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AMPK deficiency in cardiac muscle results in dilated cardiomyopathy in the absence of changes in energy metabolism

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Aims AMP-activated protein kinase (AMPK) is thought to be a central player in regulating myocardial metabolism and its activation has been shown to inhibit cardiac hypertrophy. Recently, mice with muscle-specific deletion of AMPK β1/β2 subunits (AMPKβ1β2-deficient mice, β1β2M-KO) have been generated and possess <10% of normal AMPK activity in muscle. However, how/if dramatic AMPK deficiency alters cardiac metabolism, function, or morphology has not been investigated. Therefore, the aim of this study was to determine whether a significant loss of AMPK activity alters cardiac function, metabolism, and hypertrophy, and whether this may play a role in the pathogenesis of heart failure.

Methods and results β1β2M-KO mice exhibit an approximate 25% reduction in systolic and diastolic function compared with wild-type (WT) littermates. Despite the well-documented role of AMPK in controlling myocardial energy metabolism, there was no difference in basal glucose and fatty acid oxidation rates between β1β2M-KO and WT mice. However, there was reduced AMPK-mediated phosphorylation of troponin I in β1β2M-KO and reduced ventricular cell shortening in the presence of low Ca^{2+}, which may explain the impaired cardiac function in these mice. Interestingly, β1β2M-KO mice did not display any signs of compensatory cardiac hypertrophy, which could be attributed to impaired activation of p38 MAPK.

Conclusions β1β2M-KO mice display evidence of dilated cardiomyopathy. This is the first mouse model of AMPK deficiency that demonstrates cardiac dysfunction in the absence of pathological stress and provides insights into the role of AMPK in regulating myocardial function, metabolism, hypertrophy, and the progression to heart failure.

Keywords AMPK • Remodelling • Hypertrophy • Heart failure

1. Introduction

AMP-activated protein kinase (AMPK) controls multiple catabolic and anabolic pathways that help maintain adequate cellular ATP levels in times of energetic and/or cellular stress.[1,2] In the heart, AMPK has been most extensively characterized as a modulator of energy metabolism where it regulates fatty acid and glucose transport/utilization during both physiological and pathophysiological conditions.[2] It is in this context of maintaining cellular ATP that AMPK also acts to decrease energy-consuming pathways such as protein synthesis. Several molecular pathways controlling cell growth are regulated by AMPK,[3–5] and it has been shown that impaired cardiac AMPK activity both pathologically[6,7] and in cardiac LKB1 null mice[8] is associated with promoting hypertrophy. While these findings suggest that the loss of AMPK activity is pro-hypertrophic, a variety of genetic mouse models of AMPK deficiency do not spontaneously develop cardiac hypertrophy,[9–11] thus challenging this concept. In addition, recent work in AMPKγ2 mutant mice has shown that AMPK activation is associated with cardiac

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hypertrophy.[12] Taken together, these findings suggest that the role AMPK plays in the regulation of cardiac hypertrophy and the progression to heart failure has yet to be definitively described.

To assist in our understanding of the role of AMPK in the regulation of cardiac hypertrophy and progression to heart failure, we studied muscle-specific AMPKβ1β2-deficient mice (β1β2M-KO) and their wild-type (WT) littermates. β1β2M-KO mice were generated using the muscle creatine kinase (MCK)-Cre promoter that is expressed in skeletal muscle as well as in the heart.[13,14] We speculated that, in addition to the skeletal muscle dysfunction observed in the β1β2M-KO mouse,[13,14] these mice may also have impaired cardiac metabolism/function. In agreement with this, we found that hearts from muscle-specific β1β2M-KO mice exhibit cardiac dysfunction and impaired cell shortening. However, these mice lack the ability to undergo adaptive left ventricular (LV) remodelling and do not display altered cardiac energy metabolism. This lack of compensatory cardiac remodelling despite the loss of AMPK, which is thought to act as a negative regulator of hypertrophy, suggests a new role of AMPK and/or AMPKβ1/β2 isoforms in the control of cardiomyocyte growth and supports the idea that pharmacological activation of AMPK may be of benefit in the setting of heart failure.[15]

2. Methods

2.1 Generation of β1β2M-KO mice

All protocols involving mice were approved by McMaster University or the University of Alberta Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (eighth edition; revised 2011). They adhere to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and comply with the Canadian Council on Animal Care guidelines. Mice with a skeletal muscle- and heart-specific deletion of AMPK β1/β2, here referred to as β1β2M-KO or KO mice, were generated as previously described.[13] In brief, we generated AMPK β1- and β2-floxed mice on a C57Bl6 background and crossed these mice with C57Bl6 mice expressing Cre-recombinase under the control of the MCK promoter to drive transcription in skeletal and heart muscle. After two generations, we obtained homozygous AMPK β1 fl/fl (β1M-WT) and AMPK β1 MCK-Cre (β1M-KO) and homozygous AMPK β2 fl/fl (β2M-WT) and AMPK β2 MCK-Cre (β2M-KO) mice (*Figure 1A*). These mice were then crossed for two generations to obtain homozygous AMPK β1β2 fl/fl (β1β2M-WT) and AMPK β1β2 MCK-Cre (β1β2M-KO) mice (expected one of four progeny for males). WT and β1β2M-KO mice were group-housed on a 12 h light : 12 h dark cycle with *ad libitum* access to chow diet (#5001 from Lab Diet, St Louis, MO, USA with 13.5% kcal from fat) and water. For all experiments, littermate WT mice were used as controls. Male and female WT ($n = 31$) and β1β2M-KO ($n = 24$) mice were studied at 16–20 weeks of age. Unless otherwise stated, conscious mice were euthanized by decapitation in order to exclude effects of chemical agents on tissue glucose and fatty acid handling/metabolism.

2.2 *In vivo* echocardiography

Mice were mildly anaesthetized using isoflurane (3% induction and 1–1.5% maintenance), and transthoracic echocardiography was performed using a Vevo 770 high-resolution imaging system equipped with a 30 MHz transducer (RMV-707B; VisualSonics) as described previously.[16,17]

2.3 *Ex vivo* heart perfusions

Mice were euthanized in the random fed state, and hearts were dissected and subsequently perfused. Hearts were perfused in the working heart mode at 11.5 mmHg preload and 50 mmHg afterload with Krebs–Henseleit buffer containing 0.8 mmol/L of palmitate prebound to 3% delipidated BSA, 5 mmol/L of glucose, and 50 μU/mL of insulin. Hearts were aerobically perfused for 30 min and at the end of aerobic perfusion; hearts were immediately frozen in liquid N_2 with a Wollenberger clamp and stored at −80°C until analysis as described previously.[16] For metabolic measurements, palmitate and glucose were labelled using a combination of [9,10-^{3}H] palmitate and [U-^{14}C] glucose (for determination of fatty acid and glucose oxidation, respectively).[16,18]

2.4 Gene expression

Gene expression analysis was performed on ventricular tissues collected from WT and β1β2M-KO mice by quantitative RT-PCR as previously described.[17,19]

2.5 Immunoblot analysis

Heart and skeletal muscle lysates were analysed by SDS–PAGE and immunoblotting was performed as described previously.[17]

2.6 Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using the GraphPad Prism software. Pairwise comparisons between two groups were made by unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

An expanded Methods section is available in Supplementary material online, Methods.

3. Results

3.1 Muscle-specific reduction of AMPK β1/β2 expression leads to a significant reduction in phosphorylation of AMPK and acetyl CoA carboxylase

Homozygous AMPK β1β2 fl/fl (β1β2M-WT or WT) and AMPK β1β2 MCK-Cre (β1β2M-KO or KO) mice were generated as previously described (*Figure 1A*).[13] Hearts and/or isolated ventricular myocytes were studied from WT and β1β2M-KO mice at 16–20 weeks of age. As expected, expression levels of AMPK β2 were nearly undetectable in ventricular myocytes isolated from hearts of β1β2M-KO mice (*Figure 1B*). However, AMPK β1 was only reduced by 50% in ventricular myocytes (*Figure 1B*), which may be the result of the MCK promoter being less efficient at expressing Cre in the cardiac muscle compared with skeletal muscle.[20] That said, it is yet unknown why there are differences between the expression levels of the two β isoforms. In addition, protein levels of the β1 and β2 subunits of AMPK were unchanged in non-cardiac muscle cells including cardiac fibroblasts (see Supplementary material online, *Figure S1*), demonstrating muscle-specific effects. As the β-subunit acts as a scaffold for the α and γ subunits,[21] the loss of β subunits in the cardiomyocytes resulted in greatly reduced total protein levels of the AMPK α catalytic subunit and nearly undetectable levels of AMPK threonine (Thr)172 phosphorylation (which is indicative of covalent activation) in hearts from β1β2M-KO mice compared with WT controls (*Figure 1C*), which is consistent with our previous findings.[13] The phosphorylation of acetyl CoA carboxylase (ACC) is an important downstream target of AMPK signaling[22] and is considered to be the best marker of cellular AMPK activity as it takes into account both the allosteric and covalent regulation of AMPK activity.[1] Consistent with large reductions in AMPK Thr172 phosphorylation, the phosphorylation of ACC was reduced by >95% in hearts from β1β2M-KO mice

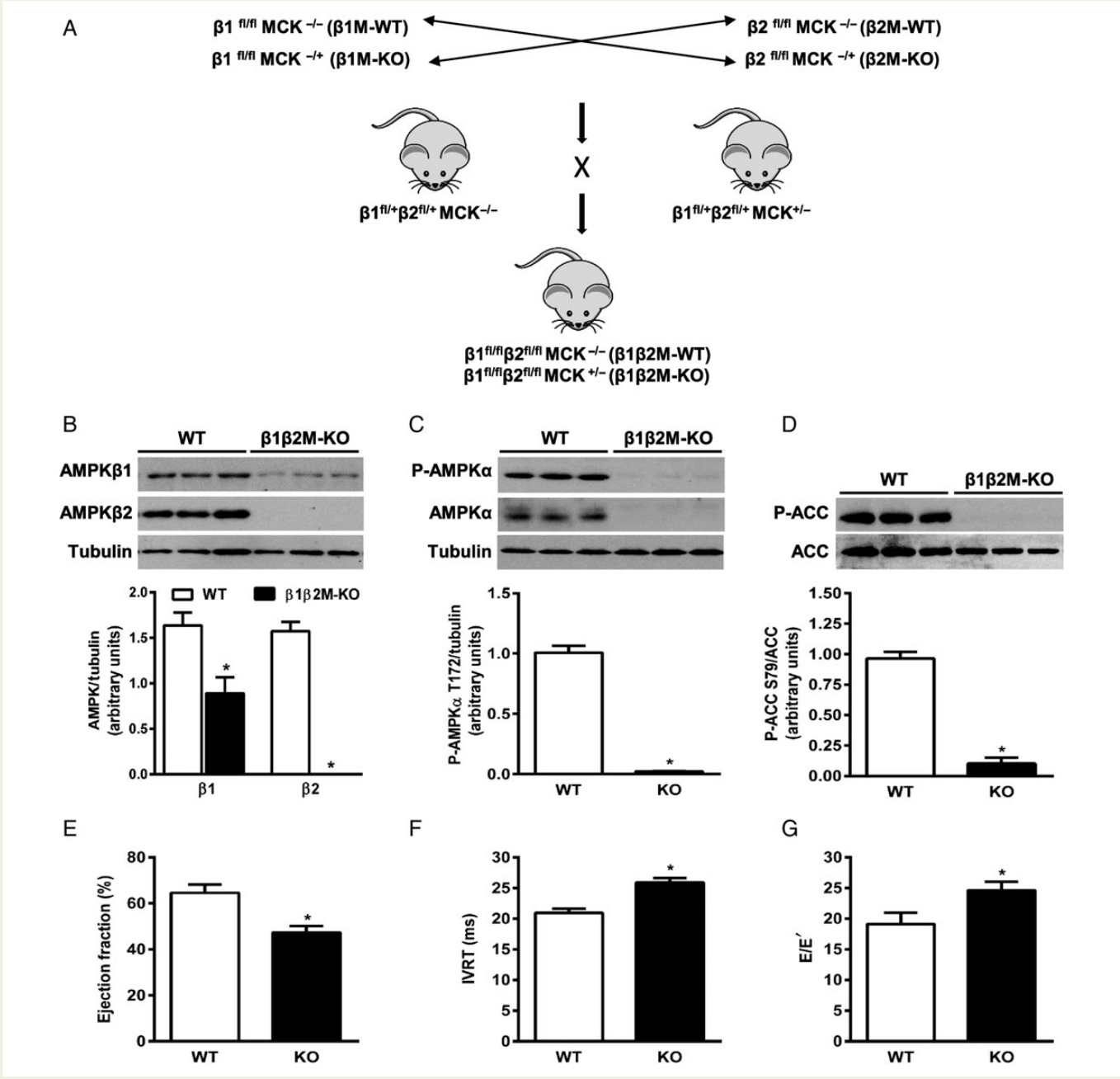

Figure 1 Muscle-specific reduction in AMPK subunit expression and AMPK phosphorylation in β1β2M-KO mice result in cardiac dysfunction. (*A*) Breeding strategy for the generation of WT and muscle-specific β1β2M-KO mice from AMPK β1- and β2-floxed (fl/fl) mice. (*B*) Protein levels of AMPK β1/β2 subunits in ventricular myocytes isolated from WT and β1β2M-KO mice. Immunoblot analysis of AMPK β1/β2 subunits was normalized to tubulin levels as a loading control ($n = 3–6$). Immunoblot analysis of the (*C*) phosphorylation status of AMPKα (Thr 172) normalized to tubulin levels ($n = 3$) and (*D*) phosphorylation status of ACC (Ser 79) normalized to total ACC levels in ventricular homogenates ($n = 3$). Representative immunoblot images shown above respective graphs. (*E*) Ejection fraction (%EF), (*F*) IVRT, and (*G*) *E/E′* by tissue Doppler were reduced in β1β2M-KO mice compared with WT mice ($n = 10–11$). *$P < 0.05$ by Student's *t*-test vs. WT.

compared with WT controls (*Figure 1D*), indicating a dramatic reduction in cardiac AMPK activity.

3.2 Muscle-specific reduction of AMPK β1/β2 expression results in cardiac dysfunction *in vivo*

To determine the effect of muscle-specific reduction of AMPKβ1/β2 expression on *in vivo* cardiac function under normal physiological conditions, we performed transthoracic echocardiography on mildly anaesthetized mice. There were reductions in percent ejection fraction (%EF) (*Figure 1E*), fractional shortening (*Table 1*), and cardiac output (*Table 1*) by 20–30% in hearts from β1β2M-KO mice compared with WT controls, demonstrating that the dramatic decrease in AMPK signalling is associated with cardiac systolic dysfunction. In addition, diastolic function was impaired in hearts from β1β2M-KO mice compared with WT mice, as shown by an increase in isovolumic relaxation time (IVRT; *Figure 1F*) and *E/E′* (*Figure 1G*), as well as increased *E/A*

Table 1 Echocardiographic assessment of *in vivo* heart function in WT and β1β2M-KO mice (*N* = 10–11)

	WT	β1β2M-KO
Systolic function		
HR (bpm)	428.8 ± 6.98	427.8 ± 9.76
FS (%)	35.50 ± 2.78	23.60 ± 1.60*
CO (mL/min)	18.07 ± 0.82	14.03 ± 0.74*
SV (μL)	42.16 ± 1.55	34.00 ± 1.21*
LVEDV (μL)	64.49 ± 4.16	74.08 ± 4.40
LVESV (μL)	24.06 ± 3.36	39.99 ± 4.34*
Diastolic function		
Tei Index	0.64 ± 0.02	0.80 ± 0.03*
Mitral *E/A*	1.49 ± 0.11	3.57 ± 0.67*
Wall dimensions		
LV mass (mg)	79.12 ± 2.98	85.56 ± 2.48
IVSd (mm)	0.75 ± 0.02	0.73 ± 0.04
LVIDd (mm)	3.85 ± 0.16	4.08 ± 0.10
LVPWd (mm)	0.73 ± 0.04	0.73 ± 0.02
IVSs (mm)	1.17 ± 0.07	1.01 ± 0.04*
LVIDs (mm)	2.51 ± 0.24	3.13 ± 0.14*
LVPWs (mm)	1.13 ± 0.07	0.94 ± 0.04*

HR, heart rate; FS, fractional shortening; CO, cardiac output; SV, stroke volume; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; IVS, interventricular septal wall thickness; LVID, left ventricular internal diameter; LVPW, left ventricular posterior wall thickness.
*$P < 0.05$ vs. WT by unpaired Student's *t*-test.

ratio (*Table 1*), which is suggestive of restrictive LV filling and impaired compliance. Furthermore, Tei index, which reflects global cardiac dysfunction, was also increased in β1β2M-KO mice compared with WT mice (*Table 1*), providing further evidence of both systolic and diastolic dysfunction in β1β2M-KO mice.

3.3 Reduction of AMPK β1/β2 expression does not result in alterations in baseline cardiac fatty acid and glucose metabolism

To examine whether cardiac dysfunction could be the result of alterations in cardiac energy metabolism, we directly assessed these parameters in hearts from WT and β1β2M-KO mice using *ex vivo* working heart perfusions. Despite a significant reduction in AMPK activation in β1β2M-KO mice (*Figure 1C*), both rates of cardiac fatty acid oxidation (*Figure 2A*) and glucose oxidation (*Figure 2B*) were similar between WT and β1β2M-KO mice. This lack of effect on major contributors to cardiac ATP supply did not alter levels of total acetyl CoA production from exogenously supplied substrates in these hearts (*Figure 2C*). Taken together, these data suggest that alterations in basal energy metabolism do not contribute to cardiac dysfunction observed in β1β2M-KO mice.

Interestingly, expression of peroxisome proliferator activator (PPAR)α was significantly increased in hearts from β1β2M-KO mice compared with WT mice (*Figure 2D*). However, levels of malonyl CoA decarboxylase (MCD; *Figure 2E*; $P = 0.16$, $n = 5–7$) and FOXO-1 (*Figure 2F*) were not changed. These data suggest that long-term ablation of AMPK can result in PPARα activation, which may help explain how cardiac energy metabolism remains unaltered despite reduction/ablation of AMPK β1/β2 subunits. Furthermore, the lack of change in cardiac energy metabolism was not associated with alterations in mitochondrial number or function, as evidenced by similar levels of oxidative phosphorylation (OXPHOS) complexes (*Figure 2G*) and citrate synthase activity (*Figure 2H*) in hearts from WT and β1β2M-KO mice. As AMPK is known to regulate glycogen content, we found that myocardial glycogen levels were modestly decreased in β1β2M-KO mice (*Figure 2I*).

3.4 AMPK deficiency in β1β2M-KO mice does not promote ventricular hypertrophy

Since AMPK has been shown to play a role in the control of cardiac hypertrophy,[3,4,6,7] we studied overall cardiac structure and morphology in β1β2M-KO mice (*Figure 3A*). Despite the fact that hearts from β1β2M-KO mice displayed significantly impaired function compared with WT mice, neither LV posterior wall thickness (LVPW; *Figure 3B*), interventricular wall thickness (IVS; *Table 1*), nor LV internal diameter (LVID; *Figure 3C*) was different between groups during diastole. However, during systole, LV chamber diameter and volume were modestly increased and LVPW and IVS were reduced in β1β2M-KO mice (*Table 1*). Furthermore, left atrial diameter as measured by echocardiography was significantly increased in hearts from β1β2M-KO mice compared with WT mice (*Figure 3A* and *D*), suggesting restrictive LV filling. Consistent with no apparent wall thickening in hearts from β1β2M-KO mice, ventricular myocyte size was similar between WT and β1β2M-KO mice (*Figure 3E*). In addition, ventricular tissue levels of collagen type 1 were unchanged between groups (*Figure 3F*), showing that hearts from β1β2M-KO mice do not develop cardiac fibrosis. In addition, transcript levels of atrial natriuretic factor (*anf*) are significantly increased and there is a trend towards increased B-type natriuretic peptide (*bnp*) in β1β2M-KO mice (*Figure 3G*), further supporting the presence of cardiac dysfunction. Interestingly, although gene expression of the sarcoplasmic reticulum Ca^{2+} re-uptake protein SERCA2 was modestly reduced (*Figure 3G*), this did not translate into reductions in protein levels in hearts from β1β2M-KO mice (*Figure 3H*). Taken together, these data demonstrate that β1β2M-KO mice do not develop ventricular hypertrophy however; cardiac dysfunction in these mice is associated with LV dilatation and wall thinning at systole.

3.5 Cardiac dysfunction in β1β2M-KO mice is associated with impaired regulation of pathways that contribute to cardiomyocyte cell shortening

Recently, it has been proposed that AMPK modulates cardiac contractile function by phosphorylating troponin I (Ser 151) leading to increased Ca^{2+} sensitivity and cardiomyocyte force development.[23–25] Consistent with this, we show that the loss of AMPK activity results in an ~50% decrease in phosphorylated troponin I (Ser 151; *Figure 4A*). There was no significant difference in % change in cell shortening at an extracellular Ca^{2+} concentration of 2 mM (*Figure 4B* and *C*). However, by reducing the transmembrane gradient for Ca^{2+} ion influx to limit Ca^{2+} availability for contraction, any differences in Ca^{2+} sensitivity may be unmasked by changes in cell shortening. Consistent with this, exposure to a 100-fold lower Ca^{2+} concentration (20 μM) revealed a 50% reduction in cell shortening in cardiomyocytes isolated from β1β2M-KO mice compared with WT mice (*Figure 4B* and *C*). These results are in agreement with a potential reduction in Ca^{2+} sensitivity of contractile proteins in β1β2M-KO cardiomyocytes

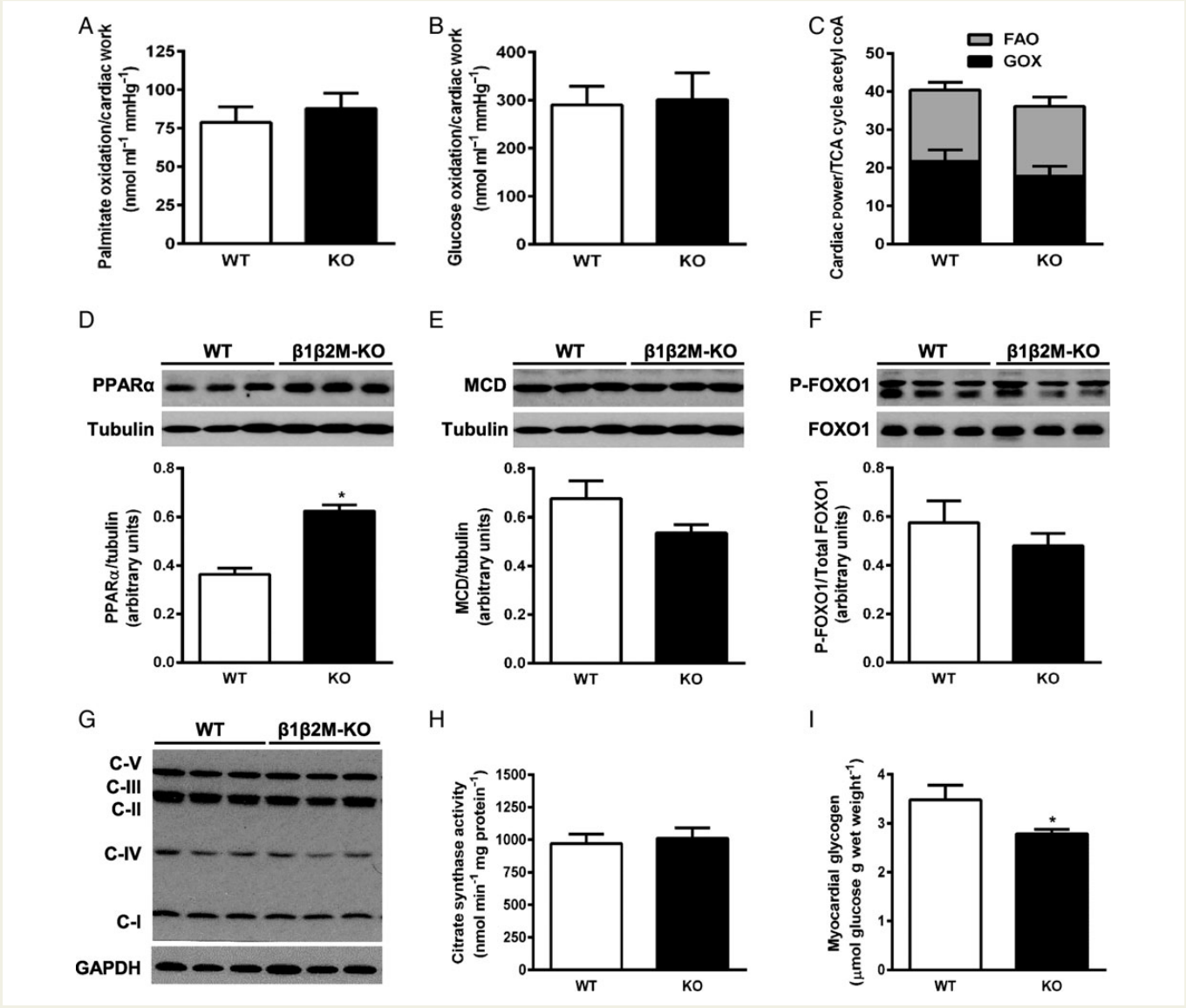

Figure 2 *Ex vivo* myocardial energy metabolism in aerobically perfused WT and β1β2M-KO mice. (*A*) Palmitate oxidation rates normalized to cardiac work ($n = 10$ WT and 6 KO), (*B*) glucose oxidation rates normalized to cardiac work ($n = 14$ WT and 8 KO), and (*C*) cardiac power/TCA cycle acetyl CoA production ($n = 6–10$). (*D*) Immunoblot analysis of PPARα and (*E*) MCD normalized to tubulin from hearts. (*F*) Immunoblot analysis of phosphorylated FOXO-1 normalized to total FOXO-1 and (*G*) OXPHOS complexes of the electron transport chain. (*H*) Citrate synthase activity in isolated myocytes and myocardial glycogen content in ventricular tissue ($n = 6$). *$P < 0.05$ vs. WT by Student's *t*-test.

compared with WT cardiomyocytes. Furthermore, the maximal rates of contraction (*Figure 4D*) and relaxation (*Figure 4E*) were also reduced in cardiomyocytes from β1β2M-KO mice. Interestingly, ECG recordings show that β1β2M-KO mice exhibit atrial fibrillation, which is consistent with the marked atrial enlargement (*Figure 4F* and *Table 2*). Owing to the presence of atrial fibrillation, PR interval could not be reliably measured in these mice. As well, the QT interval could not be consistently measured due to variability in the RR interval in the β1β2M-KO mice. In addition, β1β2M-KO mice display signs of conduction system abnormalities consistent with severe first-degree (1°) atrioventricular (AV) block, which may be due to AV nodal dysfunction. However, a full characterization of the conduction system abnormalities remains to be defined. Also, since alterations in AMPK γ subunits have been linked to arrhythmias, we examined cardiac expression of AMPK γ2 and found a 50% reduction in γ2 levels in β1β2M-KO mice compared with WT mice (*Figure 4G*). Taken together, these data suggest that ablation of AMPK is associated with impaired cardiomyocyte cell shortening and electrical conduction abnormalities.

3.6 Hypertrophic signalling pathways are impaired in hearts from WT and β1β2M-KO mice

Since AMPK is a negative regulator of pathways controlling protein synthesis and cardiac hypertrophic growth,[3,4] we examined whether hypertrophic signalling pathways are activated in response to the loss of AMPK activity. Consistent with the finding that heart size and wall thicknesses were not increased in β1β2M-KO mice, pro-hypertrophic

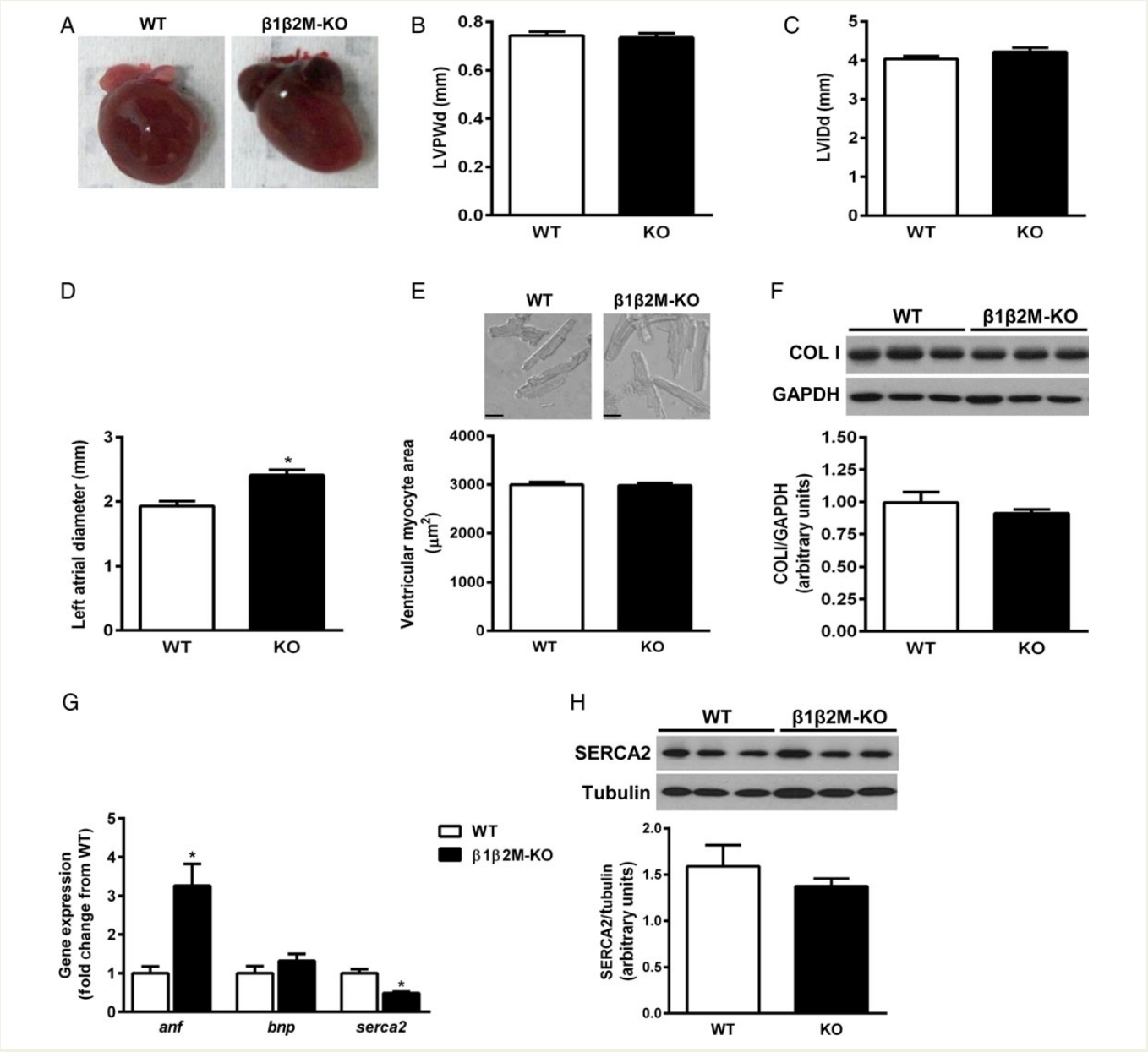

Figure 3 Hearts from β1β2M-KO mice fail to undergo hypertrophic remodelling. (*A*) Representative image of hearts from WT and β1β2M-KO mice showing enlarged atria. (*B*) LV posterior wall thickness in diastole (LVPWd), (*C*) left ventricular internal diameter in diastole (LVIDd), and (*D*) left atrial (LA) diameter as measured by transthoracic echocardiography ($n = 10–11$). (*E*) Ventricular myocyte area ($n = 237–244$ cells per group) with representative images of isolated ventricular myocytes shown above. Scale bars indicate 25 μm. (*F*) Immunoblot analysis of collagen type I (COL1) in ventricular tissue from WT and β1β2M-KO mice normalized to GAPDH as a loading control ($n = 10$ WT and 4 KO). (*G*) Gene expression analysis of *anf*, *bnp*, and *serca2* from ventricular tissue ($n = 5–7$). (*H*) Western blot analysis of SERCA2 protein levels in isolated ventricular myocytes from WT and β1β2M-KO mice ($n = 4–6$). *$P < 0.05$ vs. WT by Student's *t*-test.

signalling pathways such as Akt (*Figure 5A*) and mammalian target of rapamycin (mTOR) (*Figure 5B*) were also unchanged in hearts from WT and β1β2M-KO mice, despite AMPK inactivation. Interestingly, although we have previously shown that AMPK indirectly regulates protein synthesis via actions on the pro-hypertrophic mTOR/p70S6 kinase signalling cascade,[3,4] phosphorylation status of p70S6 kinase at both the Thr 389 and Thr 421/4 sites were not different between WT and β1β2M-KO hearts (*Figure 5C*). Taken together, these data demonstrate that hearts from β1β2M-KO mice fail to activate protein synthesis despite the loss of AMPK activity.

To explain why the loss of AMPK activity does not activate pro-hypertrophic signalling events, we examined the response of p38 MAPK in hearts from β1β2M-KO mice. As p38 MAPK activation has been proposed to be involved in promoting cardiac hypertrophy/ventricular remodelling[26–28] and AMPK has been shown to activate p38 MAPK,[29] we investigated the phosphorylation status of p38 MAPK at Thr 180/Tyr 182. We show that p38 MAPK phosphorylation is dramatically decreased in hearts from β1β2M-KO mice compared with WT mice (*Figure 5D*). Interestingly, cardiac p38 MAPK activation was unchanged in other models of AMPK deficiency including AMPKα2 KO

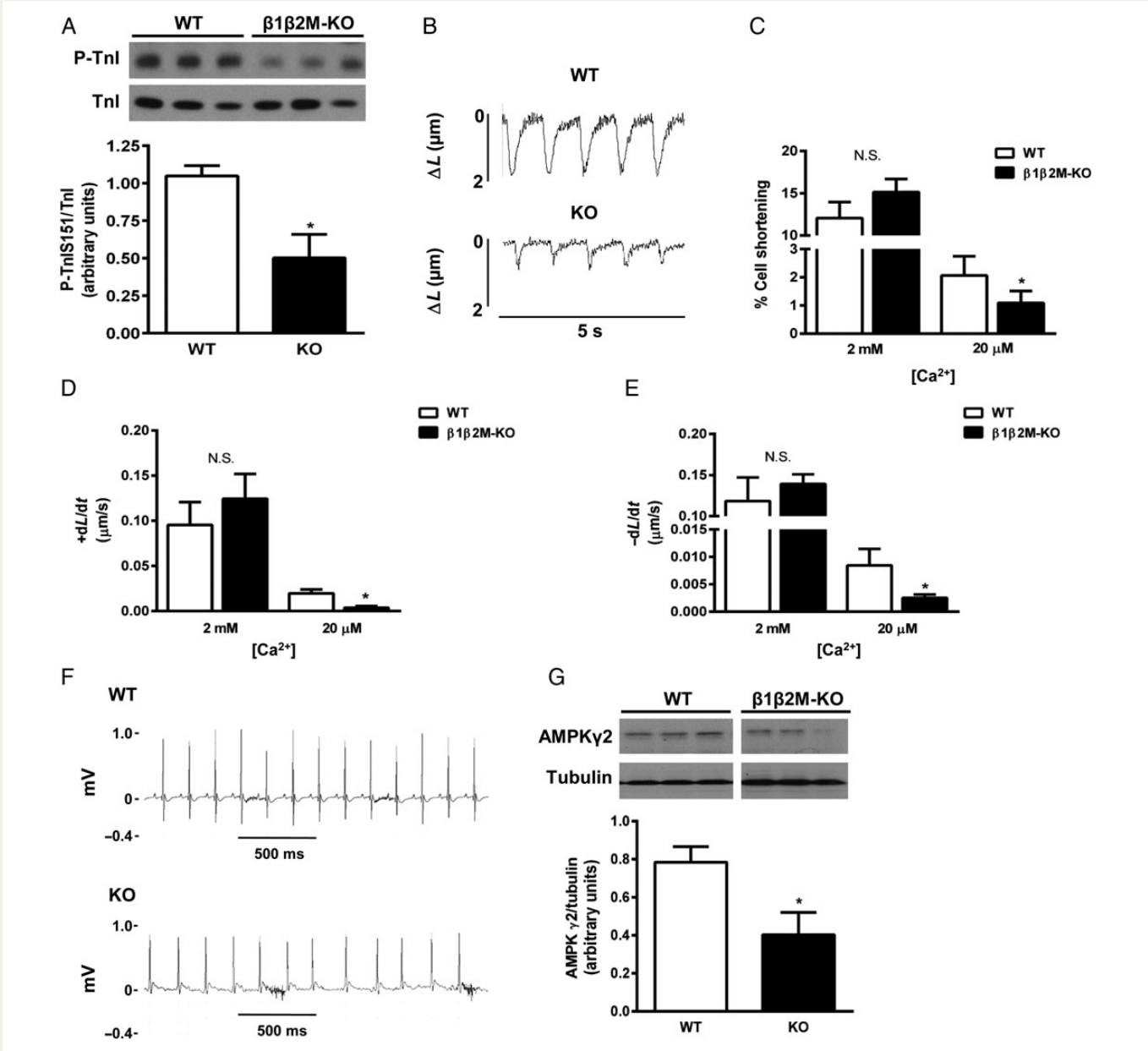

Figure 4 Reduced troponin I phosphorylation and impaired cardiomyocyte cell shortening. (*A*) Phosphorylated troponin I (TnI; Ser 151) normalized to total TnI in ventricular tissue ($n = 3–7$). (*B*) Cell shortening tracings of unloaded isolated ventricular myocytes in the presence of 2 mM and 20 μM extracellular Ca^{2+}, (*C*) cell shortening expressed as a % change in diastolic cell length, (*D*) maximal rate of contraction ($+dL/dt$), and (*E*) maximal rate of relaxation ($-dL/dt$) in the presence of 2 mM and 20 μM extracellular Ca^{2+} ($n = 5–8$ cells from 5–7 mice per group). (*F*) Representative ECG tracings from WT and β1β2M-KO mice. (*G*) Immunoblot analysis of AMPK γ2 levels normalized to tubulin from ventricular tissue ($n = 6–8$). *$P < 0.05$ vs. WT by Student's *t*-test.

mice (*Figure 5E*) and AMPK KD mice (*Figure 5F*). Importantly, these models do not display the same degree of AMPK inactivation as the β1β2M-KO mouse and also are more sensitive to hypertrophic stimuli.[30–32] Of importance, cardiac p38 MAPK was markedly reduced in LKB1 KO mice (*Figure 5G*), which do demonstrate a similar degree of cardiac AMPK inactivation as the β1β2M-KO mouse.[8] Taken together, these data suggest that the extent to which AMPK is reduced in the heart may dictate the inactivation status of p38 MAPK. Interestingly, although AMPK activity is also dramatically reduced in β1β2M-KO mouse skeletal muscle,[13] the loss of p38 MAPK activation was only observed in the heart and not in skeletal muscle (*Figure 5H*), suggesting that this is a cardiac-specific effect of a reduction in AMPK β1 and β2 subunits.

4. Discussion

AMPK β1β2M-KO mice had a dramatic reduction in cardiac AMPK activity (>95%) due to dramatic reduction in the catalytic α subunits. Since this decrease in cellular AMPK activity was much larger than previously observed in other transgenic models of cardiac AMPK deficiency,[10,30] we utilized this model to further investigate the role of AMPK in the regulation of cardiac metabolism, function, and LV

Table 2 ECG analysis in WT and β1β2M-KO mice (*N* = 6–8)

	WT	β1β2M-KO
HR (bpm)	432.76 ± 13.17	445.76 ± 19.82
RR interval (ms)	139.94 ± 4.16	137.28 ± 6.47
QRS duration (ms)	10.54 ± 0.91	10.54 ± 0.45
PR interval (ms)	42.24 ± 1.02	n.d.
QT interval (ms)	20.30 ± 1.54	n.d.
QTc (ms)	54.09 ± 3.52	n.d.

HR, heart rate; QTc, corrected QT interval; n.d., not determined.

hypertrophy under basal conditions. In our current study, β1β2M-KO mice displayed ventricular dilatation and both systolic and diastolic dysfunction, which is the first report of AMPK deficiency resulting in significant cardiac dysfunction during basal conditions. Indeed, previous studies of heart-specific AMPKα2 KD mice,[10] AMPKα2 KO mice,[11,33] and heart-specific AMPKα2-dominant negative mice[9] did not report changes in basal contractile function or cardiac hypertrophy despite having a complete loss in AMPK α2 activity. The discrepancy in cardiac phenotypes between the aforementioned AMPK-deficient mice and our β1β2M-KO mice is likely due to compensatory activity by the remaining AMPK α1 subunit, which may be sufficient to maintain normal cardiac structure and function in these other models.

Given that AMPK is a key regulator of cardiac energy metabolism,[2] we hypothesized that alterations in glucose and fatty acid metabolism would be responsible for the cardiac dysfunction observed in the β1β2M-KO mice. Indeed, we would expect that a reduction in AMPK activity and subsequent loss of ACC inhibitory phosphorylation should result in reduced fatty acid oxidation rates.[2] However, palmitate oxidation rates were unchanged in β1β2M-KO mice compared with WT mice. In addition, despite the loss of AMPK activity, cardiac glucose oxidation was not different compared with WT mice, suggesting that there was no impairment in glycolytic flux necessary to maintain rates of glucose oxidation. These findings suggest that mechanisms other than AMPK can regulate cardiac energy metabolism under normal basal conditions. This is similar to our previous findings in skeletal muscle from these mice, showing that basal skeletal muscle glucose uptake was similar between genotypes.[13] However, there was a 1.5-fold increase in expression of PPARα in these hearts, which may be upregulated in order to compensate for the chronic reduction of AMPK in β1β2M-KO mice. This increase in expression of PPARα may explain why cardiac energy metabolism remains normal in hearts from β1β2M-KO mice. Although these data were originally unexpected, we have recently shown using a mouse model with alanine knock-in mutations in both ACC1 (at Ser79) and ACC2 (at Ser212) that AMPK-dependent inhibitory phosphorylation of ACC is not essential for maintaining cardiac fatty acid metabolism.[34] Taken together, these data support the idea that AMPK-independent mechanisms can maintain cardiac fatty acid oxidation in the absence of an intact AMPK–ACC signalling pathway.

Since the loss of AMPK activity and cardiac dysfunction in the β1β2M-KO mice cannot be attributed to impaired energetics, we investigated if there were defects in AMPK-mediated pathways that contribute to cardiomyocyte contraction. As predicted, the loss of AMPK activity results in an ∼50% decrease in phosphorylated troponin I (Ser 151), which is associated with impaired myocardial contractile performance in β1β2M-KO mice. In the presence of a low extracellular Ca^{2+} concentration, cell shortening and maximal rates of contraction and relaxation were diminished in both WT and β1β2M-KO mice compared with measurements at the 2 mM Ca^{2+} concentration. However, at this lower Ca^{2+} concentration, it was observed that cell shortening and maximal rates of contraction were significantly reduced in cardiomyocytes from β1β2M-KO mice compared with WT mice, suggesting that myofilament Ca^{2+} sensitivity may have been impaired. In addition, maximal rates of relaxation in isolated myocytes were reduced in β1β2M-KO mice compared with WT mice, which may suggest that there are defects in intracellular Ca^{2+} removal. Although we did not observe changes in SERCA2 protein expression, it is possible that changes in other Ca^{2+} handling proteins are involved and mediate this impaired rate of relaxation. Our findings are supported by studies showing that AMPK regulates cardiomyocyte contraction by improving myofilament Ca^{2+} sensitivity without a change in intracellular Ca^{2+} transient, and that this effect is lost in other models of AMPK deficiency.[23,25] Although our experiments measuring cell shortening of isolated ventricular myocytes are suggestive of reduced myofilament Ca^{2+} sensitivity, this may also be the result of changes in other steps of excitation–contraction coupling and/or Ca^{2+} handling. This limitation of the current study should be addressed in the future by examining skinned myocytes or measuring cytosolic Ca^{2+} transients together with cell shortening to calculate coupling gain. Since cell shortening appeared normal at 2 mM Ca^{2+} concentration, we cannot be certain that impaired myocyte contractility is responsible for poor cardiac function *in vivo*. Indeed, it is possible that the structural changes of the myocardium and/or the *in vivo* environment in the β1β2M-KO mice result in impaired cardiac contraction. This has yet to be fully delineated. Overall, the observed reductions in troponin I phosphorylation and cell shortening provide support for AMPK playing a role in regulating cardiac contraction and are a potential mechanism for cardiac contractile dysfunction in β1β2M-KO mice.

Since AMPK is known to be a negative regulator of cardiac hypertrophy,[3,4,6,7] we investigated morphological remodelling in these hearts. Surprisingly, despite a ∼25% reduction in %EF in β1β2M-KO mice, neither heart weight, LV wall thickness, nor LVID was increased from WT mice. These findings are consistent with previous findings, indicating no change in heart weight in β1β2M-KO mice.[13] As well, ventricular myocyte size was not different between WT and β1β2M-KO mice. Furthermore, hearts from β1β2M-KO mice did not display any signs of interstitial fibrosis that would typically accompany impaired cardiac function, suggesting that hearts from these mice fail to undergo detrimental LV fibrotic remodelling. In spite of this, left atrial diameter was increased by 25% in β1β2M-KO mice, suggesting that either AMPK plays an important role in regulating atrial hypertrophy and remodelling or that the inability of LV remodelling affects atrial dilatation via simple alterations in mechanical forces. Interestingly, ECG recordings show significant atrial fibrillation and AV nodal conduction system abnormalities. Further study of the role of AMPK in mediating atrial fibrillation will be important in the future.

The cardiac phenotype observed in the β1β2M-KO mice is most similar to that of cardiac-specific LKB1 KO mice, which display bilateral atrial enlargement and impaired cardiac systolic function by 12 weeks of age.[8] However, cardiac-specific LKB1 KO mice differ to our β1β2M-KO mice in several respects. For instance, LKB1 KO mice do show modest signs of cardiac hypertrophy, an almost two-fold

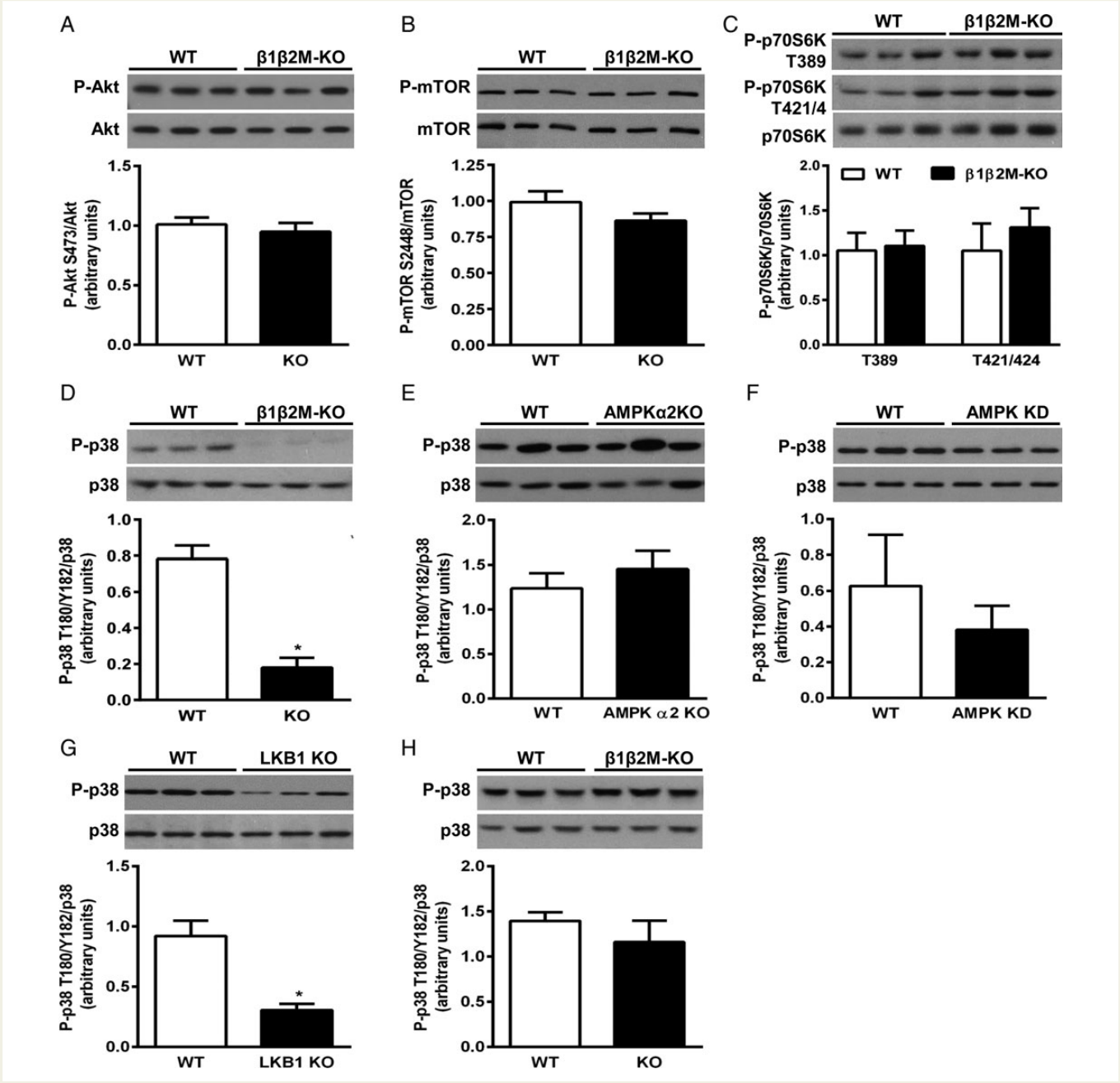

Figure 5 Pro-hypertrophic pathways not activated in hearts from β1β2M-KO mice and dramatic reduction in p38 MAPK activation. Immunoblot analysis of (*A*) phosphorylated Akt (Ser 473) normalized to total Akt levels ($n = 3$), (*B*) phosphorylated mTOR (Ser 2448) normalized to total mTOR protein levels ($n = 4–6$), (*C*) phosphorylated p70S6K (Thr 389 and Thr 421/4) normalized to total p70S6K ($n = 5–7$), and (*D*) phosphorylated p38 (Thr 180/Tyr 182) normalized to total p38 ($n = 5–7$) in ventricular tissue from WT and AMPK β1β2M-KO mice. Phosphorylated p38 MAPK (Thr 180/Tyr 182) normalized to total p38 MAPK protein levels in hearts from (*E*) WT and AMPK α2 KO ($n = 5–6$), (*F*) WT and AMPK KD ($n = 6$), and (*G*) WT and LKB1 KO mice ($n = 5–9$). (*H*) Phosphorylated p38 MAPK (Thr 180/Tyr 182) normalized to total p38 MAPK levels in gastrocnemius muscle of WT and β1β2M-KO mice ($n = 5–13$). *$P < 0.05$ vs. respective WT group by Student's *t*-test.

increase in atrial size and a more severe and progressive pathological cardiac phenotype resulting in 100% mortality of LKB1 KO mice by 5 months of age. This mortality rate has been attributed to fatal arrhythmias.[8] In contrast, the β1β2M-KO mice fail to show signs of cardiac hypertrophy and had a lower mortality rate than LKB1 KO mice. Although we did not investigate these differences in detail, they are likely due to the fact that LKB1 has multiple downstream targets in addition to AMPK that may contribute to the slightly different phenotype of these mice compared with the β1β2M-KO mice. Indeed, several cardiac- specific mouse models of LKB1 ablation also display a similar phenotype to β1β2M-KO mice with a dramatic reduction in phosphorylated ACC and similar palmitate oxidation rates between WT and KO mice.[35,36] As LKB1 is a key upstream AMPK kinase in the heart, it is not unexpected that deletion of AMPK also produces

a similar phenotype. However, our data demonstrate that the decrease in AMPK activity in LKB1 KO mice and not the loss of activity of the other LKB1 targets[37] mediates the majority of the effects observed in the LKB1 KO mouse model.

Consistent with the absence of LV hypertrophy in hearts from β1β2M-KO mice, there was no evidence of activation of pro-hypertrophic signalling pathways, including Akt, mTOR, and p70S6 kinase. However, we did observe that phosphorylation of cardiac p38 MAPK was markedly reduced by 70% in β1β2M-KO mice compared with WT mice. Importantly, activation of p38 MAPK has been associated with the development of cardiac hypertrophy and remodelling,[26–28] and is thought to be downstream of AMPK signalling.[29] Furthermore, previous work has shown that phosphorylation of p38 MAPK is decreased in cardiac fibroblasts from mice with AMPK deficiency, and that this was associated with impaired cardiac performance.[38] Notwithstanding this, myocardial p38 MAPK activation was unchanged in two other models of AMPK deficiency that we examined including AMPKα2 KO and AMPK KD mice. Although we do not have direct evidence to prove the discrepancy between our model and these other models, the AMPKα2 KO and AMPK KD mice still maintain 30–50% of total cardiac AMPK activity, suggesting that there may be a threshold of AMPK activity required to maintain normal cardiac function.[23] However, when the levels of AMPK activity decrease substantially, it is possible that this is sufficient to impair p38 MAPK signalling and a hypertrophic response. This is supported by the fact that the LKB1 KO mouse heart displays an approximate 80% loss of AMPK phosphorylation[8] as well as a significant reduction in cardiac p38 MAPK activation. This similarity between the LKB1 KO and the β1β2M-KO mice also suggests that the phenotype is induced by a dramatic loss of AMPK activity as opposed to something specific to the loss of the AMPK β subunit independent of alterations of AMPK activity. However, it is not clear if activating p38 MAPK would be desirable in cardiac disease since excessive p38 MAPK activation may be detrimental.[27]

Since activation of myocardial AMPK may be necessary in certain forms of heart disease or in heart failure,[39] whether or not existing drugs, such as metformin,[40] that are known to activate AMPK in the heart can be used as a treatment in specific cardiac diseases or cardiac injury, even in the absence of diabetes is as yet unknown. In addition, cardiac-specific AMPK activation may prove to be difficult. Although some AMPK activators such as A-769662 are thought to be AMPK β isoform specific,[41] the expression of these isoforms in other cell types and the fact that the heart expresses both β1 and β2 isoforms could make it difficult to target AMPK in a heart-specific manner for the treatment of heart failure.

5. Conclusions

Hearts from β1β2M-KO mice display significant cardiac contractile dysfunction, chamber dilatation, and lack the ability to undergo hypertrophic LV remodelling. Since AMPK activity has been shown to be inhibited in humans with advanced heart failure,[42] much of the focus of this has been on impaired energetics. However, our study shows that decreased AMPK activity also alters molecular signalling events that control cardiac hypertrophy (such as p38 MAPK) and myocardial myofilament Ca^{2+} sensitivity, and that this may also contribute to the eventual progression to heart failure. As such, our data suggest that pharmacological activation of AMPK may be of benefit in the setting of advanced heart failure by way of its ability to regulate contractile function and adaptive remodelling.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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