## PRE-CLINICAL RESEARCH

The Role of the L-Type Ca^{2+} Channel in Altered Metabolic Activity in a Murine Model of Hypertrophic Cardiomyopathy

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VISUAL ABSTRACT
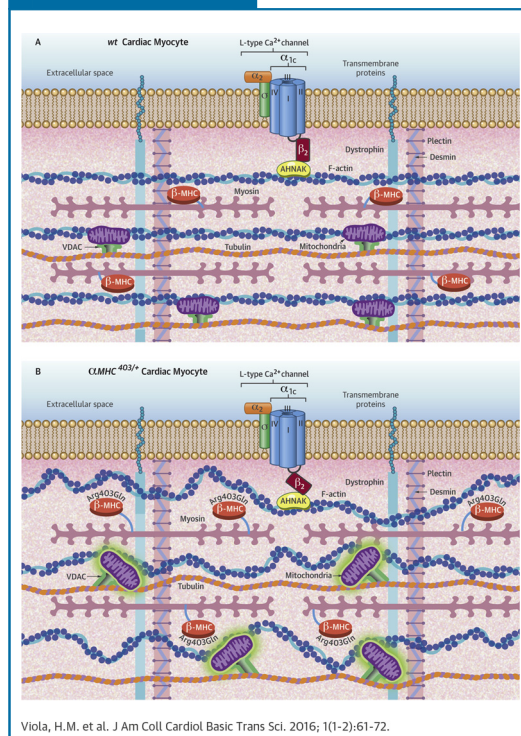
Viola, H.M. et al. J Am Coll Cardiol Basic Trans Sci. 2016; 1(1-2):61-72.

HIGHLIGHTS

- Heterozygous mice ($\alpha MHC^{403/+}$) expressing the human hypertrophic cardiomyopathy (HCM) disease causing mutation *Arg403Gln* exhibit cardinal features of HCM.

- This study investigated the role of L-type Ca2+ channel (ICa-L) in regulating mitochondrial function in *Arg403Gln* ($\alpha MHC^{403/+}$) mice.

- Activation of ICa-L in $\alpha MHC^{403/+}$ mice caused a significantly greater increase in mitochondrial membrane potential and metabolic activity when compared to wild-type mice.

- Increases in mitochondrial membrane potential and metabolic activity were attenuated with ICa-L antagonists and when F-actin or β-tubulin were depolymerized.

- ICa-L antagonists may be effective in reducing the cardiomyopathy in HCM by altering metabolic activity.

SUMMARY

Heterozygous mice ($\alpha MHC^{403/+}$) expressing the human disease-causing mutation *Arg403Gln* exhibit cardinal features of hypertrophic cardiomyopathy (HCM) including hypertrophy, myocyte disarray, and increased myocardial fibrosis. Treatment of $\alpha MHC^{403/+}$ mice with the L-type calcium channel (I_{Ca-L}) antagonist diltiazem has been shown to decrease left ventricular anterior wall thickness, cardiac myocyte hypertrophy, disarray, and fibrosis. However, the role of the I_{Ca-L} in the development of HCM is not known. In addition to maintaining cardiac excitation and contraction in myocytes, the I_{Ca-L} also regulates mitochondrial function through transmission of movement of I_{Ca-L} via cytoskeletal proteins to mitochondrial voltage-dependent anion channel. Here, the authors investigated the role of I_{Ca-L} in regulating mitochondrial function in $\alpha MHC^{403/+}$ mice. Whole-cell patch clamp studies showed that I_{Ca-L} current inactivation kinetics were significantly increased in $\alpha MHC^{403/+}$ cardiac myocytes, but that current density and channel expression were similar to wild-type cardiac myocytes. Activation of I_{Ca-L} caused a significantly greater increase in mitochondrial membrane potential and metabolic activity in $\alpha MHC^{403/+}$. These increases were attenuated with I_{Ca-L} antagonists and following F-actin or β-tubulin depolymerization. The authors observed increased levels of fibroblast growth factor-21 in $\alpha MHC^{403/+}$ mice, and altered mitochondrial DNA copy number consistent with altered mitochondrial activity and the development of cardiomyopathy. These studies suggest that the *Arg403Gln* mutation leads to altered functional communication between I_{Ca-L} and mitochondria that is associated with increased metabolic activity, which may contribute to the development of cardiomyopathy. I_{Ca-L} antagonists may be effective in reducing the cardiomyopathy in HCM by altering metabolic activity. (J Am Coll Cardiol Basic Trans Sci 2016;1:61–72) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

M yosin heavy chain (MHC) consists of a myosin carboxyl terminal rod and an amino terminal globular head that interacts with actin (1,2). During contraction, force is transduced via a hinge region between these 2 domains, allowing attachment-detachment of the myosin head with actin filaments. There are 2 cardiac-specific isoforms of MHC: α-cardiac MHC and β-cardiac MHC. In humans, β-MHC predominates in adult life, accounting for >90% of ventricular myosin (3). Genetic mutations in contractile protein β-MHC account for approximately 40% of genotyped families with hypertrophic cardiomyopathy (HCM) (4-6).

The human β-MHC mutation *MYH7 Arg403Gln* causes a severe form of HCM characterized by early-onset and progressive myocardial dysfunction with a high incidence of sudden cardiac death (7). However, the relationship between the gene mutation and phenotype is poorly understood.

Heterozygous mice expressing the human disease-causing mutation *Arg403Gln* ($\alpha MHC^{403/+}$) exhibit hallmark features of the cardiomyopathy, including hypertrophy, myocyte disarray, and increased myocardial fibrosis (8). We have previously demonstrated that treatment of $\alpha MHC^{403/+}$ mice with the L-type calcium channel (I_{Ca-L}) antagonist

diltiazem decreases left ventricular anterior wall thickness, cardiac myocyte hypertrophy, disarray, and fibrosis (9,10). In addition, administration of diltiazem to patients with HCM improves left ventricular end-diastolic diameter and left ventricular wall thickness-to-dimension ratio (10). However, the role of I_{Ca-L} in development of the cardiomyopathy is currently unknown.

The cytoskeleton consists of microtubules composed of tubulin, microfilaments composed of actin, and intermediate filaments, and is recognized as a modulator of cell morphology, motility, intracytoplasmic transport, and mitosis (11,12). Cytoskeletal proteins also regulate the function of proteins in the plasma membrane (13,14). I_{Ca-L} is anchored to F-actin and β-tubulin that regulate I_{Ca-L} activation and inactivation kinetics (15-18). In addition, the mitochondrial outer membrane contains docking sites for cytoskeletal proteins that can regulate mitochondrial function (12,19,20).

Calcium influx through the I_{Ca-L} or dihydropyridine channel is critical to cardiac excitation and contraction. I_{Ca-L} can also regulate mitochondrial function. Activation of I_{Ca-L} with voltage clamp of the plasma membrane or with application of the dihydropyridine receptor agonist Bay K8644 (BayK(-)) is sufficient to increase cytosolic and mitochondrial calcium, in addition to NADH production, superoxide generation, and metabolic activity in a calcium-dependent

manner (21,22). Activation of I_{Ca-L} can also increase mitochondrial membrane potential (Ψ_m) in a calcium-independent manner (21). The response is reversible upon inactivation of I_{Ca-L} and is in part dependent on F-actin filaments because depolymerization of F-actin prevents the response (21). The beta subunit (β_2) of I_{Ca-L} is tethered to cytoskeletal proteins. Preventing movement of the β_2 subunit with application of a peptide derived against the alpha-interacting domain of I_{Ca-L} attenuates the increase in Ψ_m (21). Therefore, I_{Ca-L} influences metabolic activity through transmission of movement of I_{Ca-L} via cytoskeletal proteins.

We and others have demonstrated that $\alpha MHC^{403/+}$ mice exhibit increased actin–myosin sliding velocity, force generation, increased ATPase activity, and ADP concentration (23,24). Here, we sought to identify whether the *Arg403Gln* mutation leads to mitochondrial dysfunction in cardiac myocytes isolated from 30- to 50-week-old $\alpha MHC^{403/+}$ mice with established cardiomyopathy (8,25). Specifically, we investigated whether the mutation resulted in altered communication between the I_{Ca-L} and mitochondria, and subsequently, altered metabolic activity.

METHODS

MOUSE MODELS. Male 30- to 50-week-old and 10- to 15-week-old mice expressing the human disease-causing mutation *Arg403Gln* ($\alpha MHC^{403/+}$) were generated (8) and studied. The mice develop cardiomyopathy by 30 to 50 weeks as evidenced by echocardiography and heart weight to body weight measurements (Supplemental Table 1). Genotype-negative littermate age-matched male mice were used as wild-type (*wt*) controls. Hearts were extracted 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* (NH&MRC, 8th Edition, 2013). Cardiac myocytes were isolated as previously described (26,27). Detailed methods are provided in the Supplemental Methods.

DATA ACQUISITION FOR PATCH CLAMP STUDIES. Whole-cell configuration of the patch clamp technique was used to measure changes in I_{Ca-L} currents in intact cardiac myocytes as described previously (28,29). Detailed methods are provided in the Supplemental Methods.

FLUORESCENT STUDIES. All studies were performed in intact mouse cardiac myocytes at 37°C as previously described. Intracellular calcium ($[Ca^{2+}]_i$) was monitored using Fura-2 (29). Superoxide generation

was assessed using dihydroethidium (29). Fluorescent indicator JC-1 was used to measure Ψ_m (29). Flavoprotein autofluorescence was used to measure flavoprotein oxidation (30). Detailed methods are provided in the Supplemental Methods.

MTT ASSAY. The rate of cleavage of the tetrazolium salt MTT to formazan by the mitochondrial electron transport chain was measured spectrophotometrically as previously described (21,26). Detailed methods are provided in the Supplemental Methods.

MITOCHONDRIAL RESPIRATION STUDIES AND DNA COPY NUMBER. Mitochondrial respiration was measured in mitochondria isolated from 3 pooled *wt* and 3 pooled $\alpha MHC^{403/+}$ mouse hearts at 37°C as previously described (31). Detailed methods are provided in the Supplemental Methods. Mitochondrial DNA copy number was determined by quantitative reverse-transcription polymerase chain reaction as previously described (32).

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION. Transcript abundance of mitochondrial transcription factor A (TFAM), peroxisome proliferator-activated receptor gamma (PPARγ) and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 was measured as previously described (33). Detailed methods are provided in the Supplemental Methods.

SAMPLE PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY AND CONFOCAL IMAGING. For transmission electron microscopy, cardiac tissue samples were imaged on a JEOL JEM-2100 electron microscope (JEOL, Akishima, Japan). For confocal imaging, cardiac myocytes tripled stained with MitoTracker (mitochondria), phalloidin (F-actin) and DAPI (nuclei) (Thermo Fisher Scientific, Massachusetts) were imaged on an Olympus IX71 inverted fluorescent microscope (Olympus, Tokyo, Japan).

Detailed methods are provided in the Supplemental Methods.

STATISTICAL ANALYSIS. Results are reported as mean ± SEM or SD where indicated. Statistical comparisons of parametric data were made using the unpaired Student *t* test (GraphPad Prism version 5.04, GraphPad Software, La Jolla, California). Statistical comparisons of non-parametric data were made using the Mann-Whitney U test, or Kruskal-Wallis test (GraphPad Prism version 5.04).

RESULTS

$\alpha MHC^{403/+}$ CARDIAC MYOCYTES EXHIBIT ALTERED I_{Ca-L} INACTIVATION KINETICS. Using the patch clamp technique, we measured I_{Ca-L} currents in

FIGURE 1   Myocytes Isolated From $\alpha MHC^{403/+}$ Hearts Exhibit Altered Inactivation Kinetics
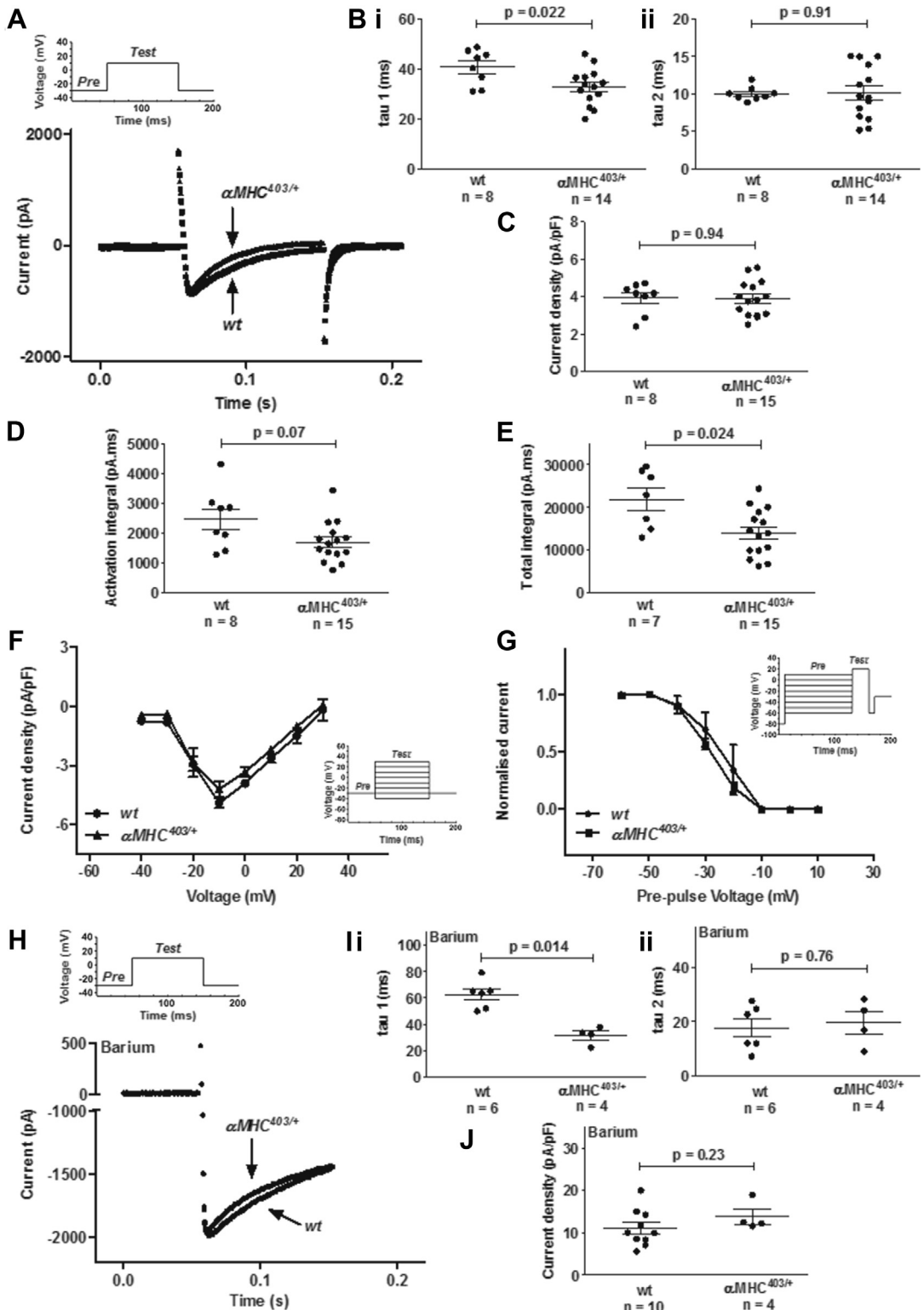
$\alpha MHC^{403/+}$ myocytes (**Figure 1A**). We found no difference in I_{Ca-L} current density recorded in $\alpha MHC^{403/+}$ versus *wt* myocytes ($\alpha MHC^{403/+}$ 3.86 ± 0.26 pA/pF vs. *wt* 3.91 ± 0.30, p = NS) (**Figures 1C and 1F**). These data suggest that I_{Ca-L} expression is not altered in $\alpha MHC^{403/+}$ myocytes. To further confirm this, we probed immunoblots of I_{Ca-L} protein isolated from $\alpha MHC^{403/+}$ hearts with an antibody directed against the pore-forming α_{1C} subunit. Densitometry analysis indicated a slight increase (8.2 ± 0.6%) in α_{1C} subunit expression in $\alpha MHC^{403/+}$ hearts (Supplemental Figures 1A and 1B), but this did not appear to be sufficient to increase peak inward current and current density (**Figures 1A, 1C, and 1F**). However, inactivation of the current was significantly faster in $\alpha MHC^{403/+}$ versus *wt* myocytes ($\alpha MHC^{403/+}$: τ1 = 32.76 ± 1.96 versus *wt*: τ1 = 40.68 ± 2.49, p < 0.05) (**Figure 1B**). Similar results were obtained when barium was used as the charge carrier indicating that changes in calcium were not mediating alterations in current inactivation (**Figures 1H to 1J**). The total integral of current in $\alpha MHC^{403/+}$ myocytes were significantly less compared with *wt* myocytes, whereas no difference in activation integral was observed (**Figures 1D and 1E**). No difference in steady-state inactivation was observed in $\alpha MHC^{403/+}$ versus *wt* myocytes (**Figure 1G**). Consistent with our results indicating no difference in I_{Ca-L} current density or peak inward current in $\alpha MHC^{403/+}$ myocytes, we found no difference in intracellular calcium ($[Ca^{2+}]_i$) in $\alpha MHC^{403/+}$ versus *wt* myocytes (Supplemental Figures 2A to 2C).

The β_2 subunit of I_{Ca-L} is bound to the α_{1C} subunit of I_{Ca-L} and plays an important role in I_{Ca-L} kinetics (34). We probed immunoblots of I_{Ca-L} protein with an antibody directed against the β_2 subunit. No significant alteration in β_2 subunit expression was observed in $\alpha MHC^{403/+}$ versus *wt* hearts (Supplemental Figures 1C and 1D). Because the β_2 subunit of I_{Ca-L} is tethered to F-actin filaments that also tightly regulate the function of I_{Ca-L} (15-17), these data suggest that cytoskeletal architecture rather than altered α_{1C} sub-

unit or β_2 subunit expression may be responsible for altered inactivation of I_{Ca-L} current in $\alpha MHC^{403/+}$ myocytes.

$\alpha MHC^{403/+}$ CARDIAC MYOCYTES EXHIBIT A SIGNIFICANTLY LARGER INCREASE IN Ψ_m FOLLOWING ACTIVATION OF I_{Ca-L}.
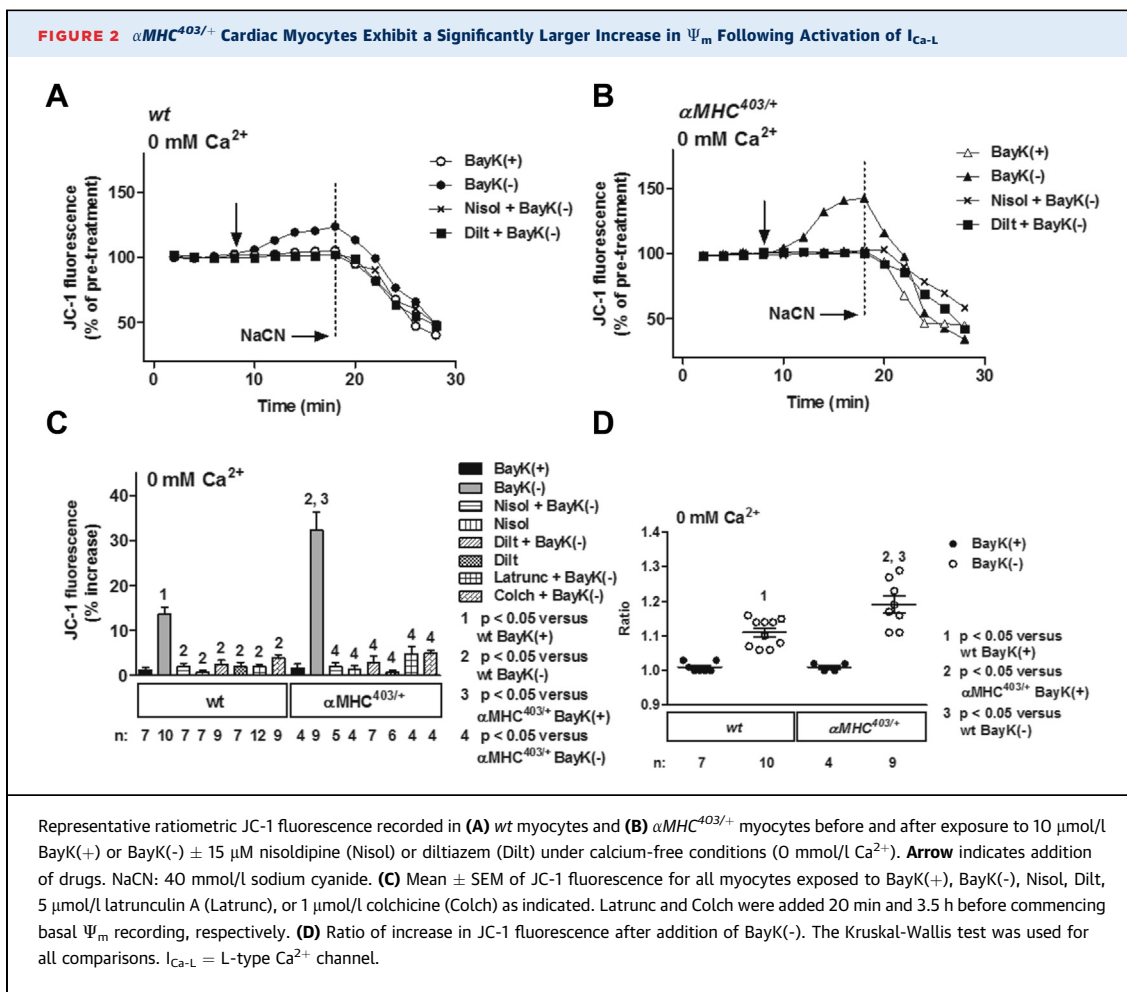
Increased mitochondrial Ca^{2+} uptake is associated with an increase in Ψ_m. However, Ψ_m can function independently of changes in $[Ca^{2+}]_i$ in the range of 0 to 400 nmol/l (35). We have previously shown that adult guinea pig cardiac myocytes exhibit increased Ψ_m following activation of I_{Ca-L} under calcium-free conditions (21). The response is dependent upon an intact cytoskeletal architecture (21).

Here, we find that application of BayK(-) elicits a significant increase in Ψ_m in $\alpha MHC^{403/+}$ and *wt* myocytes pre-incubated in calcium-free and EGTA containing HEPES-Buffered Solution for at least 3 hours (assessed as changes in JC-1 fluorescence) (**Figures 2A to 2C**). The responses were similar to those recorded in 2.5 mmol/l calcium containing Hepes-Buffered Solution (Supplemental Figure 3A). However the ratio of the response was significantly larger in $\alpha MHC^{403/+}$ versus *wt* myocytes (**Figure 2D**). The responses could be prevented with application of I_{Ca-L} antagonists nisoldipine or diltiazem (**Figures 2A to 2C**). Application of BayK(+) did not significantly alter Ψ_m in $\alpha MHC^{403/+}$ or *wt* myocytes (**Figures 2A to 2C**). Sodium cyanide was added to collapse Ψ_m demonstrating that the signal was mitochondrial and indicative of Ψ_m (**Figures 2A and 2B**). No difference was observed in basal Ψ_m in $\alpha MHC^{403/+}$ versus *wt* myocytes (Supplemental Figures 3B and 3C). These data demonstrate that activation of I_{Ca-L} causes a significantly greater increase in Ψ_m in myocytes isolated from $\alpha MHC^{403/+}$ hearts compared with *wt* myocytes, and the response does not require calcium.

The β_2 subunit of I_{Ca-L} is tethered to F-actin via subsarcolemmal stabilizing protein AHNAK (15). Mitochondria also associate with F-actin via mitochondrial docking proteins (36-38). We have previ-

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FIGURE 1 Continued

(A) Representative I_{Ca-L} current traces from $\alpha MHC^{403/+}$ (130 pF) and wild-type (*wt*) (120 pF) myocytes. **(Inset)** Pulse protocol. **(B)** Mean ± SEM rate of inactivation (tau) of current for $\alpha MHC^{403/+}$ and *wt* myocytes fitted with 2 exponential functions (*i*, τ 1; and *ii*, τ 2). Mean ± SEM of **(C)** current density, and **(D)** activation integral and **(E)** total integral of current for $\alpha MHC^{403/+}$ and *wt* myocytes. **(F)** Current/voltage (I–V) relationship and **(G)** voltage dependency of steady-state inactivation measured in $\alpha MHC^{403/+}$ and *wt* myocytes. **(Insets)** Pulse protocols. **(H)** Representative I_{Ca-L} current traces recorded from $\alpha MHC^{403/+}$ (100 pF) and *wt* (100 pF) with barium as charge carrier. **(Inset)** Pulse protocol. **(I)** Mean ± SEM of inactivation (tau) of current for $\alpha MHC^{403/+}$ and *wt* myocytes fitted with 2 exponential functions with barium as charge carrier (*i*, τ 1; and *ii*, τ 2). **(J)** Mean ± SEM of current density for all myocytes with barium as charge carrier. The unpaired Student *t* test was used for comparisons in **B and C**; Mann-Whitney test was used for comparisons in **D to G and I to J**.
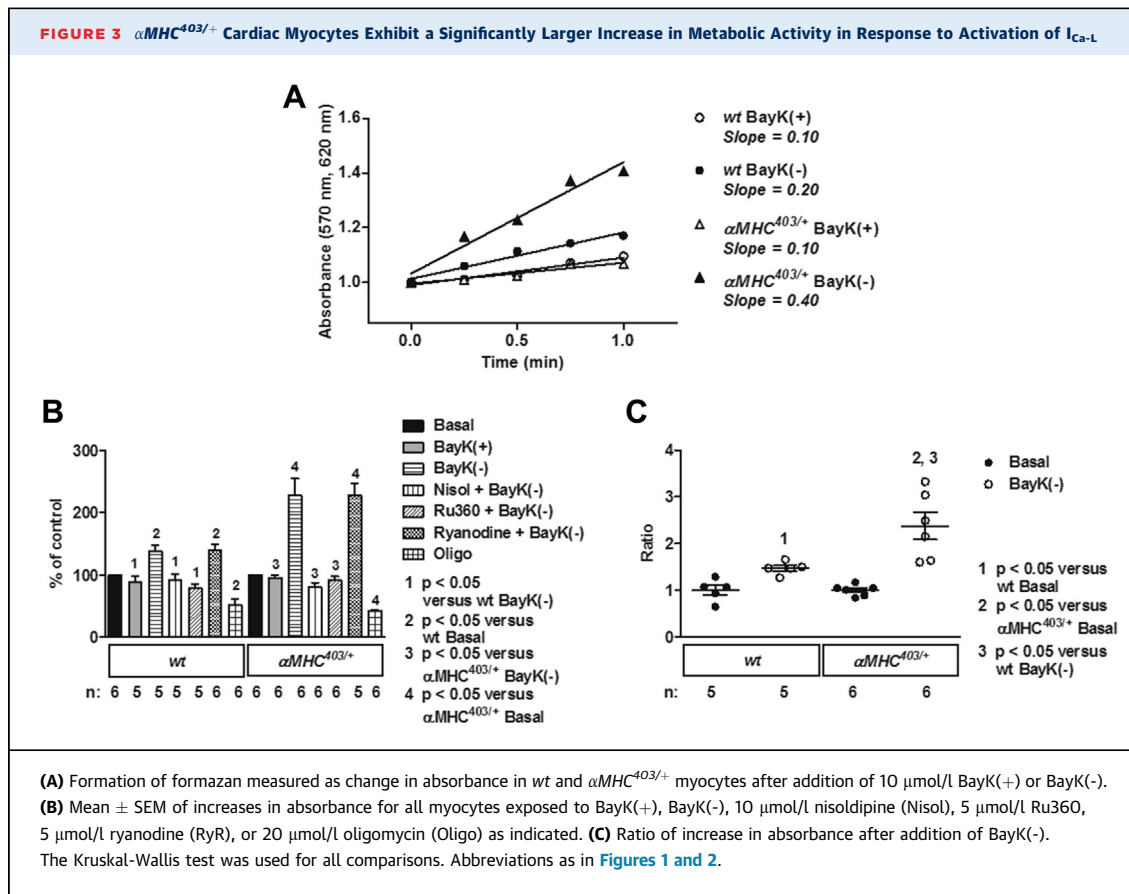
FIGURE 2 $\alpha MHC^{403/+}$ Cardiac Myocytes Exhibit a Significantly Larger Increase in Ψ_m Following Activation of $I_{Ca\text{-}L}$

Representative ratiometric JC-1 fluorescence recorded in **(A)** *wt* myocytes and **(B)** $\alpha MHC^{403/+}$ myocytes before and after exposure to 10 µmol/l BayK(+) or BayK(-) ± 15 µM nisoldipine (Nisol) or diltiazem (Dilt) under calcium-free conditions (0 mmol/l Ca^{2+}). **Arrow** indicates addition of drugs. NaCN: 40 mmol/l sodium cyanide. **(C)** Mean ± SEM of JC-1 fluorescence for all myocytes exposed to BayK(+), BayK(-), Nisol, Dilt, 5 µmol/l latrunculin A (Latrunc), or 1 µmol/l colchicine (Colch) as indicated. Latrunc and Colch were added 20 min and 3.5 h before commencing basal Ψ_m recording, respectively. **(D)** Ratio of increase in JC-1 fluorescence after addition of BayK(-). The Kruskal-Wallis test was used for all comparisons. $I_{Ca\text{-}L}$ = L-type Ca^{2+} channel.

ously demonstrated that $I_{Ca\text{-}L}$ regulates mitochondrial function due to an association between $I_{Ca\text{-}L}$ and the mitochondria via cytoskeletal protein F-actin (21,26). We exposed $\alpha MHC^{403/+}$ myocytes to F-actin depolymerizing agent latrunculin A. Under calcium-free conditions, the increase in Ψ_m in response to BayK(-) was attenuated in $\alpha MHC^{403/+}$ and *wt* myocytes (**Figure 2C**). These data indicate that elevated Ψ_m in response to activation of $I_{Ca\text{-}L}$ is dependent on cytoskeletal protein F-actin.

Regulation of Ψ_m is in part dependent on the mitochondrial voltage-dependent anion channel (VDAC) (39,40). We have previously demonstrated that directly blocking VDAC (and anion transport from the outer mitochondrial membrane) mimics the effect of BayK(-) on Ψ_m in *wt* mouse myocytes (26,41). In addition, it is known that the cytoskeletal protein β-tubulin associates with and regulates the function of VDAC (42). Therefore, we examined whether BayK(-)-induced alterations in Ψ_m were dependent

upon β-tubulin in $\alpha MHC^{403/+}$ myocytes by incubating myocytes in the β-tubulin depolymerizing agent colchicine. Under calcium-free conditions, the increase in Ψ_m in response to BayK(-) was attenuated in $\alpha MHC^{403/+}$ and *wt* myocytes (**Figure 2C**). These data indicate that the increase in Ψ_m in response to activation of $I_{Ca\text{-}L}$ is dependent on cytoskeletal protein β-tubulin.

$\alpha MHC^{403/+}$ CARDIAC MYOCYTES EXHIBIT A SIGNIFICANTLY LARGER INCREASE IN METABOLIC ACTIVITY IN RESPONSE TO ACTIVATION OF $I_{Ca\text{-}L}$. Metabolic activity is dependent upon oxygen consumption and electron flow down the inner mitochondrial membrane. Application of BayK(-) elicited a significant increase in metabolic activity in both $\alpha MHC^{403/+}$ and *wt* myocytes (**Figures 3A and 3B**). However, the ratio of the response was significantly larger in $\alpha MHC^{403/+}$ versus *wt* myocytes (**Figure 3C**). Both responses could be prevented with application of nisoldipine or the mitochondrial Ca^{2+} uniporter inhibitor Ru360, but
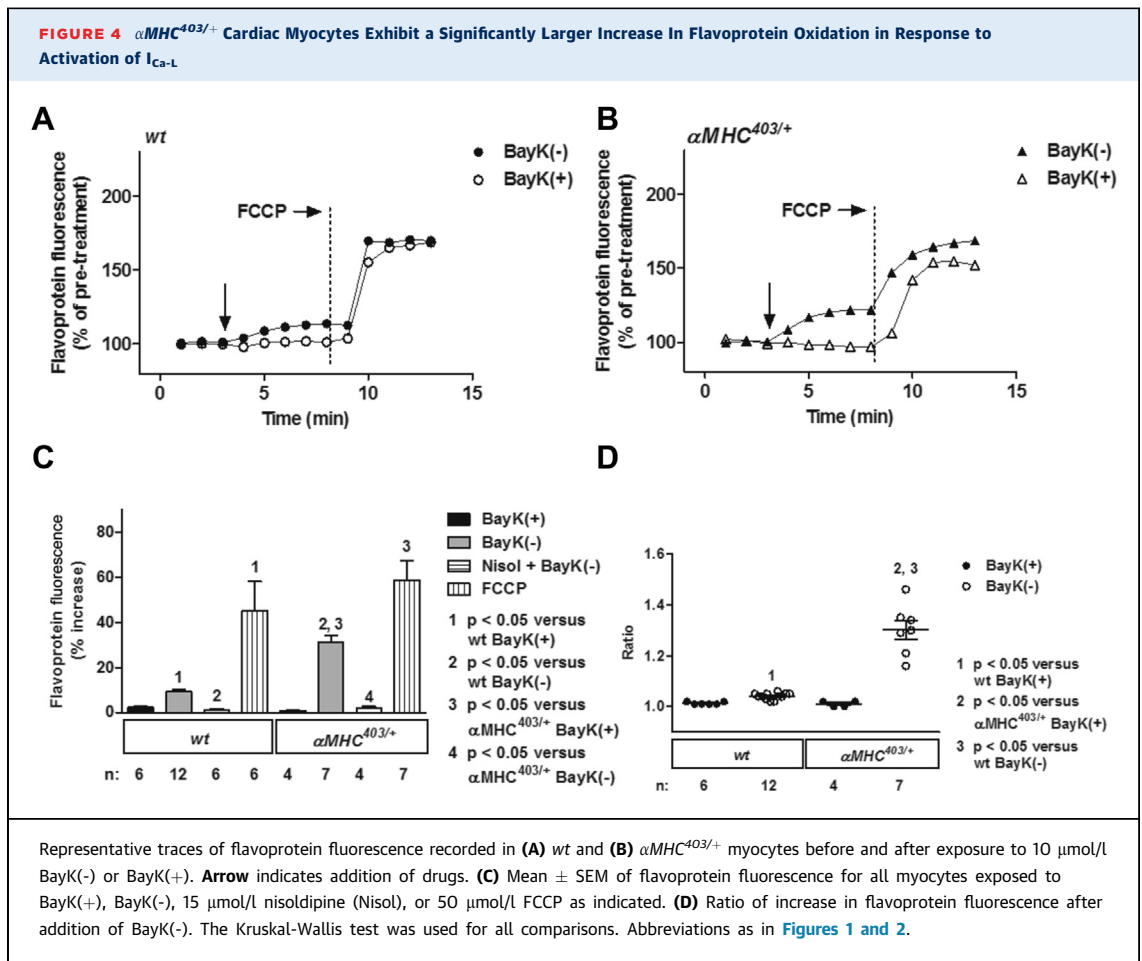
FIGURE 3 $\alpha MHC^{403/+}$ **Cardiac Myocytes Exhibit a Significantly Larger Increase in Metabolic Activity in Response to Activation of** I_{Ca-L}

(A) Formation of formazan measured as change in absorbance in *wt* and $\alpha MHC^{403/+}$ myocytes after addition of 10 μmol/l BayK(+) or BayK(-).
(B) Mean ± SEM of increases in absorbance for all myocytes exposed to BayK(+), BayK(-), 10 μmol/l nisoldipine (Nisol), 5 μmol/l Ru360,
5 μmol/l ryanodine (RyR), or 20 μmol/l oligomycin (Oligo) as indicated. **(C)** Ratio of increase in absorbance after addition of BayK(-).
The Kruskal-Wallis test was used for all comparisons. Abbreviations as in **Figures 1 and 2**.

not ryanodine (**Figure 3B**). Application of BayK(+) did not significantly alter metabolic activity in $\alpha MHC^{403/+}$ or *wt* myocytes (**Figures 3A and 3B**). Application of ATP synthase blocker oligomycin significantly decreased metabolic activity in $\alpha MHC^{403/+}$ and *wt* myocytes confirming the cells were metabolically active (**Figure 3B**).

We examined changes in mitochondrial electron transport by measuring alterations in flavoprotein oxidation in myocytes isolated from $\alpha MHC^{403/+}$ hearts in response to activation of I_{Ca-L}. Application of BayK(-) caused a significant increase in flavoprotein oxidation in $\alpha MHC^{403/+}$ and *wt* myocytes (**Figures 4A to 4C**). The ratio of the increase in flavoprotein oxidation was significantly larger in $\alpha MHC^{403/+}$ versus *wt* myocytes (**Figure 4D**). Both responses could be prevented with application of nisoldipine (**Figure 4C**). Application of BayK(+) did not significantly alter flavoprotein oxidation in $\alpha MHC^{403/+}$ or *wt* myocytes (**Figures 4A to 4C**). FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone) was added at the end of each experiment to increase flavoprotein signal confirming the signal was mitochondrial in origin (**Figures 4A to 4C**). These

data indicate that activation of I_{Ca-L} causes a significantly greater increase in metabolic activity in myocytes isolated from $\alpha MHC^{403/+}$ compared to *wt* hearts.

RESPIRATORY COMPLEX ACTIVITY IS SIMILAR IN MITOCHONDRIA ISOLATED FROM HEARTS OF $\alpha MHC^{403/+}$ **AND** *wt* **HEARTS.** We performed respiratory electron transport chain complex activity and oxygen consumption measurements on mitochondria isolated from $\alpha MHC^{403/+}$ and *wt* hearts. No differences were observed in mitochondria isolated from $\alpha MHC^{403/+}$ versus *wt* hearts (**Figure 5A**). These data suggest that alterations in mitochondrial function observed in $\alpha MHC^{403/+}$ myocytes were cell intrinsic, and not secondary to hypertrophic remodeling of the ventricle.

MITOCHONDRIAL DNA COPY NUMBER AND GENE EXPRESSION ARE ALTERED IN $\alpha MHC^{403/+}$ **HEARTS.** We found that mitochondrial DNA copy number was increased in $\alpha MHC^{403/+}$ versus *wt* hearts (**Figure 5B**). This correlated with increased expression of nuclear encoded regulators of the mitochondrial genome TFAM, PPARγ, and PGC-1 (**Figure 5C**). Because fibroblast growth factor 21 (FGF21) is a marker for

FIGURE 4 $\alpha MHC^{403/+}$ Cardiac Myocytes Exhibit a Significantly Larger Increase In Flavoprotein Oxidation in Response to Activation of I_{Ca-L}

Representative traces of flavoprotein fluorescence recorded in **(A)** *wt* and **(B)** $\alpha MHC^{403/+}$ myocytes before and after exposure to 10 μmol/l BayK(-) or BayK(+). **Arrow** indicates addition of drugs. **(C)** Mean ± SEM of flavoprotein fluorescence for all myocytes exposed to BayK(+), BayK(-), 15 μmol/l nisoldipine (Nisol), or 50 μmol/l FCCP as indicated. **(D)** Ratio of increase in flavoprotein fluorescence after addition of BayK(-). The Kruskal-Wallis test was used for all comparisons. Abbreviations as in **Figures 1 and 2**.

mitochondrial dysfunction in myocytes (43,44), we measured FGF21 in circulating blood from the mice. We measured a significant increase in FGF21 levels in $\alpha MHC^{403/+}$ versus *wt* mice, correlating with the onset of cardiomyopathy (**Figure 5D**). Transmission electron microscopy imaging revealed disordered mitochondrial distribution in $\alpha MHC^{403/+}$ versus *wt* hearts (**Figures 5E and 5F**). Additionally, confocal imaging revealed disordered mitochondrial distribution and disorganization of F-actin in $\alpha MHC^{403/+}$ versus *wt* myocytes (**Figures 5G and 5H**).

PRE-CARDIOMYOPATHIC $\alpha MHC^{403/+}$ CARDIAC MYOCYTES EXHIBIT ALTERED I_{Ca-L} INACTIVATION KINETICS AND LARGER INCREASES IN Ψ_m AND METABOLIC ACTIVITY FOLLOWING ACTIVATION OF I_{Ca-L}. We assessed alterations in I_{Ca-L} inactivation kinetics and mitochondrial responses in cardiac myocytes from 10- to 15-week-old $\alpha MHC^{403/+}$ hearts that had not yet developed cardiomyopathy (Supplemental Table 1) (8,25). Using the patch clamp technique, we

measured I_{Ca-L} currents in the myocytes (Supplemental Figure 4A). Similar to 30- to 50-week-old myocytes, we found no difference in I_{Ca-L} current density in 10- to 15-week-old $MHC^{403/+}$ versus age-matched *wt* myocytes (Supplemental Figure 4C). However, inactivation of the current was significantly faster in $\alpha MHC^{403/+}$ myocytes (Supplemental Figure 4B). Additionally, application of BayK(-) elicited a significantly larger increase in Ψ_m and flavoprotein oxidation in 10- to 15-week-old $MHC^{403/+}$ versus age-matched *wt* myocytes (Supplemental Figure 5). The responses could be prevented with I_{Ca-L} antagonist nisoldipine. These data suggest that altered communication between I_{Ca-L} and the mitochondria *precedes* the development of $\alpha MHC^{403/+}$ cardiomyopathy.

DISCUSSION

The L-type Ca^{2+} channel plays an important role in cardiac excitation and contraction. It can also

FIGURE 5 Respiration Is Normal, but Gene Expression Is Altered in Mitochondria Isolated From $\alpha MHC^{403/+}$ Hearts
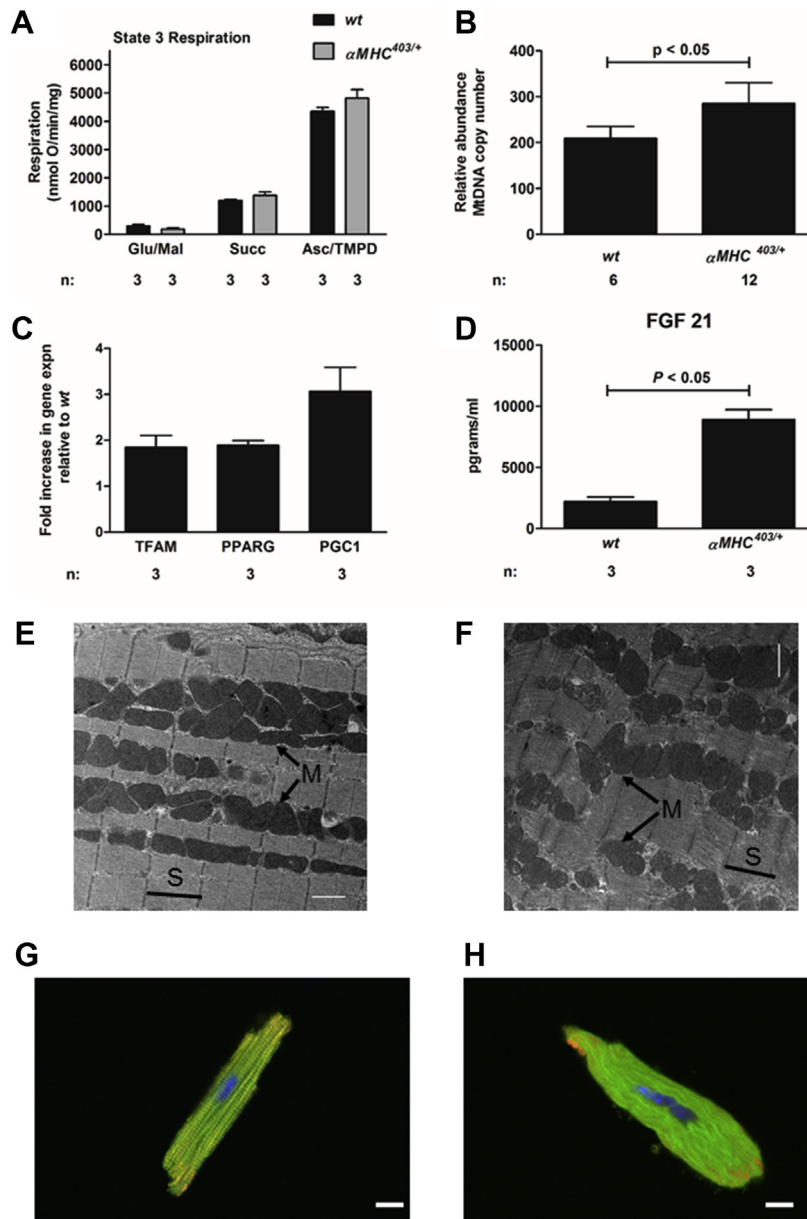
(A) Respiration and mitochondrial electron transport chain complex activity in mitochondria isolated from 3 pooled *wt* and 3 pooled $\alpha MHC^{403/+}$ hearts. (B) Mean ± SD of MtDNA copy number normalized to 18S rDNA. (C) Mean ± SD of gene expression of $\alpha MHC^{403/+}$ MtDNA copy number regulators TFAM, PPARγ and PGC1 relative to *wt*. (D) Mean ± SD of FGF21. (E and F) Representative TEM images demonstrating disordered distribution of mitochondria in $\alpha MHC^{403/+}$ (F) versus *wt* (E) heart sections. M = mitochondria; S = sarcomere. Scale = 1 μm. (G and H) Confocal imaging demonstrating disordered mitochondrial distribution and disorganization of F-actin in representative $\alpha MHC^{403/+}$ (H) versus *wt* (G) myocytes. Mitochondria shown in **red** (MitoTracker), F-actin shown in **green** (phalloidin), nuclei shown in **blue** (DAPI). Scale = 10 μm. The Mann-Whitney test was used for all comparisons. MtDNA = mitochondrial DNA; TEM = transmission electron microscopy; *wt* = wild type.

influence metabolic activity through transmission of movement of the β subunit *via* cytoskeletal proteins (21,26). We investigated whether the *Arg403Gln* mutation in contractile protein β-MHC results in impaired communication between $I_{Ca\text{-}L}$ and the mitochondria, and subsequently, altered metabolic function. We find that $I_{Ca\text{-}L}$ current inactivates more rapidly in myocytes from $\alpha MHC^{403/+}$ hearts (**Figure 1**).

This appears to occur as a result of tethering of I_{Ca-L} to cytoskeletal proteins, and is consistent with findings that dissociation of microtubules or depolymerization of actin alters I_{Ca-L} inactivation rate (16,17,45,46). Peak inward current, current density, and I_{Ca-L} expression were not significantly altered in $\alpha MHC^{403/+}$ myocytes. Consistent with this, basal and BayK(-)-stimulated increases in $[Ca^{2+}]_i$ and superoxide production were also no different from *wt* myocytes (Supplemental Figure 2). Previous studies have demonstrated that the relaxation rate of $\alpha MHC^{403/+}$ cardiac myocytes is slowed and calcium transients are smaller due to reduced expression of ryanodine receptors and calsequestrin, leading to diminished sarcoplasmic reticulum stores (9,47). Taken together, the findings demonstrate that the *Arg403Gln* mutation is associated with altered sarcoplasmic reticulum calcium cycling but does not appear to be associated with significant changes in diastolic Ca^{2+} or superoxide production in aged $\alpha MHC^{403/+}$ hearts.

One factor that influences metabolic activity and mitochondrial ATP production is electron flow down the inner mitochondrial membrane. We demonstrate that $\alpha MHC^{403/+}$ cardiac myocytes exhibit a significantly larger increase in Ψ_m, oxygen consumption and flavoprotein oxidation in response to activation of I_{Ca-L} that can be attenuated by I_{Ca-L} antagonist nisoldipine (Figures 2 to 4). The increase in Ψ_m can also be attenuated by diltiazem. These data indicate that metabolic activity in $\alpha MHC^{403/+}$ myocytes is higher versus *wt* myocytes. We demonstrate that this is dependent upon the *intact* cellular environment because respiration was normal in mitochondria isolated from $\alpha MHC^{403/+}$ hearts (Figure 5A). In support of this, desmin-null mice exhibit normal rates of maximal respiration in isolated mitochondria, but in vivo mitochondrial respiration is abnormal (20). Because alterations in mitochondrial function are observed only in the intact myocyte, we conclude that alterations to the cell's intrinsic environment (as evidenced in Figures 5G and 5H) result in altered communication between I_{Ca-L} and mitochondria, contributing to a hypermetabolic state in the $\alpha MHC^{403/+}$ cardiac myocyte.

We have demonstrated previously that I_{Ca-L} co-immunoprecipitates with many cytoskeletal proteins (21,26). We investigated how the mutation in the MHC gene leads to alterations in protein-protein interactions through the cytoskeletal network. The β_2 subunit of the L-type Ca^{2+} channel is tightly bound to the α_{1C} subunit via the alpha-interacting domain (48,49). The β_2 subunit of the channel is also tethered to F-actin via sub-sarcolemmal stabilizing protein AHNAK (15). Mitochondria also associate with actin via mitochondrial docking proteins (36-38), and with β-tubulin via VDAC (42). Here, we demonstrate that elevated Ψ_m in response to activation of I_{Ca-L} is dependent on cytoskeletal proteins F-actin and β-tubulin in the $\alpha MHC^{403/+}$ cardiac myocyte because exposure of myocytes to either F-actin depolymerizing agent latrunculin A or β-tubulin depolymerizing agent colchicine attenuates elevated Ψ_m in response to activation of I_{Ca-L} (Figure 2C). These data indicate that the *Arg403Gln* mutation is associated with altered functional communication between I_{Ca-L} and mitochondria via the cytoskeletal network, and increased cardiac metabolic activity.

To determine whether alterations in mitochondrial responses occurred before the onset of cardiomyopathy, we examined I_{Ca-L} kinetics and the effect of activation of I_{Ca-L} on Ψ_m and flavoprotein oxidation in myocytes isolated from pre-cardiomyopathic 10- to 15-week-old $\alpha MHC^{403/+}$ hearts. Similar responses were recorded to those observed in myocytes from 30- to 50-week-old $\alpha MHC^{403/+}$ hearts that had developed cardiomyopathy (Supplemental Figures 4 and 5). Because the responses were observed before the development of the cardiomyopathy, we conclude that altered communication between the I_{Ca-L} and mitochondria may contribute to the histology and pathophysiology, specifically altered energy reserve and hypercontractility, which has been identified in patients with HCM (50). The I_{Ca-L} antagonist diltiazem is effective in preventing the development of cardiomyopathy in $\alpha MHC^{403/+}$ mice and in some patients with identified *MYH7* gene mutations. Our findings indicate that targeting I_{Ca-L} may be effective in the treatment of cardiomyopathy by modulating the activity of the I_{Ca-L} and decreasing/restoring metabolic activity. We speculate that early intervention involving treatment with I_{Ca-L} antagonist diltiazem may prove beneficial in regulating metabolic activity and subsequently, preventing the development of cardiomyopathy in "at-risk" patients with identified *MYH7* gene mutations.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Mutations in contractile protein β-myosin heavy chain account for approximately 40% of genotyped families with HCM. Altered energy reserve has been identified in patients with HCM, however the relationship between the gene mutation and phenotype is poorly understood. L-type Ca^{2+} channel antagonists are used clinically to treat patients but the role of the L-type Ca^{2+} channel in the development of the cardiomyopathy is unknown. Here we find that the β-myosin heavy chain mutation *Arg403Gln* leads to altered functional communication between the L-type Ca^{2+} channel and mitochondria that is associated with increased cardiac metabolic activity. This may contribute to the development of the cardiomyopathy because the response is present *prior* to the development of cardiomyopathy.

TRANSLATIONAL OUTLOOK: Further studies are needed to determine cardiac metabolic activity in "at risk" patients with identified *MYH7* gene mutations before the development of HCM. On the basis of our findings, we speculate that utilizing L-type Ca^{2+} channel antagonists as a means of modulating cardiac metabolic activity may prove beneficial in early intervention and subsequent prevention of HCM in at-risk patients with identified *MYH7* gene mutations.

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APPENDIX For expanded Methods and Results sections, as well as a supplemental table and figures, please see the supplemental appendix.