Inhibition of Mammalian Target of Rapamycin With Rapamycin Reverses Hypertrophic Cardiomyopathy in Mice With Cardiomyocyte-Specific Knockout of PTEN

Xihui Xu, Nathan D. Roe, Mary C.M. Weiser-Evans, Jun Ren

Abstract—The role of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) in the maintenance of cardiac homeostasis still remains controversial. This study was designed to evaluate the role of cardiomyocyte-specific PTEN in the maintenance of cardiac homeostasis and the underlying mechanisms involved with a focus on autophagy, an evolutionarily conserved pathway for protein degradation. Cardiomyocyte-specific PTEN+/- α-myosin heavy chain Cre mice, henceforth referred to as CM-PTENKO, were generated by crossing the floxed PTEN mice with α-myosin heavy chain Cre mice driven by a Cre recombinase promoter. The adult PTEN-/- mice displayed the phenotype of established hypertrophic cardiomyopathy, including unfavorable geometric, functional, and histological changes. Furthermore, cardiomyocyte-specific PTEN knockout mice exhibited increased cardiac mammalian target of rapamycin although suppressed autophagy. Treatment with rapamycin (2 mg/kg per day, IP), an inhibitor of mammalian target of rapamycin, for 1 month effectively reversed the established hypertrophic cardiomyopathy in CM-PTENKO mice. With rapamycin treatment, autophagy activity was significantly restored in the heart of CM-PTENKO mice. Taken together, our results demonstrate an essential role for cardiomyocyte PTEN in maintaining cardiac homeostasis under physiological condition. Cardiomyocyte-specific deletion of PTEN results in the development of hypertrophic cardiomyopathy possibly through a mechanism associated with mammalian target of rapamycin hyperactivation and autophagy suppression. (Hypertension. 2014;63:729-739.) ● Online Data Supplement

Key Words: autophagy ▪ hypertrophic cardiomyopathy ▪ PTEN ▪ rapamycin

Cardiovascular disease is one of the leading causes of morbidity and mortality in the United States and other developed countries. Among such, hypertrophic cardiomyopathy, a genetically predisposed heart disease, contributes to the increased prevalence of heart failure and sudden cardiac death (or resuscitated cardiac arrest) in young adults. Ample evidence has confirmed the cause of hypertrophic cardiomyopathy mainly from mutations in genes encoding essential protein components of the sarcomere, namely β cardiac myosin heavy chain (MHC) and myosin binding protein C. However, approximately one fourth of hypertrophic cardiomyopathy may be attributed to mutations in other genes, most of which unidentified. Recent evidence has depicted an indispensable although conflicting role for phosphatase and tensin homolog deleted from chromosome 10 (PTEN) in the maintenance of cardiac homeostasis. PTEN depletion was shown to facilitate the development of cardiac hypertrophy accompanied with compromised cardiomyocyte contractile function although the underlying mechanism remains essentially elusive. However, loss of PTEN was demonstrated to be cardioprotective against pressure overload–induced cardiac hypertrophy and heart failure.

As a downstream target of PTEN, mammalian target of rapamycin (mTOR) activity has been demonstrated to be tightly regulated by PTEN through tuberous sclerosis complex (TSC). PTEN deficiency is capable of promoting hyperactivation of mTOR in animal models and patients. It is widely conceived that mTOR orchestrates cardiac hypertrophy given its role in governing protein synthesis. Aberrant activation of mTOR has been shown to contribute to the onset and development of cardiac hypertrophy. More importantly, inhibition of mTOR using rapamycin has shown some promise in the reversal of established cardiac hypertrophy in animal models. In addition to regulation of protein synthesis, mTOR also serves as the primary suppresser for autophagy, an evolutionarily conserved pathway for the maintenance of intracellular homeostasis. Macroautophagy, herein referred to as autophagy, is a catabolic process involved in recycling intracellular proteins and organelles and generation of ATP for energy homeostasis. In the past decade, accumulating

Received September 30, 2013; first decision October 21, 2013; revision accepted December 24, 2013.

From the Center for Cardiovascular Research and Alternative Medicine, School of Pharmacy, University of Wyoming College of Health Sciences, Laramie (X.X., N.D.R., J.R.); and Division of Renal Disease and Hypertension, Department of Medicine, University of Colorado Denver (M.C.M.W.-E.). The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.02526/-/DC1.

Correspondence to Jun Ren, Center for Cardiovascular Research and Alternative Medicine, University of Wyoming College of Health Sciences, Laramie, WY 82071. E-mail jren@uwyo.edu

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.113.02526

729
studies have consolidated an indispensable role for autophagy in the maintenance of cardiac geometry and function under both physiological and pathological conditions.\textsuperscript{22,23} It has been shown that basal levels of autophagy are essential for cardiac homeostasis under physiological conditions. Atg (autophagy-related gene) 5 deficiency results in loss of amino acids and ATP availability in the heart, thus leading to neonatal death.\textsuperscript{24} The indispensable role for basal autophagy in cardiac homeostasis is further consolidated by the fact that conditional knockout of Atg5 or Atg7 gene prompts cardiac hypertrophy and dampens cardiac function.\textsuperscript{25} To this end, the present study was designed to examine the role of autophagy, a downstream component of the PTEN–mTOR signaling cascade, in cardiomyocyte-specific deletion of PTEN–induced cardiac anomalies and more importantly the therapeutic potential of autophagy induction using rapamycin in PTEN deletion–induced cardiac defect, if any.

Materials and Methods
Generation of Cardiomyocyte-Specific PTEN Knockout Mice
All animal procedures performed in this study were approved by the Animal Care and Use Committee at the University of Wyoming (Laramie, WY) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Cardiomyocyte-specific PTEN\textsuperscript{flox/flox}/\textit{α}-MHC Cre mice, henceforth referred to as CM-PTENKO, were generated by crossing the floxed PTEN mice with \textit{α}-MHC Cre mice driven by a Cre recombinase promoter (Jackson Laboratories, Stock No. 009074, Bar Harbor, ME). The wild-type (WT) PTEN (+/−), heterozygous PTEN (+/+), and floxed PTEN (+/-) alleles were analyzed using polymerase chain reaction with tail DNA when mice were 4-week-old. The Cre recombinase transgene was analyzed using polymerase chain reaction as previously described.\textsuperscript{26} Only PTEN\textsuperscript{flox/flox} littermates negative for \textit{α}-MHC Cre were used as WT. WT and CM PTEN\textsuperscript{−/−} mice with or without rapamycin

![Figure 1](http://hyper.ahajournals.org/)

Figure 1. Generation of cardiomyocyte-specific phosphatase and tensin homolog deleted from chromosome 10 (PTEN) knockout mice. A, Representative polymerase chain reaction gel bands from mice containing floxed PTEN and the \textit{α}-myosin heavy chain (\textit{α}-MHC) Cre transgene. B, Representative gel blots depicting protein levels of PTEN in heart, skeletal (Sk) muscle, brain, and liver; protein tyrosine phosphatase 1B (PTP1B) and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase type 2 (SHIP2) in heart from CM-PTENKO mice. GAPDH was used as the loading control. C–H, Quantification of PTEN expression in the heart, Sk muscle, brain, and liver; PTP1B and SHIP2 in heart from CM-PTENKO mice. Mean±SEM, n=5 to 6 mice per group, *P<0.05 vs wild-type (WT) group. CM-PTENKO indicates cardiomyocyte-specific knockout of phosphatase and tensin homolog deleted from chromosome 10, \textit{α}-MHC Cre-PTEN\textsuperscript{flox/flox}.}
Mice were housed in a climate-controlled environment (22.8±2.0°C; 45%–50% humidity) with a 12/12 light/dark cycle with ad libitum access to tap water and regular diet (No. D12450B, Research Diets Inc, New Brunswick, NJ).

Additional methods are in the online-only Data Supplement.

Results

Generation of CM-PTENKO Mice

To generate CM-PTENKO mice, homozygous floxed PTEN mice were bred with the α-MHC Cre transgenic mice. Mice were born with few notable defects and survived to adulthood. The offspring were genotyped and screened for the recombinant allele using conventional polymerase chain reaction analysis of tail genomic DNA (Figure 1A). Western blot analysis further confirmed that majority of PTEN protein was depleted in the heart but not in any other organs from CM-PTENKO mice (Figure 1B–1F).

CM-PTENKO Causes Hypertrophic Cardiomyopathy in Mice

Although cardiomyocyte-specific knockout of PTEN did not affect survival in mice, it prompted a hypertrophic cardiomyopathy. The hearts of CM-PTENKO mice showed increased wall thickness and septal thickness compared to wild-type mice (Figure 2A–2E). Furthermore, CM-PTENKO mice had larger left ventricular end systolic diameter (LVESD), left ventricular end diastolic diameter (LVEDD), and fractional shortening (Figure 2B–2D).

Figure 2. Hypertrophic cardiomyopathy in CM-PTENKO mice. A–E, Echocardiographic indices in wild-type (WT) and CM-PTENKO mice, including left ventricular (LV) wall thickness, septal thickness, LV end systolic diameter (LVESD), LV end diastolic diameter (LVEDD), and fractional shortening. F, Representative gross images of hearts from WT and CM-PTENKO mice. G, Heart weight normalized to body weight in WT and CM-PTENKO mice. H, Representative cross-sectional images of cardiomyocytes stained with lectin (green). I, Quantitative analysis of cardiomyocyte cross-sectional (transverse) area (≈100 cells from 3–4 mice per group). J–L, Expression of cardiac fetal genes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) from WT and CM-PTENKO mice. The ratio of ∆∆Ct was analyzed using GAPDH as a control. M, Representative Masson Trichrome staining micrograph from WT and CM-PTENKO mice. N, Quantitative analysis of fibrosis area in each view area using measurement of ≈100 cells from 3 to 4 mice per group. O, Representative TUNEL staining of frozen LV sections from WT and CM-PTENKO mice. Triple immunofluorescence is used for labeling of nuclei (blue), TUNEL (green), and cardiomyocytes (red). Arrows indicate colocalization of nuclei and TUNEL-positive staining. P, Quantitative analysis of the percentage of TUNEL-positive nuclei measurement of ≈100 nuclei. Mean±SEM, n= 8 to 9 mice per group, *P<0.05 vs WT group. CM-PTENKO indicates cardiomyocyte-specific knockout of phosphatase and tensin homolog deleted from chromosome 10, α-MHC Cre-PTEN(flox/flox).
Hypertension phenotype in adult mice. Echocardiography analysis depicted that CM-PTENKO overtly increased left ventricular (LV) wall thickness, LV end diastolic diameter, and LV end systolic diameter and decreased fractional shortening (Figure 2A–2E). Concordantly, the heart-to-body weight ratio was significantly greater in CM-PTENKO mice compared with the WT mice (Figure 2F and 2G). Histological analysis further confirmed that cardiomyocyte-specific knockout of PTEN dramatically increased the cross-sectional area of cardiomyocytes (Figure 2H and 2I). Cardiomyocyte-specific knockout of PTEN–facilitated cardiac hypertrophy was substantiated by a dramatic increase in mRNA expression of cardiac fetal genes, including atrial natriuretic peptide, brain natriuretic peptide, and β-MHC, in CM-PTENKO mouse hearts (Figure 2J–2L).

In addition, we noted overt interstitial fibrosis in the heart of CM-PTENKO mice (Figure 2M and 2N). Furthermore, our immune-confocal microscopy analysis demonstrated that deletion of PTEN dramatically triggered cardiomyocyte apoptosis characterized by the TUNEL (in situ terminal dUTP nick end-labeling)-positive nuclei in cardiomyocytes stained with Desmin (Figure 2O and 2P). Consistently, protein level of the antiapoptotic marker Bcl-2 was significantly decreased, whereas the proapoptotic marker Bax was dramatically increased in CM-PTENKO mouse hearts (Figure 3A–3C). Next, we examined mitogen-activated protein kinase signaling cascade, considering its essential role in the development of pathological hypertrophic cardiomyopathy. Neither extracellular signal–regulated kinase 1/2 nor jun N-terminal kinase 1/2 was significantly activated in response to cardiomyocyte-specific knockout of PTEN (Figure 3A, 3D, 3E, 3F, 3G, 3H, 3I, 3J, and 3K), reminiscent of the previous finding. To further consolidate a role of PTEN in the regulation of cardiomyocyte function, mechanical properties were evaluated in cardiomyocytes isolated from CM-PTENKO and WT mice. Although loss of PTEN failed to significantly increase cell length in cardiomyocytes, it dramatically dampened cardiomyocyte contractile function, as evidenced by decreased velocity of shortening and relengthening (±dL/dt), peak shortening, and prolonged time-to-90% relengthening without affecting time-to-peak shortening (Figure S1A–S1F in the online-only Data Supplement). To further explore the possible mechanism(s) behind impaired contractile dysfunction in PTEN−/− cardiomyocytes, intracellular Ca2+ handling was evaluated. Our data revealed that loss of PTEN failed...
to affect basal level of intracellular Ca$^{2+}$ although it dramatically disturbed intracellular Ca$^{2+}$ homeostasis as manifested by decreased intracellular Ca$^{2+}$ release in response to electric stimulus (AFFI [fura-2 fluorescence intensity]) and prolonged intracellular Ca$^{2+}$ decay in cardiomyocytes (Figure S1G–S1I).

Next, Western blot analysis was performed to examine the essential intracellular Ca$^{2+}$ regulatory proteins, including sarco/endoplasmic reticulum Ca$^{2+}$-ATPase type 2a and phospholamban. Our data showed that protein expression of sarco/endoplasmic reticulum Ca$^{2+}$-ATPase type 2a was significantly decreased, whereas phospholamban was markedly increased in CM-PTENKO mouse hearts (Figure 3A, 3L, and 3M).

PTEN Deficiency in Cardiomyocytes Induces Akt–mTOR Signaling Pathway Activation

Earlier study has indicated hyperactivated cardiac PKB (protein kinase B)/Akt signaling in the muscle-specific knockout of PTEN en route to cardiac hypertrophy. Coincidentally, Akt phosphorylation, but not pan protein expression, was found to be elevated in the heart of CM-PTENKO mice (Figure 4A–4C). Next, myocardial TSC2, a direct downstream target of Akt, was evaluated. Our data revealed that PTEN deletion dramatically increased myocardial TSC2 phosphorylation/activation, without affecting the pan protein expression of TSC2 (Figure 4A, 4D, and 4E). Subsequently, myocardial mTOR, which is directly regulated by TSC, was hyperactivated in CM-PTENKO heart, as manifested by elevated phosphorylation of mTOR and pS6K (Figure 4A, 4F, and 4H). Pan protein expression of mTOR and S6K was unaffected by cardiomyocyte-specific deletion of PTEN (Figure 4A, 4G, and 4I).

Cardiomyocyte-Specific Depletion of PTEN Inhibits Myocardial Autophagy Pathway

Given that mTOR serves as the primary inhibitory regulator of autophagy, we evaluated autophagy in the heart from WT
and CM-PTENKO mice. As an important mediator governing initiation of autophagy, the activity of uncoordinated 51-like kinase 1 (Ulk1) was suppressed by mTOR through Ser757 phosphorylation.28 As expected, our data displayed that loss of PTEN markedly increased the phosphorylation of Ulk1 at Ser757 in the heart (Figure 5A and 5B). Total protein level of Ulk1 was unaffected by PTEN knockout (Figure 5A and 5C).

The formation of microtubule-associated protein light chain 3 B type II, a mammalian homolog of yeast Atg8, is one of the most widely used markers for autophagosomes.29 A dramatic decrease in light chain 3 B type II was observed in the heart from CM-PTENKO mice (Figure 5A, 5E, and 5F). Because the decrease in autophagosome formation may have resulted from either a decrease in the generation of autophagosome (inhibited initiation) or an increase of autophagosome degradation (facilitated clearance),29 we next examined the level of p62, a selective autophagy cargo adaptor protein.30 A significant accumulation of p62 was noted in the heart of CM-PTENKO mice (Figure 5A and 5G). Furthermore, our data revealed that myocardial levels of Beclin1 and Atg7 were downregulated in CM-PTENKO mice (Figure 5A, 5H, and 5I). Taken together, these findings favor the notion that cardiomyocyte-specific knockout of PTEN suppresses myocardial autophagy activity (dampened initiation and degradation).

Rapamycin Rescues Hypertrophic Cardiomyopathy in CM-PTENKO Mice

To further consolidate a role of autophagy in cardiomyocyte-specific PTEN deletion–induced cardiac anomalies, rapamycin, a widely used specific mTOR complex 1 inhibitor,31 was administered to promote autophagy (2 mg/kg per day, IP for 30 days).15,16 Our data revealed that chronic treatment with rapamycin dramatically improved cardiac geometry and function in CM-PTENKO mice without affecting cardiac geometry in WT mice (Figure 6A–6E). Rapamycin treatment reversed cardiac hypertrophy developed in CM-PTENKO mice without affecting cardiac geometry in WT mice (Figure 6F and 6G). To further confirm that rapamycin ameliorated loss of PTEN-induced cardiac hypertrophy, cross-sectional area in cardiomyocytes was evaluated. Our results indicated that rapamycin dramatically attenuated the cross-sectional area of cardiomyocytes in CM-PTENKO mice without affecting that in WT mice.
Next, our real-time polymerase chain reaction data revealed that although rapamycin did not affect myocardial fetal gene expression in WT mouse hearts, it significantly alleviated increased mRNA expression of atrial natriuretic peptide, brain natriuretic peptide, and β-MHC in CM-PTENKO mouse hearts (Figure 6J–6L). In addition, Masson trichrome staining was examined to evaluate interstitial fibrosis. Loss of PTEN-induced cardiac interstitial fibrosis was significantly attenuated by the treatment with rapamycin. Rapamycin treatment did not affect cardiac fibrosis in WT mice (Figure 6M and 6N).

Consistent with echocardiographic findings, our results showed that rapamycin treatment effectively rescued PTEN deletion–induced cardiomyocyte contractile dysfunction (Figure S2A–S2F). Furthermore, analysis of intracellular Ca²⁺ handling further consolidated a beneficial effect of rapamycin against PTEN deletion–induced intracellular Ca²⁺ abnormalities in cardiomyocytes. In line with the cardiomyocyte mechanical responses, rapamycin treatment failed to overly affect intracellular Ca²⁺ handling property in cardiomyocytes from WT mice (Figure S2G–S2I).

Rapamycin Restores Myocardial mTOR-Mediated Autophagy Activity in CM-PTENKO Mice

To examine the possible mechanisms underlying the cardioprotective effect of rapamycin in CM-PTENKO mice, myocardial autophagy was evaluated using Western blot analysis. Our data revealed that treatment with rapamycin significantly inhibited the activity of mTOR pathway in the heart from WT and CM-PTENKO mice, as evidenced by reduced levels of phosphor-mTOR and phosphor-S6K (Figure 7A, 7B, and 7D). Not surprisingly, inhibition of mTOR promoted basal autophagy in the heart from WT mice, as evidenced by increased expression of Atg7, Beclin1, and light chain 3 B type II, and decreased accumulation of phosphor-Ulk1 and p62 (Figure 7A, 7F, 7G, 7H, 7I, 7J, 7K, 7L, and 7M). Importantly, on rapamycin treatment, the inhibited autophagy pathway in the heart from CM-PTENKO mice was dramatically ameliorated.
Restoration of Cardiac Autophagy With Rapamycin Treatment Attenuated Myocardial Apoptosis in CM-PTENKO mice

We went on to examine the effect of rapamycin treatment on apoptosis in the heart using TUNEL staining and Western blot analysis. As shown in Figure 8, the loss of PTEN significantly promoted cardiomyocyte apoptosis as evaluated by both TUNEL staining and Western blot analysis, the effects of which were mitigated rapamycin. Rapamycin treatment did not affect cardiomyocyte apoptosis by itself.

Discussion

The salient findings from our current study suggested that cardiomyocyte-specific PTEN deletion triggers pronounced hypertrophic cardiomyopathy (echocardiographic remodeling and dampened myocardial function) probably through inhibition of basal myocardial autophagy. In particular, cardiomyocyte-specific knockout of PTEN significantly increases cardiomyocyte cross-sectional area, fetal gene expression, interstitial fibrosis, and apoptosis in the heart. Furthermore, cardiomyocyte-specific PTEN deletion compromises cardiomyocyte contractile function, intracellular Ca\textsuperscript{2+} handling, and intracellular Ca\textsuperscript{2+} regulatory protein expression. Our study depicted a role of mTOR-mediated autophagy in the indispensable action of PTEN in the maintenance of cardiac homeostasis. More importantly, our results provided the proof-of-concept evidence for mTOR-dependent autophagy in PTEN-governed cardiac homeostasis as treatment with rapamycin, a specific inhibitor for mTOR, restored hypertrophic cardiomyopathy and myocardial autophagy in CM-PTENKO mice. Last but not least, treatment with rapamycin significantly attenuated cardiomyocyte apoptosis in the heart of CM-PTENKO mice.

Earlier evidence has depicted an indispensable role for PTEN in the maintenance of cardiac homeostasis and myocardial contractile function.\textsuperscript{6} However, the precise role for PTEN in the regulation of cardiac geometry and function still remains controversial.\textsuperscript{6,7} However, recent evidence from Oudit et al showed that deletion of PTEN may be beneficial...
against pressure overload–induced heart failure probably through preserving myocardial angiogenesis and metabolic genes.\(^7\) In these earlier studies, the muscle-specific conditional PTEN knockout mice were used to elucidate the role of PTEN in the heart.\(^6,7\) To better understand the role of PTEN in the maintenance of cardiac homeostasis, we generated a cardiomyocyte-specific PTEN knockout mouse model. Reminiscent of previous reports,\(^6\) our data demonstrated that loss of PTEN prompts development of hypertrophic cardiomyopathy and cardiomyocyte contractile dysfunction.\(^6,7\) Cardiomyocyte-specific loss of PTEN leads to significantly increased LV wall thickness, LV end systolic diameter, and LV end diastolic diameter and reduced fractional shortening. Consistently, cardiomyocyte-specific PTEN deletion increases cardiomyocyte cross-sectional area, interstitial fibrosis, and cardiomyocyte apoptosis. More importantly, loss of PTEN triggers cardiomyocyte intracellular Ca\(^{2+}\) handling and contractile anomalies, reminiscent of earlier report.\(^6\) Our observation further revealed hyperactivated myocardial PI3K (phosphatidylinositide 3-kinases)–Akt signaling cascade with cardiomyocyte-specific loss of PTEN, as manifested by Akt phosphorylation. Our data further depicted an essential role of mTOR in the development of hypertrophic cardiomyopathy in CM-PTENKO mice. In our hands, basal mTOR activity is overtly elevated in the heart from CM-PTENKO mice. As the direct upstream regulator of mTOR, TSC2 phosphorylation is also significantly elevated in the heart of CM-PTENKO mice. It has been shown that mTOR is tightly regulated by the TSC1/2 complex, whereas loss of TSC1/2 activity (through phosphorylation) directly results in mTOR hyperactivation.\(^13\)

The activity of mTOR is influenced by various molecular modulators, among which Akt acts as the primary drive.\(^10\) Through phosphorylation of TSC2, Akt interrupts the ability of TSC2 to inhibit mTOR signaling, leading to phosphorylation of mTOR.\(^10,32\) Accumulating studies have suggested that mTOR is a key regulator for cell size in various organs including heart.\(^5,15–17,33\) Loss of TOR has been shown to reduce cell size in mosaic Drosophila.\(^33\) In various models of cardiac hypertrophy, the activity of mTOR is found to be significantly elevated.\(^16,19\) More importantly, treatment with rapamycin, a specific mTOR inhibitor, may effectively reverse cardiac hypertrophy induced by various triggers including pressure overload, adrenergic receptor agonist phenylephrine, and genetic mutation.\(^5,15–17\)

Observation from our present study favored a unique role for mTOR in PTEN depletion–induced hypertrophic cardiomyopathy. A pivotal role of mTOR is appreciated not only in the process of protein synthesis but also in autophagy.\(^11,31,34\) Autophagy is an evolutionarily conserved pathway involved in bulk degradation of intracellular organelles and proteins.\(^22,23\)
Over the past decades, the role of autophagy in the governance of cardiac homeostasis under physiological and pathophysiological conditions has been extensively explored. Under physiological conditions, it is conceived that basal autophagy prevents abnormal protein aggregation and degrades long-lived proteins and organelles, providing free amino acids and fatty acids for the maintenance of cellular ATP levels and anabolic pathways. The indispensable role of basal autophagy in the maintenance of cardiac geometry and function is highly appreciated by development of cardiac hypertrophy and dysfunction on inactivation of either Atg5 or Atg7 autophagy gene. Furthermore, neonatal mice deficient in Atg5 displayed less availability of amino acid and ATP in the heart and plasma, thus provoking embryonic lethality within a day after birth. Our data revealed suppressed myocardial autophagy in hearts from CM-PTENKO mice in an mTOR-dependent manner. Treatment with rapamycin rescued myocardial autophagy in the heart of CM-PTENKO mice. Through forming a ternary complex with the FK506-binding protein 12 and the FRB (FKBP12-rapamycin-binding) domain of mTOR, rapamycin is capable of inhibiting the activity of mTOR complex 1. mTOR complex 1 is an inhibitor of autophagy probably through phosphorylation of the Atg1–Ulk1 complex. Treatment with rapamycin has been widely used for autophagy induction through inhibiting mTOR activity in various organ systems including brain, liver, and heart. Autophagy induction by rapamycin has been shown to be an avenue in the therapeutic remedies against cardiac dysfunction under several pathological conditions such as aging and LMNA (lamin A and C) cardiomyopathy. PTEN was previously demonstrated to function as a mechanosensing signal transducer for myosin II in Dictyostelium cells. Although it is beyond the scope of our current study, other possible mechanisms such as mechanosensing may not be excluded at this time.

In conclusion, our study provides evidence for the first time that mTOR complex 1–mediated autophagy suppression contributes to PTEN deletion–triggered hypertrophic cardiomyopathy. This is supported by the observation that myocardial autophagy is suppressed in the heart of CM-PTENKO mice and that treatment with rapamycin restored myocardial autophagy and rescued hypertrophic cardiomyopathy. Although it is somewhat premature to consolidate a unique role of autophagy in the cardiomyocyte-specific PTEN deletion–induced hypertrophic cardiomyopathy, our results should shed some lights toward a better understanding for the role of PTEN in the maintenance of myocardial geometry and function. Human studies have confirmed the presence of PTEN haploinsufficiency. Findings from our current study suggest that individuals with PTEN deficiency may be prone to the development of hypertrophic cardiomyopathy. More importantly, rapalogs, derivatives of rapamycin, are in clinical practice for treatment of human diseases. Our findings have shown promise in clinical therapeutic strategy using rapamycin in the management of hypertrophic cardiomyopathy possibly resulting from PTEN mutations.

Perspectives

The findings from our study revealed overt hypertrophic cardiomyopathy in adult mice with cardiomyocyte-specific knockout of PTEN. PTEN deficiency increases cardiac mTOR and suppresses autophagy. Treatment with rapamycin, a specific mTOR inhibitor and an autophagy inducer, effectively rescued the established hypertrophic cardiomyopathy in CM-PTENKO mice. Currently, derivatives of rapamycin are used in clinical practice for treatment of human diseases. Human studies have indicated the presence of PTEN haploinsufficiency. It is likely that stimulation of autophagy with rapamycin may possess potential clinical use in the treatment of hypertrophic cardiomyopathy possibly resulting from PTEN mutations.

Sources of Funding
This work was supported in part by grants from the National Center for Research Resources (SP20RR016474-12) and the National Institute of General Medical Sciences (8P20GM103432-12).

Disclosures
None.

References

Xu et al  PTEN, Hypertrophic Cardiomyopathy, and Rapamycin  739


Novelty and Significance

**What Is New?**

- For the first time, we generated mice with cardiomyocyte-specific knockout of phosphatase and tensin homolog deleted from chromosome 10 (PTEN), α-myosin heavy chain Cre-PTEN<sup>fl/fl</sup> (CM-PTENKO).
- The adult PTEN<sup>−/−</sup> mice develop cardiac hypertrophy and cardiac dysfunction probably by inhibiting cardiac autophagy in mammalian target of rapamycin–dependent way.
- Administration with rapamycin, an inhibitor of mammalian target of rapamycin and inducer of autophagy, reverses the established hypertrophic cardiomyopathy in CM-PTENKO mice.

**What Is Relevant?**

- Cardiomyocyte PTEN regulates autophagy through inhibiting mammalian target of rapamycin to maintain cardiac homeostasis.

**Inhibiting mammalian target of rapamycin with rapamycin reverses the established hypertrophic cardiomyopathy in CM-PTENKO mice.**

**Summary**

Induction of autophagy with rapamycin may be a potential therapeutic strategy for the treatment of hypertrophic cardiomyopathy in patients with PTEN haploinsufficiency.
Inhibition of Mammalian Target of Rapamycin With Rapamycin Reverses Hypertrophic Cardiomyopathy in Mice With Cardiomyocyte-Specific Knockout of PTEN
Xihui Xu, Nathan D. Roe, Mary C.M. Weiser-Evans and Jun Ren

_Hypertension_. 2014;63:729-739; originally published online January 20, 2014;
doi: 10.1161/HYPERTENSIONAHA.113.02526

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/4/729

An erratum has been published regarding this article. Please see the attached page for:
/content/67/3/e5.full.pdf

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/01/20/HYPERTENSIONAHA.113.02526.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org/subscriptions/
In the article by Xu et al (Xu X, Roe ND, Weiser-Evans MCM, Ren J. Inhibition of mammalian target of rapamycin with rapamycin reverses hypertrophic cardiomyopathy in mice with cardiomyocyte-specific knockout of PTEN. *Hypertension*. 2014;63:729–739. doi: 10.1161/HYPERTENSIONAHA.113.02526), which published online ahead of print January 20, 2014, and appeared in the April 2014 issue of the journal, a correction was needed.

The authors of the above-mentioned paper noticed an error in Figure 1. During the assembly of representative gel bands, a single GAPDH band was mistakenly used to normalize PTEN levels and represent loading control for various tissues. The summary graphs have been updated using the correct GAPDH gel bands for each individual tissue type and the conclusion remains unchanged. The authors sincerely regret the error and wish to thank American Heart Association for publishing the correction. The corrected figure is shown below.

This correction has been made to the current online version of the article, which is available at http://hyper.ahajournals.org/content/63/4/729.full.

(Hypertension. 2016;67:e5. DOI: 10.1161/HYP.000000000000040.)
© 2016 American Heart Association, Inc.
*Hypertension* is available at http://hyper.ahajournals.org DOI: 10.1161/HYP.000000000000040
Supplementary Materials

Inhibition of mTOR with Rapamycin Reverses Hypertrophic Cardiomyopathy in Mice with Cardiomyocyte-Specific Knockout of PTEN

Xihui Xu1, Nathan D. Roe1, Mary C.M. Weiser-Evans2, Jun Ren1
1Center for Cardiovascular Research and Alternative Medicine, School of Pharmacy, University of Wyoming College of Health Sciences, Laramie, WY 82071 USA; 2Department of Medicine, Division of Renal Disease and Hypertension, University of Colorado Denver, Denver, CO 80262 USA

Running title: PTEN, Hypertrophic Cardiomyopathy, Rapamycin

Supplemental Experimental Procedures

METHODS

Echocardiographic assessment: Cardiac geometry and function were evaluated in anesthetized (ketamine 80 mg/ kg and xylazine 12 mg/ kg, i.p.) mice using the two-dimensional guided M-mode echocardiography (Philips SONOS 5500) equipped with a 15-6 MHz linear transducer (Phillips Medical Systems, Andover, MD). The chests were shaved and mice were placed in a shallow left lateral position on a heating pad. Using the 2-dimensional (2-D) parasternal short-axis image obtained at a level close to papillary muscles as a guide, a 2-D guided M-mode trace crossing the anterior and posterior wall of LV was obtained at a sweep speed of 50 mm/sec. The echocardiographer was blind to the treatment of the mice. Caution was taken to avoid excessive pressure over the chest, which could induce bradycardia and deformation of the heart. Left ventricular (LV) anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode using method adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (EDD) and end-systolic (ESD) diameters using the equation (EDD-ESD)/EDD*100. Heart rates were averaged over 10 consecutive cycles.

Isolation of murine cardiomyocytes: Hearts were rapidly removed from anesthetized mice and mounted onto a temperature-controlled (37°C) Langendorff system. After perfusion with a modified Tyrode's solution (Ca²⁺ free) for 2 min, the heart was digested with a Ca²⁺-free KHB buffer containing liberase blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN) for 20 min. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, MgCl₂ 1.0, HEPES 10, NaH₂PO₄ 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO₂–95% O₂. The digested heart was then removed from the cannula and left ventricle was cut into small pieces in the modified Tyrode's solution. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca²⁺ was added incrementally back to 1.20 mM over 30 min. A yield of at least 60-70% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Only rod-shaped myocytes with clear edges were selected for contractile and intracellular Ca²⁺ studies.
Cell shortening/relengthening: Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA). IonOptix SoftEdge software was used to capture changes in cardiomyocyte length during shortening and re-lengthening. In brief, cardiomyocytes were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (1 ml/min at 25 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with supra-threshold voltage at a frequency of 0.5 Hz (unless otherwise stated), 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS) – the amplitude myocytes shortened on electrical stimulation, which is indicative of peak ventricular contractility; time-to-PS (TPS) – the duration of myocyte shortening, which is indicative of contraction duration; time-to-90% relengthening (TR$_{90}$) – the duration to reach 90% re-lengthening, which represents cardiomyocyte relaxation duration (90% rather 100% re-lengthening was used to avoid noisy signal at baseline concentration); and maximal velocities of shortening (+ dL/ dt) and relengthening (− dL/dt) – maximal slope (derivative) of shortening and relengthening phases, which are indicative of maximal velocities of ventricular pressure rise/fall.

Intracellular Ca$^{2+}$ transient measurement: Cardiomyocytes were loaded with fura-2/AM (0.5 µM) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Cardiomyocytes were placed on an Olympus IX-70 inverted microscope and imaged through a Fluor × 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca$^{2+}$ concentration were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths (360/380). Fluorescence decay time was measured as an indication of the intracellular Ca$^{2+}$ clearing rate. Both single and bi-exponential curve fit programs were applied to calculate the intracellular Ca$^{2+}$ decay constant.

Histological examination: Histological analysis was performed as described. Following anesthesia, hearts were arrested in diastole with saturated KCl, excised and fixed in 10% neutral-buffered formalin at room temperature for 24 hrs. The specimen was processed through graded alcohols, cleared in xylenes, embedded in paraffin, serial sections were cut at 5 µm and stained with FITC-tagged wheat germ agglutinin to examine cardiomyocyte size and Masson’s trichrome to evaluate interstitial fibrosis. Cardiomyocyte cross-sectional areas in cells with clear myofiber outlines and collagen volume fraction were measured on a digital microscope (x 400) using the Image J (version1.34S) software.
**TUNEL staining:** Mouse hearts were frozen immediately after euthanasia, and 7 μm thickness sections were obtained using a Leica, cryomicrotome (Model CM3050S, Leica Microsystems, Buffalo Grove, IL). Sections were stained with *in situ* terminal dUTP nick end-labeling (TUNEL) staining kit (Roche Diagnostics Corporation, 11684795910) to detect apoptotic cells according to the manufacturer’s instructions. Cardiomyocytes were further stained with Desmin antibody (Cell Signaling Technology, #4042). Nuclei were stained with DAPI. After coverslipped, the cells were imaged with an inverted laser-scanning confocal microscope at ×100 magnification (Zeiss 710, Thornwood, NY). The percentage of TUNEL positive nuclei was quantified using ImageJ software.

**Western blot analysis:** Murine hearts, brain, liver and skeletal (Sk) muscle were flash-frozen in liquid nitrogen and stored at -80 °C before protein extraction. For protein extraction, heart tissues were homogenized and sonicated in RIPA buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Roche Diagnostics). Heart homogenates containing equal amount of proteins were resolved by SDS-polyacrylamide gels in a mini-gel apparatus (Mini-PROTEAN II, Bio-Rad) and the proteins were transferred to nitrocellulose membranes, incubated overnight with primary antibody at 4°C. After being washed 3 times, the membrane was incubated with horseradish peroxidase (HRP)-coupled secondary antibody for 1 hr at room temperature. The membrane was washed again for 3 times 10 min each time, and the signal was detected quantified with a Bio-Rad Calibrated Densitometer and the intensity of immunoblot bands was normalized to that of GAPDH. For reprobing, membranes were tripped with 50mmol/ L Tris-HCl, 2% SDS and 0.1 mol/ L β-mercaptoethanol (pH 6.8). Polyclonal rabbit antibodies against PTEN, SHIP2, PTP1B, Bcl-2, Bax, phosphorylated ERK at Thr202/Tyr204, ERK, phosphorylated JNK at Thr183/Tyr185, JNK, SERCA2a, PLB, phosphorylated AMPK (pAMPKα) at Thr172, total AMPKα, phosphorylated mTOR (pmTOR) at Ser2448, total mTOR, Atg5, Atg7, Beclin 1, LC3B, and GAPDH (1: 1,000; Rabbit; Cell Signaling Technology, Danvers, MA); and p62 (1: 1,000; Guinea Pig; Enzo Life Sciences, Plymouth Meeting, PA) were examined by standard western immunoblotting. Membranes were probed respective antibodies with GAPDH or α-tubulin serving as the loading control.

**mRNA Analysis by Real-time PCR:** RNA was isolated from frozen shole heats using TRIZol® regent (Invitrogen) and RNAeasy (Qiagen). cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad). Concentration and purity of the RNA were assessed by spectrophotometry (SmartSpec™ 3000 Spectrophotometer; Bio-Rad). Quantitative real-time PCR was performed with iQ™ SYBR® Green Supermix (Bio-Rad). Gene expression analyses for ANP, BNP and β-MHC were carried out according to the manufacturer’s instructions. Each measurement was obtained from 6 mice per group, and each reaction was duplicated. GAPDH expression was employed as the control. Comparative C_T method was used for data quantification and analysis.

**Real-time PCR Primers and Information**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP -FWD</td>
<td>5’ – ACA GCC AAG GAG GAA AAG GC – 3’</td>
</tr>
<tr>
<td>ANP -REV</td>
<td>5’ – CCA CAG TGG CAA TGT GAC CA – 3’</td>
</tr>
</tbody>
</table>
PCR primers were commercially purchased from INTEGRADED DNA TECHNOLOGIES (IA, USA) 4.

Administration of rapamycin: Rapamycin treatment was performed as previously described 4-6. In brief, rapamycin (LC Laboratories) was dissolved in ethanol and resuspended in vehicle (0.25% PEG, 0.25% Tween-80) at a final concentration of 1 mg/ ml. Rapamycin (i.p., 2 mg/kg/d) or vehicle was given to adult male CM-PTENKO and WT mice (12 weeks old) for 4 weeks. The dose of rapamycin and treatment course were based on the literatures in which rapamycin was used to reverse mouse cardiac hypertrophy 4-6.

Data analysis: Data were expressed as Mean ± SEM. Statistical significance (p < 0.05) was estimated by one-way analysis of variation (ANOVA) followed by a Tukey’s post hoc test or a 2-way repeated-measures of ANOVA where appropriate. All statistics was performed with GraphPad Prism 4.0 software (GraphPad, San Diego, CA).

REFERENCES

Fig. S1: Contractile and intracellular Ca$^{2+}$ handling properties of cardiomyocytes isolated from WT and CM PTENKO mice. A: Resting cell length; B: Maximal velocity of shortening (+ dL/dt); C: Maximal velocity of relengthening (− dL/dt); D: Peak shortening (PS, normalized to resting cell length); E: Time-to-90% relengthening ($T_{R90}$); F: Time-to-PS (TPS); G: Resting fura-2 fluorescence intensity (FFI); H: Electrically-stimulated rise in FFI (ΔFFI); and I: Single exponential intracellular Ca$^{2+}$ decay rate. Mean ± SEM, n = 100 – 130 cells from 5 mice per group, * p < 0.05 vs. WT group.
Fig. S2: Contractile and intracellular Ca\(^{2+}\) handling properties of cardiomyocytes isolated from WT and CM PTENKO mice treated with or without rapamycin. A: Resting cell length; B: Maximal velocity of shortening (+ dL/dt); C: Maximal velocity of relengthening (− dL/dt); D: Peak shortening (PS, normalized to resting cell length); E: Time-to-90% relengthening (TR\(_{90}\)); F: Time-to-PS (TPS); G: Resting fura-2 fluorescence intensity (FFI); H: Electrically-stimulated rise in FFI (ΔFFI); and I: Single exponential intracellular Ca\(^{2+}\) decay rate. Mean ± SEM, n = 100 – 130 cells from 5 mice per group, * p < 0.05 vs. WT group.