max}) and minimum (F_{min}) fluorescent values, respectively. Fluorescent values recorded over 3 min before and 8 min following addition of drugs were averaged to give basal and treatment fluorescent values (F), as a percentage of full size changes in fluorescence according to the following equation:

\[
\%NADH = \left[ \frac{(F - F_{min})}{(F_{max} - F_{min})} \right] \times 100
\]

where F_{min} = 0.66 ± 0.04 (wt, n = 9) and 0.66 ± 0.02 (mdx, n = 9); and F_{max} = 1.14 ± 0.02 (wt, n = 9) and 1.08 ± 0.01 (mdx, n = 9).

**Measurement of superoxide.** Superoxide generation was assessed in intact mouse cardiac myocytes using fluorescent DHE (5 μM; 515- to 560-nm ex filter and 590 long pass em) at 37°C as previously described (37). Fluorescent images were taken at 1-min intervals with 200-ms exposure. Fluorescence was reported as the slope of the signal measured at 1–15 min (basal) and 16–30 min (treatment). Slope values for each treatment group were also plotted relative to basal slope values assigned a value of 1.0.

**Fig. 1.** Direct activation of L-type Ca\(^{2+}\) channel (I_{Ca-L}) increases intracellular Ca\(^{2+}\). Representative traces of intracellular calcium recorded in wt (A) myocytes and mdx (B) myocytes before and after exposure to 10 μM BayK(−) or −10 μM BayK(+) show no significant difference in calcium influx. Nisoldipine, 10 μM nisoldipine, Dant, 10 μM dantrolene; RyR, ryanodine receptor; Thaps, thapsigargin; KB-R7943, Na\(^{+}/Ca\(^{2+}\) exchanger inhibitor. D: ratio of increase in intracellular Ca\(^{2+}\) after addition of BayK(−) for wt and mdx myocytes; n: number of myocytes. E: representative current traces from a wt (a) (110 pF) and mdx (b) myocyte (112 pF) over 100-ms time course as indicated. Data are means ± SE of 50-ms inactivation integral of current (set top right), and activation integral of current (set top left). F: mean ± SEM of total integrated current (at 200 ms) for all myocytes as indicated. G: representative current traces from a wt (a) (168 pF) and mdx (b) myocyte (167 pF) over a 500-ms time course as indicated. Data are means ± SEM of total integrated current (set top right) for all wt and mdx myocytes. H: current/voltage (I-V) relationship measured in wt (n = 4) and mdx (n = 8) myocytes during voltage steps from −40 to +20 mV (P = NS).

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Data acquisition for patch-clamp studies. The whole cell configuration of the patch-clamp technique was used to measure changes in L-type Ca\textsuperscript{2+} channel currents in intact ventricular myocytes (14, 37). Microelectrodes with tip diameters of 3–5 μm and resistances of 0.5–1.5 MΩ contained the following (in mM): 115 CsCl, 10 HEPES, 10 EGTA, 20 tetraethylammonium chloride, 5 MgATP, 0.1 Tris-GTP, 10 phosphocreatine, and 1 CaCl\textsubscript{2} (pH adjusted to 7.05 at 37°C with CsOH). Currents were measured in extracellular modified Tyrode’s solution containing the following (in mM): 140 NaCl, 5.4 CsCl, 2.5 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 5.5 HEPES, and 11 glucose (pH adjusted to 7.4 with NaOH). All experiments were performed at 37°C. Macroscopic currents were recorded 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\textsuperscript{+} channels and T-type Ca\textsuperscript{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\textsuperscript{2+} conductance were monitored by applying a 100-ms test pulse to 10 mV once every 10 s.

Mitochondrial respiration studies. Mitochondria were isolated from three pooled wt hearts and from three pooled mdx hearts (8), and approximately 50–100 μg were resuspended in 0.25 ml mitomedium B (0.5 mM EGTA, 3 mM MgCl\textsubscript{2}, 20 mM taurine, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES, 1 g/l fatty acid-free BSA, 60 mM lactobionate, 110 mM mannitol, and 0.3 mM DTT, pH 7.1 with KOH) and added to 0.5 ml 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 μg/ml) and waiting for 5 min for the oxygen consumption to decline. Respiration on 0.5

Fig. 2. Direct activation of $I_{\text{Ca,L}}$ results in an increase in mitochondrial Ca\textsuperscript{2+}. Representative traces of Rhod-2 fluorescence (%Rhod-2) recorded from wt myocytes (A) and mdx myocytes (B) before and after exposure to 10 μM BayK (−) or 10 μM BayK (+). Vertical arrow indicates when drug was added. Representative traces of response of (A, inset right) wt and (B, inset right) mdx myocytes to 5 μM ionomycin and 40 μM oligomycin, then 50 μM carbonyl cyanide p-(tri-fluoromethoxy)phenylhydrazone (FCCP). C: means ± SE of changes in Rhod-2 fluorescence for all myocytes exposed to treatments as indicated; 10 μM Ru360. D: ratio of increase in Rhod-2 fluorescence after addition of BayK (−) for wt and mdx myocytes; n, number of myocytes. 

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mM TMPD and 2 mM ascorbate was measured in the permeabilized membranes (state 3 respiration).

Statistical analysis. Results are reported as means ± SE. Statistical comparisons of responses between unpaired data were made using the Student’s t-test or between groups of cells using one-way ANOVA and the Tukey’s post hoc test (GraphPad Prism version 5.04).

RESULTS

Activation of ICa-L significantly increases intracellular Ca2+ in myocytes from mdx hearts and alters mitochondrial Ca2+. We measured intracellular Ca2+ in myocytes isolated from wt hearts before and after activation of ICa-L with the dihydropyridine agonist BayK(−). Intracellular Ca2+ increased from 208 ± 11 to 432 ± 51 nM after activation of ICa-L (Fig. 1, A and C). The increase in intracellular Ca2+ could be prevented with application of the ICa-L blocker nisoldipine but was unaffected by the ryanodine receptor antagonists dantrolene and ryanodine, the sarcoplasmic reticulum Ca2+-ATPase inhibitor thapsigargin, and the plasma membrane Na+/Ca2+ exchanger inhibitor KB-R7943 (Fig. 1C). We also exposed wt myocytes to the (+)-enantiomer of BayK that does not act as an agonist [BayK(+)]. Exposure of wt myocytes to BayK(+) did not significantly alter intracellular Ca2+ (Fig. 1, A and C).

There is good evidence that resting calcium is elevated in mdx cardiac myocytes as a result of increased transient receptor potential channel and reverse mode Na+/Ca2+ exchanger activity (2, 16). Consistent with these findings, resting Ca2+ was higher in mdx myocytes than in myocytes from wt hearts (247 ± 13 nM; Fig. 1, B and C). Addition of BayK(−) caused a significantly further increase in intracellular Ca2+ in mdx myocytes (681 ± 40 nM; Fig. 1, B and C) such that the ratio of the increase in intracellular Ca2+ was greater compared with that of wt myocytes (wt = 1.69 ± 0.15 and mdx = 2.27 ± 0.18; P < 0.05; Fig. 1D). The increase in intracellular Ca2+ in response to BayK(−) could be prevented with application of nisoldipine but was unaffected by dantrolene, ryanodine, thapsigargin, and KB-R7943 (Fig. 1C). Exposure of mdx myocytes to BayK(+) did not significantly alter intracellular Ca2+ (Fig. 1, B and C).

![Graph](http://ajpheart.physiology.org/)

**Fig. 3.** Direct activation of ICa-L results in an increase in mitochondrial NADH. Representative traces of NADH autofluorescence (%NADH) recorded from wt myocytes (A) and mdx (B) myocytes before and after exposure to 10 μM BayK(−) or 10 μM BayK(+). Vertical arrow indicates when drug was added; 10 μM FCCP were added as indicated to confirm that the signal was mitochondrial in origin. C: means ± SE of changes in NADH fluorescence for all myocytes exposed to treatments as indicated. Nisol, 10 μM nisoldipine. D: ratio of increase in Rhod-2 fluorescence after addition of BayK(−) for wt and mdx myocytes; n, number of myocytes.
We patch clamped the myocytes and measured the rate of inactivation of the macroscopic current. Consistent with previous reports (18, 41), the inactivation rate of $I_{\text{Ca-L}}$ current was $24.2 \pm 1.3\%$ slower in myocytes from mdx hearts ($n = 16$) compared with currents recorded in wt myocytes recorded over a 100-ms time course ($n = 13$; Fig. 1E). Consistent with a delayed inactivation rate, the 50-ms inactivation integral of current and total integral of current were significantly greater in myocytes from mdx hearts compared with myocytes from wt hearts recorded over a 100 ms time course (Fig. 1E, inset top and inset right). Similarly,

![Graphs and images showcasing data from experiments on myocytes.](image)

Fig. 4. Direct activation of $I_{\text{Ca-L}}$ results in an increase in mitochondrial superoxide. Representative traces of dihydroethidium (DHE) fluorescence (DHE fluorescence) recorded from (A) wt myocytes and (B) mdx myocytes before and after exposure to 10 $\mu$M BayK(-) or 10 $\mu$M BayK(+). Vertical arrow indicates when drug was added. Slopes of the signals are indicated in brackets at right. Data are means ± SE of changes in DHE fluorescence for all (C) wt myocytes and (D) mdx myocytes exposed to treatments as indicated: Nisol, 10 $\mu$M nisoldipine; 10 $\mu$M Ru30; Myx, 10 $\mu$M myxothiazol; 10 $\mu$M gp91ds-tat peptide. E: ratio of increase in DHE fluorescence after addition of BayK(-) for wt and mdx myocytes. F: ratio of increase in DHE fluorescence after addition of gp91ds-tat peptide; $n$, number of myocytes; a.u., arbitrary units.
total integral of current was significantly greater in myocytes from mdx vs. wt myocytes recorded over a 50-ms time course (Fig. 1G). No difference was recorded in the activation integral of current in mdx and wt myocytes (Fig. 1E, inset right). Current density in mdx myocytes was not different from current density recorded in wt myocytes (Fig. 1, F and H) and cell size did not differ (116.8 ± 5.4, n = 30 vs. 107.8 ± 4.6, n = 41; NS), indicating that I_{Ca-L} expression was not altered. In addition, resting membrane potential does not differ between wt and mdx myocytes (41). Therefore, the slower inactivation rate appeared to be contributing to a greater influx of calcium and the significantly higher intracellular Ca^{2+} recorded in mdx myocytes after activation of the channel.

We assessed mitochondrial Ca^{2+} as changes in Rhod-2 fluorescence in the myocytes. Resting mitochondrial Ca^{2+} was significantly higher in myocytes from mdx hearts (Fig. 2, B and C). Rhod-2 fluorescence increased after addition of BayK(+) in myocytes from wt and mdx hearts. Alterations in mitochondrial membrane potential can mediate alterations in cardiac myocyte mitochondrial calcium (31). Application of the electron transport chain uncoupler FCCP causes depolarization of the mitochondrial membrane potential, resulting in reduced uptake of calcium by the mitochondria. Conversely, exposure of cardiac myocytes to oligomycin results in hyperpolarization of the mitochondrial membrane potential and increased uptake of calcium by the mitochondria. We confirmed that the Rhod-2 signal was mitochondrial in origin with application of ionomycin and oligomycin followed by the mitochondrial electron chain uncoupler FCCP. After addition of oligomycin, the Rhod-2 signal increased consistent with inhibition of ATP synthase and application of FCCP caused a decrease in Rhod-2 signal (Fig. 2, A, inset and B, inset). The mitochondrial Ca^{2+} uniporter inhibitor Ru360 attenuated the increase in Rhod-2 fluorescence in both wt and mdx myocytes (Fig. 2C). Exposure of wt and mdx myocytes to BayK(+) did not significantly alter Rhod-2 fluorescence (Fig. 2C). Although resting mitochondrial Ca^{2+} was higher in mdx myocytes, the ratio of the increase in Rhod-2 fluorescence in response to activation of I_{Ca-L} was similar to that of wt myocytes (wt = 1.97 ± 0.17 and mdx = 1.88 ± 0.25; NS; Fig. 2D).

Increased uptake of calcium into the mitochondria triggers activation of the tricarboxylic acid (TCA) cycle. Increased TCA cycle activity causes an increase in production of NADH from nicotinamide adenine dinucleotide (NADH). Therefore, the production of NADH from NADH + is a calcium dependent process. Consistent with a higher resting mitochondrial Ca^{2+}, the reduction of NADH to NADH + is a calcium dependent process. Consistent with a higher resting mitochondrial Ca^{2+}, the reduction of NADH to NADH + is a calcium dependent process. Consistent with a higher resting mitochondrial Ca^{2+}, the reduction of NADH to NADH + is a calcium dependent process. Consistent with a higher resting mitochondrial Ca^{2+}, the reduction of NADH to NADH + is a calcium dependent process.

Activation of I_{Ca-L} increases superoxide production in mitochondria from mdx hearts. Previous studies have demonstrated increased NAD(P)H-oxidase expression and superoxide production in the mdx heart and skeletal muscle (16, 38, 40). Consistent with this, we found that basal cellular superoxide (assessed as changes in DHE fluorescence) was significantly higher in mdx myocytes and a component of this was due to increased NAD(P)H-oxidase activity because application of gp91ds-tat peptide [that inhibits activity of the oxidase by preventing association of gp47phox with gp91phox in NAD(P)H-oxidase] decreased basal superoxide levels (Fig. 4, B). We measured the effect of activation of I_{Ca-L} on cellular superoxide. Application of BayK(−) further increased superoxide in wt myocytes, and the response was attenuated with prior exposure of cells to nisoldipine (Fig. 4, A and C). The increase in superoxide could also be prevented with application of mitochondrial calcium uniporter blocker Ru360 and complex III inhibitor myxothiazol but was unaffected by application of gp91ds-tat peptide, implicating the mitochondria as the source of increased superoxide production in response to activation of I_{Ca-L} (Fig. 4, C and F). Exposure of wt myocytes to BayK(+) did not significantly alter superoxide production (Fig. 4, A and C). BayK(−) further increased superoxide in mdx myocytes and the increase was attenuated with Ru360 and myxothiazol but unaltered by application of gp91ds-tat peptide (Figs. 4, B and D). Exposure of mdx myocytes to BayK(+) did not significantly alter superoxide production (Fig. 4, B and D). The ratio of the increase in DHE signal response to application of BayK(−) was similar in wt and mdx myocytes (wt = 1.38 ± 0.07 and mdx = 1.42 ± 0.06; NS; Fig. 4E). These data indicate that activation of I_{Ca-L} can further increase mitochondrial superoxide production.

Mitochondrial function is altered in the intact cell but not in isolated mitochondria. We isolated mitochondria from 8-wk-old wt and mdx hearts and examined the activity of the mitochondrial respiratory complexes. In 8-wk-old mdx mice that have not yet developed overt cardiomyopathy (26), there was no difference in state 3 respiration for each of the complexes compared with isolated mitochondria from wt mice (Fig. 5). Therefore our data suggest that alterations in mitochondrial function only occur in the intact myocyte. Consistent with previous studies (17) we propose that altered mitochondrial function precedes the development of cardiomyopathy.

**DISCUSSION**

The cytoskeletal environment plays a significant role in stabilizing cell proteins. However, the cytoskeleton also influ-
ences protein function. In this study, we exploited the murine model of Duchenne muscular dystrophy (mdx) to examine the effect of the absence of dystrophin and associated cytoskeletal disarray (3, 26, 30) on regulation of mitochondrial function by \( I_{Ca-L} \).

In agreement with previous studies, we find that basal (resting) \( Ca^{2+} \) is increased in myocytes from mdx hearts (Fig. 1) (2, 16, 39). This is proposed to occur as a result of \( Ca^{2+} \) influx through stretch-activated channels such as transient receptor potential (TRP) channels. We examined the effect of alterations in \( I_{Ca-L} \) current on cytoplasmic calcium in myocytes from mdx hearts. We demonstrate that \( Ca^{2+} \) influx is increased through the channel in myocytes from mdx hearts as a result of delayed inactivation of \( I_{Ca-L} \) (Fig. 1) rather than an increase in channel expression because current density did not differ. This contributes to a further increase in cytoplasmic \( Ca^{2+} \) in the mdx myocyte. The delay in inactivation that we recorded is consistent with the delay in current inactivation that is observed with dissociation of microtubules or depolymerization of actin (10, 20, 28, 30).

As a consequence of a higher resting level of cytoplasmic \( Ca^{2+} \), resting mitochondrial \( Ca^{2+} \) (Fig. 2), NADH (Fig. 3), and mitochondrial superoxide (Fig. 4) were higher in myocytes from mdx hearts. Activation of \( I_{Ca-L} \) further increased mitochondrial \( Ca^{2+} \), NADH, and superoxide as a result of increased uptake of \( Ca^{2+} \) into the mitochondria. The ratio of the increase was similar in mdx and wt myocytes, suggesting that \( Ca^{2+} \) uptake by the mitochondria is similar in mdx hearts.

We find that respiration and mitochondrial electron transport chain complex activity in mitochondria isolated from myocytes from 8-wk-old mdx mice are similar to mitochondria isolated from wt myocytes (Fig. 5). At 8 wk, mdx myocytes have disrupted cytoskeletal architecture and are vulnerable to mechanical stress but the hearts have not yet developed overt cardiomyopathy (7, 26). Therefore, alterations in metabolic activity appear to be dependent on the cytoskeletal environment. Consistent with this argument, mitochondria isolated from desmin-null mice demonstrate similar rates of maximal respiration to mitochondria isolated from wt mice but in vivo mitochondrial respiration is abnormal (6). Similarly, in the working ex vivo mdx heart, alterations in mitochondrial citric acid cycle intermediates and decreased aconitase activity contribute to the decline in metabolic activity and function in the 8-wk-old heart (17). Therefore, our data confirm that altered mitochondrial function is present in the mdx myocyte before the development of cardiomyopathy.

It is well recognized that the mdx heart is oxidatively stressed (40) and mechanical stretch exacerbates the increase in reactive oxygen species (ROS) production from NAD(P)H-oxidase (16). Here we demonstrate that the production of mitochondrial superoxide is higher in the mdx myocyte in response to activation of \( I_{Ca-L} \) (Fig. 4). These data suggest that in addition to \( Ca^{2+} \) influx contributed by stretch-activated channels, \( Ca^{2+} \) homeostasis in the mdx myocyte is further perturbed by increased influx through \( I_{Ca-L} \).

In cardiac tissue, persistent increases in ROS are associated with pathological remodeling and myocardial dysfunction (29). Mitochondria are a major source of ROS production within cardiac myocytes (4). There is good evidence that elevated mitochondrial production of ROS is associated with the progression toward cardiac hypertrophy (21). The production of ROS is a calcium-dependent process (5). Small increases in ROS have been associated with progression toward pathological remodeling and cardiac hypertrophy due to activation of a number of calcium-dependent signaling pathways including nuclear factor of activated T cells, serine-threonine and tyrosine kinases, CaMK, and MAPK (9). It is proposed that ROS and calcium act as signaling molecules that participate as partners in the initiation of pathological remodeling and progression toward cardiac hypertrophy.

Duchenne muscular dystrophy pathology involves disruption of the cytoskeleton, as well as poor oxygen consumption and energy supply by the mitochondria. However, the mechanisms by which cytoskeletal disruption leads to abnormal mitochondrial function and compromised cardiac function are unknown. Here we find that isolated mitochondria from the mdx heart exhibit normal mitochondrial function; however, intact myocytes from the mdx heart exhibit increased cytosolic and mitochondrial \( Ca^{2+} \) handling and associated superoxide production after activation of \( I_{Ca-L} \). Since alterations in mitochondrial function are observed in the intact myocyte but not in isolated mitochondria, we conclude that disruption of the cytoskeleton results in altered communication between \( I_{Ca-L} \) and mitochondria, contributing to alteration in mitochondrial calcium handling in the mdx cardiac myocyte. This may further contribute to the pathophysiology associated with the development of cardiomyopathy in the mdx heart.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.M.V. and S.M.D. performed experiments; H.M.V., S.M.D., A.F., and L.C.H. edited and revised manuscript; H.M.V. and L.C.H. drafted manuscript; H.M.V., S.M.D., A.F., and L.C.H. analyzed data; H.M.V., S.M.D., A.F., and L.C.H. contributed equally to the work and writing of the paper; H.M.V., S.M.D., A.F., and L.C.H. contributed equally to the writing of the paper. The manuscript was reviewed and approved by all authors.

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