AMPK attenuates microtubule proliferation in cardiac hypertrophy

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AMPK attenuates microtubule proliferation in cardiac hypertrophy. Am J Physiol Heart Circ Physiol 304: H749–H758, 2013. First published January 11, 2013; doi:10.1152/ajpheart.00935.2011.—Cell hypertrophy requires increased protein synthesis and expansion of the cytoskeletal networks that support cell enlargement. AMPK limits anabolic processes, such as protein synthesis, when energy supply is insufficient, but its role in cytoskeletal remodeling is not known. Here, we examined the influence of AMPK in cytoskeletal remodeling during cardiomyocyte hypertrophy, a clinically relevant condition in which cardiomyocytes enlarge but do not divide. In neonatal cardiomyocytes, activation of AMPK with 5-aminoimidazole carboxamide ribonucleotide (AICAR) or expression of constitutively active AMPK (CA-AMPK) attenuated cell area increase by hypertrophic stimuli (phenylephrine). AMPK activation had little effect on intermediate filaments or myofilaments but dramatically reduced microtubule stability, as measured by detyrosinated tubulin levels and cytoskeletal tubulin accumulation. Importantly, low-level AMPK activation limited cell expansion and microtubule growth independent of mTORC1 or protein synthesis repression, identifying a new mechanism by which AMPK regulates cell growth. Mechanistically, AICAR treatment increased Ser-915 phosphorylation of microtubule-associated protein 4 (MAP4), which reduces affinity for tubulin and prevents stabilization of microtubules (MTs). RNAi knockdown of MAP4 confirmed its critical role in cardiomyocyte MT stabilization. In support of a pathophysiological role for AMPK regulation of cardiac microtubules, AMPK α2 KO mice exposed to pressure overload (transverse aortic constriction; TAC) demonstrated reduced MAP4 phosphorylation and increased microtubule accumulation that correlated with the severity of contractile dysfunction. Together, our data identify the microtubule cytoskeleton as a sensitive target of AMPK activity, and the data suggest a novel role for AMPK in limiting accumulation and densification of microtubules that occurs in response to hypertrophic stress.

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- AMP-activated protein kinase; cytoskeleton; hypertrophy; microtubule-associated protein 4; microtubule trafficking (29), mRNA transport (22, 27), and intracellular organization (38, 40, 43), and disruption of these microtubule functions by aberrant stabilization in pressure overload may also contribute to adverse remodeling and heart failure. In support of a role for MT stabilization in contractile dysfunction, we found that chronic (every other day) treatment with colchicine to reduce MT densification attenuated hypertrophy and improved contractile function in Balb-c mice exposed to pressure overload produced by transverse aortic constriction (TAC) (7). Identification of endogenous pathways that regulate cardiac microtubule dynamics may thus provide pharmacological targets for attenuating microtubule-related ventricular dysfunction in the hypertrophied heart.

AMPK is a metabolic sensor that limits anabolic processes when ATP production is insufficient to meet the energetic demands of the cell. Activated by increased AMP/ATP ratio, AMPK reduces energy-consuming processes, such as protein synthesis by inhibiting the mTOR signaling pathway (10, 13, 28). In the heart, deletion of the AMPK α2 catalytic subunit exacerbates cardiac hypertrophy and heart failure in response to pressure overload, and this is associated with up-regulated mTOR signaling (46). While mTOR signaling is important for the hypertrophic response (31), recent evidence suggests mTOR also has antiapoptotic, anti-inflammatory, and metabolic functions that may be important for adaptive hypertrophy (30, 33, 45). Thus, while increased mTOR signaling may contribute to cardiac hypertrophy, it is not sufficient to explain the worsened cardiac function in AMPK α2 KO hearts after TAC.

In addition to regulating protein synthesis, AMPK can regulate the cytoskeleton. AMPK is a member of the MARK/PAR kinase subfamily (microtubule affinity-regulating kinase), which phosphorylates MAPs (microtubule-associated proteins) to influence MT dynamics and cell polarity (18). AMPK was recently shown to directly phosphorylate the microtubule-associated protein tau and alter its binding to neuronal microtubules (39). In addition, AMPK phosphorylation of the plus end tracking protein clip-170 increased MT polymerization speed and was found necessary for directed cell migration, while reduced CLIP-170 phosphorylation by AMPK resulted in less dynamic MTs (20). Whether AMPK regulates MT dynamics in cardiomyocytes, however, and how this influences the hypertrophic response is not known.

Here, we investigated AMPK regulation of cardiac microtubules during hypertrophy of cultured rat neonatal cardiomyocytes and in pressure overload-induced cardiac hypertrophy using the TAC model in mice. Our data identify, for the first time, the cardiac microtubule cytoskeleton as a sensitive target of AMPK activity, and suggest a novel role for AMPK in limiting accumulation and densification of microtubules in response to pressure overload.

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Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from 2-day-old Sprague-Dawley rats by enzymatic digestion and separated from nonmuscle cells on a discontinuous Percoll gradient, as previously described (46). Left ventricular (LV) diameter, shortening fraction, and wall thickness were measured from two-dimensional guided short-axis M-Mode views of the LV.

Echocardiography. Mice were anesthetized with 1.5% isoflurane, and echocardiographic images were obtained with a Visualsonics high-resolution Veve 660 system, as previously described (46). Left and echocardiographic images were obtained with a Visualsonics.

RESULTS

AMPK regulation of cell size, protein synthesis, and microtubule dynamics. Cell enlargement requires increased protein synthesis, as well as coordinated expansion of the cytoskeletal networks that support the new cell dimensions. While investigating the role of AMPK in cardiomyocyte hypertrophy, we observed that AICAR treatment was able to limit cell area at a low dose (0.1 mM) that did not block protein synthesis (Fig. 1D–F). This suggests an AICAR effect on cell spreading or cell shape that may occur independently of the well-known AMPK inhibitory effects on protein synthesis. Because cell spreading is mediated by changes in cytoskeletal dynamics, we examined expression and partitioning (cytosolic vs. cytoskeletal) of cardiac myosin heavy chain (MHC) (Abcam, Cambridge, MA) were used for detection of proteins mediating by changes in cytoskeletal dynamics, we examined expression and partitioning (cytosolic vs. cytoskeletal) of cardiac myosin heavy chain (MHC) (Abcam, Cambridge, MA) were used for detection of proteins.
Fig. 1. AMPK regulation of microtubule (MT) stability, cell size, and protein synthesis in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were treated with phenylephrine (50 µM) and increasing doses of 5-aminoimidazole carboxamide ribonucleotide (AICAR). Triton-soluble and insoluble fractions were collected after 24 h of treatment, and the expression and partitioning of the indicated proteins were analyzed by Western blot analysis (A) and quantified (B). Immunofluorescent staining of β-tubulin (green) and detyrosinated tubulin (Glu-tubulin; red) and DNA (Hoescht; blue) were examined at 24 h of treatment (C). For [3-H]leucine incorporation (Leu inc; D) and cell area (E), 0.1 µM nocodazole was also included to determine effects of MT disruption. For measuring cell area, fixed cells were stained with Coomassie blue (F). Graphs represent protein levels relative to PE-treated cardiomyocytes averaged from three to six experiments *P < 0.05 compared with PE-treated cells.
PE-induced assembly of myofilaments (cask-sarcomeric actin or β-actin) or intermediate filaments (cask-desmin), while it dose-dependently reduced microtubule (cask-tubulin) levels (Fig. 1, A–C). Examination of stabilized MTs using antibodies to detyrosinated tubulin (the c-terminus tyrosine is cleaved in longer-lived polymerized microtubules, leaving a c-terminal glutamine, Glu-tubulin) confirmed that microtubule longevity was much reduced in the presence of AICAR (Fig. 1, A–C). Interestingly, MT stability was more sensitive to AMPK activation than inhibition of mTOR, EEF2, or protein synthesis, as AICAR attenuated microtubule stability and cell area (Fig. 1, A, B, and E and F) at doses (0.1 mM) that did not block mTORC1 signaling, EEF2 phosphorylation (Fig. 1, A and B), or protein synthesis (Fig. 1D). Similar to low-dose AICAR treatment, nocodazol inhibition of microtubule growth reduced cell area without inhibiting protein synthesis (Fig. 1, D and E), indicating that blocking microtubule proliferation can attenuate cell spreading, independent of overall changes in protein synthesis. One explanation for the decreased cell area despite equal levels of protein synthesis is that MT-dependent distribution of proteins or organelles to the cell periphery requires more persistent elongated microtubule tracks, which were diminished in the presence of AICAR. This possibility is supported by Coomassie staining of cellular protein distribution in fixed cells (Fig. 1F), which shows proteins more highly concentrated in perinuclear regions of cells treated with AICAR or nocodazole, while cells treated with PE alone have more evenly distributed proteins that reach the cell periphery.

To rule out the possibility that reduction in MT stability is an indirect effect of less hypertrophy, cardiomyocytes were treated with phenylephrine for 72 h to establish hypertrophy and microtubule densification, then treated briefly (3 h) with AICAR. Three hours of AICAR treatment reduced Glu-tubulin levels (Fig. 2, A–C), but it did not significantly reduce overall cytoskeletal tubulin, indicating that AMPK reduction of microtubule stability can occur independent of AMPK effects on hypertrophy, or on overall microtubule levels.

To determine whether the reduction of MTs is due to activated AMPK rather than an indirect effect of AICAR, cells were infected with adenovirus expressing constitutively active AMPK (CA-AMPK) for 24 h, then treated with phenylephrine for an additional 48 h. Similar to AICAR treatment, CA-AMPK reduced microtubule density and Glu-tubulin levels in phenylephrine-treated cells as demonstrated by immunofluorescence staining (Fig. 2D). Similar to low-dose AICAR treatment, CA-AMPK reduced cytoskeletal tubulin levels (Fig. 2E) and cell area (Fig. 2F) without inhibiting protein synthesis (Fig. 2G). These data indicate that AMPK regulates MT dynamics, which can influence cell spreading independent of protein synthesis.

To further examine the role of AMPK in microtubule regulation, we overexpressed wild-type AMPK-α2 in neonatal cardiomyocytes and examined whether this influences phenylephrine-induced MT stabilization and accumulation. Adenoviral overexpression of WT AMPK resulted in spontaneously increased AMPKThr172 phosphorylation, a reduction in stabilized microtubules (det tyrosinated tubulin), and a reduction in tubulin association with the Triton-insoluble cytoskeleton, similar to the effects of activating AMPK using AICAR (Fig. 2, H and I). These data are further evidence that AMPK regulates microtubule stability in cardiomyocytes.

The finding that MTs are a sensitive target of AMPK in neonatal cardiomyocytes led us to investigate whether AMPK may influence cardiac MT dynamics in vivo, where microtubule densification is believed to contribute to pressure overload-induced heart failure.

AMPK regulation of MT dynamics in pressure overload hypertrophy. We have previously demonstrated that TAC-induced hypertrophy, LV dysfunction, and heart failure are exacerbated by AMPK α2 KO (46). Because we observed that AMPK activation can reduce MT stabilization in neonatal cardiomyocytes exposed to hypertrophic signals, we examined the influence of AMPK α2 (AMPK α2 KO), the predominant AMPK catalytic subunit in the heart (34), on cardiac microtubule dynamics in mice under basal conditions or hypertrophic stress (transverse aortic constriction, TAC) (Fig. 3, A and B). Interestingly AMPK α2 KO mice had ~60% increase in polymerized tubulin levels compared with WT mice under basal conditions. Three weeks of TAC resulted in further increases in free and polymerized tubulin levels in both WT and AMPK α2 KO mice, but the increase in polymerized and free tubulin was significantly greater in the AMPK α2 KO mice. Detyrosinated tubulin (Glu-tubulin) levels were also significantly higher in AMPK α2 KO mice exposed to TAC, indicating an increase in microtubule stability. Interestingly, in AMPK KO mice, the levels of polymerized tubulin exhibited a striking correlation with increased lung to body weight ratio (indicative of pulmonary congestion) and reduced ejection fraction (indicative of contractile dysfunction) (Fig. 3, C and D). Taken together, these results suggest AMPK α2 plays a role in limiting pressure overload-induced accumulation and stabilization of cardiac microtubules, and they identify an association between increased MT accumulation and left ventricular dysfunction.

AMPK regulation of microtubule-associated protein 4. To investigate mechanism(s) of AMPK regulation of cardiac MTs,
3 hr AICAR treatment reduces stabilized microtubules in pre-hypertrophied cardiomyocytes

A

B

C

D

E

F

G

H

I

Scale bar = 10 µM

Scale bar = 25 µM
we examined MAP4. MAP4 binds to and stabilizes microtubules in response to pressure overload, but its affinity for microtubules is reduced by phosphorylation at Ser-914 (ms) [equivalent to Ser-915 (rat), Ser-924 (cat), and Ser-941 (hu) (4, 6, 12)]. To determine whether MAP4 expression or phosphorylation is regulated by AMPK α2 in vivo, we analyzed the Triton-soluble and insoluble ventricular lysates of WT and AMPK α2 KO mice exposed to sham or TAC conditions. In Triton X-100 tissue homogenates, mechanical homogenization in the presence of detergent, phosphatase inhibitors, and low temperature causes most of the tubulin to fractionate with the Triton-soluble fraction. Interestingly, most of the MAP4 protein partitioned in the Triton-insoluble fraction (not shown). This suggests that MAP4 also associates with other more stable or Triton-insoluble cytoskeletal elements of the cell in addition to microtubules, consistent with a recent report demonstrating MAP4 also binds to actin (19). Under basal conditions, insoluble MAP4 levels were significantly higher in AMPK α2 KO hearts than WT, while phosphorylation of insoluble MAP4 relative to total MAP4 was reduced in AMPK α2 KO samples (Fig. 4, A and B). These differences in expression level or phosphorylation were not observed after TAC, however, whereas MAP4 expression increased in both WT and AMPK α2 KO (albeit with a higher relative increase in WT hearts compared with the low levels under sham conditions). In response to TAC, phosphorylation of Triton-insoluble MAP4Ser914 was reduced as previously observed in cells (4), with no significant difference between WT and KO. In the Triton-soluble fraction, however, MAP4Ser914 phosphorylation was increased in WT samples, while the increase in MAP4Ser914 phosphorylation was significantly attenuated in AMPK α2 KO, consistent with a role for AMPK α2 in promoting MAP4 phosphorylation and reducing MT stability.

To determine whether activation of AMPK can increase MAP4 phosphorylation, we treated prehypertrophied (after 48 h of phenylephrine exposure) neonatal cardiomyocytes for an additional 24 h with phenylephrine in the absence or presence of 0.2 mM AICAR. AICAR exposure resulted in increased MAP4 phosphorylation and a trend toward reduced total MAP4 levels. At the same time, stabilized MTs, but not total MTs, in the same fraction were dramatically reduced, consistent with a role for MAP4 in MT stabilization. (Fig. 5, A and B). Recently, AMPK was found to phosphorylate serine-262 within the KXGS motif in the microtubule-associated protein tau (39). Ser-262 in human tau is analogous to Ser-914 in the KXGS motif in mouse MAP4 (87% homology in the 22 amino acids surrounding Ser-914). To determine whether AMPK can directly phosphorylate Ser-914 of MAP4 in vitro, we incubated active WT, AMPK α2β2/γ2 with a peptide corresponding to amino acids 903 to 925 in mouse MAP4 and used LCMS to detect phosphorylation of the peptide. LCMS analysis showed that this peptide was monophosphorylated by AMPK on serine-914 within the KXGS motif (data not shown), suggesting AMPK can directly phosphorylate MAP4 at serine-914.

To verify the role of MAP4 in cardiomyocyte microtubule stability, we used RNAi to reduce MAP4 expression. MAP4 RNAi treatment of neonatal cardiomyocytes reduced stabilized MT levels (Glu-tubulin), compared with control nontargeting RNAi (Fig. 5, C and D). Interestingly, MAP4 depletion also altered MT organization, so that microtubules were sparsely distributed in some areas of the cell, while densely aligned along the cell periphery in others. Some cells treated with MAP4 RNAi also exhibited abnormal nuclear positioning, so that nuclei were observed along the cell periphery rather than the center (Fig. 5C). MAP4 also has been shown to bind to and regulate actin filaments (19), so MAP4 may influence cytoskeletal organization and nuclear positioning through effects on actin and/or microtubules. Interestingly, AICAR treatment still further reduced stabilized MTs in MAP4-deficient cells (Fig. 5D), suggesting AMPK activation may also reduce MT stabilization through mechanisms that are independent of MAP4.
AMPK has long been recognized as a metabolic sensor that reduces energy-consuming processes (such as protein synthesis), while increasing energy production (glucose transport, fatty acid oxidation, mitochondria biogenesis). Our data suggest a novel role for cardiac AMPK in attenuating the stabilization and densification of MTs that occurs in response to hypertrophic stress. AMPK prevention of microtubule proliferation can limit expansion of cell area in cultured cells, independent of previously identified AMPK inhibitory effects on protein synthesis. More importantly, our data suggest AMPK limits microtubule stabilization that occurs in response to pressure overload, where microtubule proliferation is believed to contribute to heart failure.

Microtubules and heart failure. There is evidence that microtubule densification contributes to contractile dysfunction through mechanical impairment of contractility (15, 36, 41). Reducing the viscous load upon myofilaments imposed by microtubules in the heart may allow AMPK to improve contractile function without additional energy expenditure. However, because microtubules play an important role in intracellular transport and subcellular organization, altered microtubule dynamics in pressure overload could prove maladaptive through other mechanisms in addition to increasing myofilament load. For instance, microtubule decoration by MAP4 (which plays a role in pressure overload-induced MT stabilization) can inhibit transport of receptors (2) and mRNAs (25, 26). Disruption of mRNA and protein transport may impair compensatory hypertrophy and promote the transition to decompensation, which is associated with increased MT accumulation in pressure overload-induced heart failure (35). Microtubules also play an important role in organelle transport and distribution (1, 9, 38, 40, 44), and decoration of microtubules by MAPs may, thus, alter intracellular organization. Interestingly, microtubules were recently shown to mediate stretch-induced NADPH oxidase activation, oxidative stress, and calcium sparks (14, 23), suggesting a potential role for increased MTs in oxidative stress and altered calcium dynamics observed in heart failure. In support of a role for MT accumulation in contractile dysfunction in AMPK KO mice, we observed that polymerized microtubule levels correlated strikingly with reduced left ventricular ejection fraction and pulmonary congestion in mice exposed to TAC. Furthermore, our previous study demonstrated that periodic colchicine treatment attenuated chamber dilation and improved contractile function in Balb-c mice exposed to pressure overload (7). The present data suggest that agents that activate AMPK may similarly preserve contractile function by preventing MT densification.

How does AMPK regulate microtubules? Recently, a mechanism for MT stabilization during pressure overload hypotrophy was identified. MAP4, which is upregulated in response to pressure overload (24), binds to and promotes MT stabilization (12, 37). However, when phosphorylated at Ser-924 of the KXGS motif (equivalent to Ser-914 in mice) within the MT binding domain, this interaction is inhibited and MT stability is reduced (4). Cheng et al. (3) showed that increased phospho-

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**Fig. 4.** AMPK α2 KO reduces phosphorylation of microtubule-associated protein 4Ser914 (MAP4Ser914).

Triton-soluble and insoluble lysates from WT and AMPK α2 KO mice exposed to sham or TAC were analyzed by Western blot (A) and quantified (B) for the indicated proteins. *P < 0.05 comparing WT to KO under same conditions. †P < 0.05 comparing sham to TAC.
Fig. 5. AMPK activation increases phosphorylation of MAP4. Prehypertrophied neonatal cardiomyocytes (48-h exposure to 50 μM phenylephrine) were exposed to an additional 24 h of phenylephrine treatment in the presence or absence of 0.2 mM AICAR, prior to analysis by Western blot analysis (A) and quantification (B) of phosphor-AMPK (P-AMPK), tubulin, Glu-tubulin, MAP4, and phosphor-MAP4Ser915 (P-MAP4) (Note Ser-915 in rat MAP4 is equivalent to Ser-914 in mouse MAP4). MAP4 is important for cardiomyocyte microtubule stability and organization. C: cardiomyocytes were exposed to MAP4 RNAi or nontargeting control RNAi for 72 h and analyzed by immunofluorescence (C). Arrows point to abnormally localized nuclei in MAP4 RNAi-treated cells. D: microtubule stability was also examined by Western blot in control and MAP4 RNAi-treated cells after AICAR treatment (0.2 mM) for 24 h. *P < 0.05 compared with phenylephrine-treated cells.
tase activity during pressure overload results in dephosphorylation of MAP4Ser924, thereby promoting MT assembly and stabilization. We find that activation of AMPK increases MAP4 phosphorylation at the analogous site in rat cardiomyocytes. Conversely, MAP4 phosphorylation at this site is reduced in AMPK α2 KO mice. Thus, it appears that AMPK plays a role in counteracting the effects of PP2A on MT stability by reducing MAP4 affinity for MTs.

AMPK is a member of the MARK/PAR kinase subfamily of Ser/Thr kinases, some of which have been shown to directly phosphorylate MAP4 on KXGS motifs to reduce affinity for microtubules (5, 12). The MAP4 kinases in the heart have not been identified. It was recently demonstrated that AMPK can directly phosphorylate Ser-262 of the KXGS motif in the microtubule binding domain of tau, the neuronal MAP associated with Alzheimer’s disease (39). The 22 amino acids flanking Ser-914 in MAP4 and Ser-262 in tau are ~87% homologous (19/22). In support of direct phosphorylation of MAP4 by AMPK, we find that purified active AMPK can phosphorylate this same site in vitro. Because MAP4 phosphorylation was not completely removed in AMPK KO mice, it is likely that other kinases also regulate MAP4 phosphorylation in the heart. This could be due to AMPK α1 activity, or the activity of an AMPK-related kinase, such as MARK2. MARK2 exhibits reduced activity in response to right ventricular pressure overload in cats at the same time that MAP4 phosphorylation is reduced (3). However, in this mouse model, we did not observe reduced MARK2 activation (as indicated by phosphorylation at Ser-215 in the activation loop; not shown) in response to TAC. Although our results suggest a role for AMPK in cardiac MAP4 phosphorylation, it will be important to determine the role of other MARKs and AMPK-related kinases in cardiac MAP4 phosphorylation, as well as the counterpoised MAP4 phosphatases (PP2A), to acquire a more complete understanding of the signaling pathways that regulate MAP4 and cardiac MT dynamics.

In addition to phosphorylation of MAP4, AMPK may influence MT dynamics through MAP4-independent mechanisms. This is suggested by the reduction in MT stability by AICAR, even when MAP4 was eliminated using RNAi. AMPK has also been shown to regulate microtubule dynamics through phosphorylation of clip 170 (21). However, RNAi depletion of clip 170 did not result in obvious effects on MT stability (not shown). We did observe a slight reduction in free tubulin in cells treated with AICAR, while total tubulin was higher in AMPK KO mice after TAC. Persistently high levels of tubulin synthesis are necessary for MT growth and stability, so it is possible that AMPK reduction of tubulin heterodimer synthesis may also contribute to reduced MT levels. However, we still observe rapid MT destabilization in prehypertrophied cells, even as free or polymerized tubulin levels change only slightly, so other AMPK-dependent mechanisms of MT destabilization are also likely to be involved. While our data identify MAP4 phosphorylation as one mechanism by which AMPK activity may promote MT destabilization, additional experiments will be necessary to identify the MAP4-independent mechanisms of cardiac MT regulation by AMPK.

In summary, we have identified a new role for AMPK in limiting stabilization and accumulation of microtubules during pressure overload-induced cardiac hypertrophy. Future studies will be designed to determine whether treatments that activate cardiac AMPK can prevent microtubule accumulation during pressure overload, and the extent to which AMPK regulation of microtubule dynamics contributes to the cardioprotective effects of AMPK.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

AMPK REGULATION OF THE CARDIAC MICROTUBULE CYTOSKELETON


