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Polycystin-1 is a Cardiomyocyte Mechanosensor That Governs L-type Ca^{2+} Channel Protein Stability

Zully Pedrozo, PhD[1,2,3], **Alfredo Criollo, PhD**[1,4], **Pavan K. Battiprolu, PhD**[1], **Cyndi R. Morales, BS**[1], **Ariel Contreras, PhD**[2], **Carolina Fernández, MSc**[2], **Nan Jiang, MSc**[1], **Xiang Luo, MD, PhD**[1], **Michael J. Caplan, MD, PhD**[5], **Stefan Somlo, MD**[6,7], **Beverly A. Rothermel, PhD**[1,8], **Thomas G. Gillette, PhD**[1], **Sergio Lavandero, PhD**[1,2,3], and **Joseph A. Hill, MD, PhD**[1,8]

[1]Division of Cardiology, Department of Internal Medicine, UT Southwestern Medical Center; Dallas, TX

[2]Advanced Center for Chronic Diseases (ACCDiS) & Centro de Estudios Moleculares de la Célula (CMEC), Facultad de Medicina & Facultad de Ciencias Químicas y Farmacéuticas, Santiago, Chile

[3]Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

[4]Instituto de Investigación en Ciencias Odontológicas, Facultad de Odontología, Universidad de Chile, Santiago, Chile

[5]Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT

[6]Department of Internal Medicine, Yale University School of Medicine, New Haven, CT

[7]Department of Genetics, Yale University School of Medicine, New Haven, CT

[8]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX

Abstract

Background—L-type calcium channel (LTCC) activity is critical to afterload-induced hypertrophic growth of the heart. However, mechanisms governing mechanical stress-induced activation of LTCC activity are obscure. Polycystin-1 (PC-1) is a G-protein-coupled receptor-like protein that functions as a mechanosensor in a variety of cell types and is present in cardiomyocytes.

Methods and Results—We subjected neonatal rat ventricular myocytes (NRVMs) to mechanical stretch by exposing them to hypo-osmotic (HS) medium or cyclic mechanical stretch, triggering cell growth in a manner dependent on LTCC activity. RNAi-dependent knockdown of PC-1 blocked this hypertrophy. Over-expression of a C-terminal fragment of PC-1 was sufficient

Correspondence: Joseph A. Hill, MD, PhD, Cardiology Division, Department of Internal Medicine, UT Southwestern Medical Center, 6000 Harry Hines Blvd, Dallas, TX 75390-8573, Phone: 214-648-1400, Fax: 214-648-1450, joseph.hill@utsouthwestern.edu or: Sergio Lavandero, PhD, Advanced Center for Chronic Diseases (ACCDiS), Facultad de Ciencias Químicas & Facultad de Medicina, Universidad de Chile, Olivos 1007, Santiago 8380492, Chile, Phone/Fax: +562-2978 2919, slavander@uchile.cl.

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to trigger NRVM hypertrophy. Exposing NRVMs to HS medium resulted in an increase in α1C protein levels, a response that was prevented by PC-1 knockdown. MG132, a proteasomal inhibitor, rescued PC-1 knockdown-dependent declines in α1C protein. To test this *in vivo*, we engineered mice harboring conditional silencing of PC-1 selectively in cardiomyocytes (PC-1 KO) and subjected them to mechanical stress *in vivo* (transverse aortic constriction, TAC). At baseline, PC-1 KO mice manifested decreased cardiac function relative to littermate controls, and α1C LTCC protein levels were significantly lower in PC-1 KO hearts. Whereas control mice manifested robust TAC-induced increases in cardiac mass, PC-1 KO mice showed no significant growth. Likewise, TAC-elicited increases in hypertrophic markers and interstitial fibrosis were blunted in the knockout animals

Conclusions—PC-1 is a cardiomyocyte mechanosensor and is required for cardiac hypertrophy through a mechanism that involves stabilization of α1C protein.

Keywords

cardiac hypertrophy; polycystin-1; mechanosensor; mechanical stress; L-type calcium channel

Introduction

Living cells actively sense, integrate, and convert mechanical stimuli into biochemical signals, triggering intracellular responses.[1] In cardiomyocytes, short-term exposure to mechanical stress (MS) results in increased cardiac contractility, while long-term stresses are met by structural changes in the myocardium itself.[1] Owing to the limited regenerative potential of cardiomyocytes, cellular hypertrophy, not hyperplasia, is a major mechanism by which these cells cope with increased hemodynamic load.[1, 2] This pathological growth is provoked by abnormal metabolic, structural, and functional events that occur in hypertension, valvular heart disease, myocardial infarction, or mutations in genes coding for contractile proteins.[1, 3] However, mechanisms in cardiomyocytes underlying the transduction of extracellular mechanical signals into intracellular events (mechanotransduction) are poorly characterized. Polycystin-1 (PC-1) is the protein product of the major gene underlying autosomal dominant polycystic kidney disease (PKD), *Pkd1*.[4] Eight polycystin genes comprise a novel family of membrane-associated proteins.[4] Both PC-1 and polycystin-2 (PC-2) are expressed in many different tissues, but their function has been mainly characterized in primary cilia in kidney cells.[5, 6] It is also widely accepted that these proteins act as a complex, where PC-2 functions as a Ca^{2+} channel and PC-1 is a sensor of shear stress. However, PC-1 and PC-2 can operate independently.[7, 8] In fact, PC-1 can act as a mechanosensor independent of shear stress.[9–11] L-type Ca^{2+} channel (LTCC) activity plays a key role in the cardiomyocyte response to MS.[12] Yet, how MS regulates LTCC function and which mechanosensor(s) are involved in the heart are unclear.

Mechanisms of mechanotransduction in heart are poorly understood. Further, in cardiomyocytes, it is unknown what role, if any, PC-1 plays in physiological and pathological events. That being said, PC-1 has been implicated in pathways central to the development of cardiac hypertrophy, such as mTOR and calcineurin-NFAT[17]. Therefore, we set out to explore a possible role for PC-1 in the development of pressure overload-induced cardiac hypertrophy.

Materials and Methods

All studies conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by the Institutional Ethics Review Committees of the University of Texas Southwestern Medical Center and Universidad de Chile.

Cardiomyocyte culture and transfections

NRVMs were isolated from the ventricles of Sprague-Dawley rat pups on postnatal day 1–2. Cells were preplated to enrich for cardiomyocytes, plated and cultured for 24 hours in DMEM/M199 (3:1) containing 5% FBS, 10% horse serum, and 100 μmol/L BrdU (Sigma-Aldrich).[13] For PC-1 knockdown (PC-1 KD), NRVMs were transfected overnight with two sequence-independent siRNAs specific for PC-1 (120 nmol/L, Sigma-Aldrich) with oligofectamine (Invitrogen). Decreases in PC-1 mRNA and protein were optimized in terms of both concentration (60 to 200 nmol/L) and time (6, 12, 24 and 48 hours). Stimuli were applied 24 hour post-transfection. For lentivirus-mediated over-expression of PC-1 C-terminal peptide, cells were infected at a multiplicity of infection of 15 plaque-forming units per cell and subsequently harvested in Trizol or T-PER buffer (Thermo Scientific) containing phosphatase and protease inhibitors (Roche) for harvesting mRNA or protein, respectively.

Polycystin-1 knockout mice (PC-1 KO)

Male mice harboring a cre recombinase transgene driven by an α-MHC promoter were crossed with mice harboring floxed P*kd-1* alleles[14] to generate PC-1 KO mice. Mendelian frequencies in the birthrate were normal. Genotyping was performed by PCR. Primers used for PCR are listed in Supplemental Table 1. PC-1 silencing was confirmed by Western blot and RT-PCR for *Pkd-1* mRNA in cardiac tissue. *Pkd-2* (gene encoding PC-2) mRNA levels were also evaluated in these animals. 9- to 11-week old mice were used in this study. Both αMHC-cre and F/F mice were used as controls.

Model of pressure overload

Male α-MHC cre and PC-1 KO mice (9–11 weeks old) were subjected to pressure overload by surgical transverse aortic constriction (TAC).[15] After 3 weeks, animals were sacrificed and proteins and mRNA were harvested.

[^{3}H]Leucine incorporation

Cultured NRVMs were stimulated and incubated with [^{3}H]Leucine (1 mCi/mL; Perkin-Elmer). After treatment, NRVMs were washed three times with ice-cold PBS and incubated with 10% trichloroacetic acid (30 min, 4°C) followed by three washes with ice-cold 95% ethanol. Cells were then lysed in 1 mL 0.5 mol/L NaOH (6h, 37°C) with gentle agitation. Lysates were neutralized with 1 mL 0.5 mol/L HCl, and the total content from each well was processed by scintillation counting (Beckman).

Histology

All tissues were fixed in 4% paraformaldehyde and transferred to 1x PBS, followed by paraffin embedding. Hematoxylin/Eosin (H&E) staining was performed for morphological analyses. Wheat germ agglutinin was used for cross-sectional area measurements, quantified from at least 25 cells per section and two sections per group. Masson's trichrome staining was employed for measurements of fibrosis and quantified using ImageJ software.

RNA isolation and qRT-PCR

Total RNA was isolated from mouse tissues or NRVMs using an Aurum™ total RNA Mini Kit according to the recommendations of the manufacturer (Bio-Rad). 150 ng RNA from each sample was used for RT using the iScript cDNA synthesis kit (Bio-Rad). cDNA was diluted 10-fold with ddH_2O and used for quantitative PCR analysis (Roche). Primers used for RT-PCR are listed in Supplemental Table 2. A ΔCt method was used to calculate relative transcript abundances.

Western blot

Proteins from mouse tissue or NRVMs were separated by SDS/PAGE, transferred to a supported nitrocellulose membrane, and immunoblotted. PC-1, LTCC subunit (α-1C, β2, α2δ), and GAPDH antibodies were purchased from Santa Cruz Biotechnology. Other antibodies employed include RCAN (Sigma), β-MHC and PC-1 (Abcam), and ERK (Cell Signaling). Blots were scanned and quantified using an Odyssey Licor (version 3.0) imaging system. Results were normalized to GAPDH.

Echocardiography

Echocardiograms were performed on conscious, gently restrained mice using either a Sonos 5500 system with a 15-MHz linear probe or a Vevo 2100 system with a MS400C scanhead. LVEDD and LVESD were measured from M-mode recordings. Fractional shortening (FS) was calculated as (LVEDD - LVESD)/LVEDD and expressed as a percentage. LV mass was calculated by the cubed method as $1.05 \times ([IVS + LVID + LVPW]^3 - LVID^3)$, where IVS is interventricular septum thickness, LVID is LV internal diameter, and LVPW is LV posterior wall thickness, and expressed in milligrams.[13] All measurements were made at the level of the papillary muscles.

Protein biotinylation

Plasma membrane proteins from NRVMs were isolated using a biotinylation kit according to the recommendations of the manufacturer (Thermo Scientific). We loaded equivalent protein amounts, separated them by SDS/PAGE, and membranes were blotted with α1C LTCC antibody.

Co-immunoprecipitation

NVRMs were collected and lysed with buffer lysis containing Tris-HCl, pH 7 (50 mmol/L), NaCl (120 mmol/L), Nonidet P-40 (0.5%) and a protease inhibitor cocktail (Complete, Roche Diagnostic). The lysate was centrifuged (4°C, 10 min, 10,000 × g), and the supernatant was collected and immunoprecipitated with anti-α1C or anti-β2 LTCC and

protein A/G-Sepharose (Sigma-Aldrich). The immunoprecipitates were analyzed by Western blot with anti-α1C or antiβ2 antibodies.

Cyclic mechanical stretch

NRVMs were cultured on flexible membrane plates coated with collagen IV for adherence (Flexcell Bioflex plates). After 24 hours of culture, the cells were serum-starved for 24 hours. For mechanical stretch, we used a computer-regulated vacuum strain apparatus (Flexcell Strain Unit FX-4000 Tension plus, Flexcell International) and cycles of stretch and relaxation were applied (1 Hz, 20% stretch, 2 hours). Control cells were maintained under static conditions.

Duolink assay

Duolink staining (Sigma) was performed according to the manufacturer's guidelines and previous reports[16]. For PC-1/α1C interaction, we used either mouse anti-PC-1 (Abcam) and rabbit anti-α1C (Santa Cruz Biotechnology) or rabbit anti-PC-1 (Santa Cruz Biotechnology) and mouse anti-α1C primary antibodies. Images were obtained via confocal microscopy.

Other procedures

Lactate dehydrogenase (LDH) activity was measured in the supernatant of NRVM cultures with a CytoTox 96® Non-Radioactive Cytotoxicity Assay according to the recommendations of the manufacturer (Promega). Proteins were quantified according to the Bradford method.

Reagents

After 24 hours of serum starvation, NRVMs were exposed to hypo-osmotic stress (HS) by exposure to medium diluted 1:1 with double-distilled H_2O. The osmolality of the DMEM:M199 diminished from 250 to 125 mOsmol/Kg. Phenylephrine was used at 50 μmol/L. All inhibitors were added 30 minutes before the corresponding stimulus or measurement: nifedipine (10 μmol/L), verapamil (10 μmol/L), cycloheximide (35 μmol/L), gadolinium chloride (10 μmol/L), MG132 (1 μmol/L), SKF 96365 (10 μmol/L) and trolox (100 μmol/L).

Statistical analysis

Data are presented as mean ± S.E.M. of multiple (n) independent replicates. Data were analyzed either by Student's unpaired t-test to compare means when there were two experimental groups or by Welch's version of one-way ANOVA followed by Tukey's test to compare means among three or more groups. Data were analyzed statistically using IBM SPSS Statistics software, version 21. Differences were considered significant at $P<0.05$.

Results

PC-1 mediates cardiomyocyte hypertrophy induced by mechanical stretch *in vitro*

PC-1 has been proposed to act as a mechanosensor in a variety of cell types, regulating cell growth and differentiation.[8] Furthermore, PC-1 activity has been shown to trigger activation

of the calcineurin/NFAT pathway in both T-cells and osteoblasts.[17, 18] As the calcineurin/NFAT pathway is central to the development of pressure overload-induced hypertrophy[19], we set out to test for a possible role of PC-1 in stretch-induced cardiomyocyte growth responses. To initially examine this, we knocked down PC-1 in NRVMs using targeted siRNA (Supplemental Figures 1 A–B) and induced hypertrophy using an *in vitro* model of mechanical stretch by exposure to HS medium.[20] These results were compared with cells exposed to nonsense siRNA and with cells in which hypertrophy was induced neurohumorally with phenylephrine (PE). Hypertrophic growth was assayed as [^{3}H]Leucine incorporation.

Both PE and HS triggered an increase in [^{3}H]Leucine incorporation after 48 hours in cells transfected with control siRNA (Figure 1A). PC-1-specific siRNA treatment had no effect on the ability of PE to induce hypertrophy, but elicited a marked inhibition of HS-induced hypertrophy. Importantly, this growth-suppression effect was observed using a second, sequence-independent siRNA construct targeting PC-1 (data not shown).

We next tested for a requirement of PC-1 in the induction of markers of hypertrophy. Consistent with the protein synthesis assay, PE treatment resulted in an increase in hypertrophic markers, including an increase in both β-MHC and RCAN1.4 protein levels (Figure 1B) and mRNA levels (Figure 1C), even in the absence of PC-1. In contrast, PC-1 knockdown significantly attenuated HS-induced increases in both β-MHC and RCAN1.4 levels (Figures 1B–C). Importantly, HS treatment did not result in significant cell death during the 48 hour treatment period when compared with control (Supplemental Figure 1 C). Together, these data suggest that PC-1 is required for cardiomyocyte growth triggered by HS-induced stretch but not PE-induced cell growth. Additionally, they suggest involvement of PC-1 in activating the calcineurin/NFAT signaling cascade.

The calcineurin–NFAT and ERK1/2 signaling cascades are involved in hypertrophic growth in cardiomyocytes and appear to be interdependent.[21] During mechanical stretch, ERK1/2 is activated early after stimulus onset.[20] In our model, HS treatment also resulted in an early increase in ERK1/2 phosphorylation within 1–2 hours after stimulus onset which decreased back to baseline at 4–8 hours (Supplemental Figure 1 D). Knockdown of PC-1 suppressed this increase in ERK1/2 phosphorylation, suggesting that PC-1 may also modulate ERK1/2 activation. Together, these data are consistent with a model in which PC-1 acts as a mechanosensor in cardiomyocytes and mediates hypertrophy induced by mechanical stretch.

L-type calcium channel is involved in mechanical stretch-induced cardiomyocyte hypertrophy

One of the crucial triggers of cardiac hypertrophy is a sustained increase in cytoplasmic Ca^{2+}.[22] These increases in cytoplasmic Ca^{2+} arise, at least in part, from increased Ca^{2+} influx via LTCC activity and store-operated calcium entry (SOCE).[12, 23, 24] Given this, we tested the role of LTCC in HS-induced cardiomyocyte hypertrophy. NRVMs were exposed to HS in the presence/absence of LTCC inhibitors, and hypertrophy was measured as [^{3}H]Leucine incorporation. Exposure of NRVMs to two structurally distinct LTCC inhibitors (verapamil, nifedipine; 10 μmol/L) completely inhibited HS-induced increases in [^{3}H]Leucine incorporation, confirming a role for the LTCC in this response (Figure 2A).[25]

Interestingly, Gd^{3+}, which inhibits SOCE as well as a number of other channels, did not inhibit [^{3}H]Leucine incorporation in NRVM after HS stimulation (Figure 2B), suggesting that SOCE-induced increases in Ca^{2+} are not involved in HS-induced hypertrophy.

The requirement for LTCC activity in HS-induced hypertrophy was confirmed by measuring molecular markers of hypertrophy; HS-induced activation of both β-MHC and RCAN1.4 mRNA levels was inhibited by LTCC inhibitors (Figure 2C). These data demonstrate a requirement for LTCC activity in HS-induced hypertrophy. Furthermore, the inability of Gd^{3+} to inhibit HS-induced hypertrophy is interesting in light of our earlier report that hypertrophy induced by neurohumoral cues (PE) is inhibited by Gd^{3+} (Figure 2B).[24] Together, these data support a model in which LTCC is required to induce cardiomyocyte hypertrophy by mechanical stretch, suggesting that PC-1 modulates this pathway. They also demonstrate that HS-induced hypertrophy requires both functional LTCC activity and PC-1.

PC-1 promotes LTCC stabilization during HS stress

Ca^{2+} influx through the LTCC is regulated a number of ways.[26] Among these regulatory mechanisms, governance of channel density in the plasma membrane is prominent.[26–28] To determine whether LTCC protein abundance is altered after HS treatment, we measured steady-state levels of LTCC protein by Western blot. Interestingly, one hour of exposure to HS triggered >2-fold increases in α1C protein, a change which was maintained at least after 4 hours of HS exposure (Figure 3A). Knockdown of PC-1 resulted in a decrease in steady-state α1C levels at baseline (Figures 3A), lasting for at least 48 hours (Figure 3B). Further, PC-1 silencing blunted HS-induced increases in α1C protein levels (Figure 3A). These data demonstrate that HS-induced increases in α1C protein are dependent on PC-1 and suggest a mechanism whereby PC-1 may regulate mechanical stress-induced hypertrophy.

Whereas steady-state levels of α1C protein were increased by HS treatment, increased LTCC activity, and consequent Ca^{2+} influx, requires localization of LTCC to the plasma membrane, a process regulated by the β2 subunit.[29] To test for β2 subunit involvement, we performed immunoprecipitation assays to determine whether increases in α1C protein correlated with an increase in β2 subunit interaction. Exposure of NRVMs to HS for 2 hours resulted in an increase in the amount of β2 subunit harvested with an α1C antibody, a change which is roughly proportional to increases in α1C levels (Figure 3C). Importantly, those increases were absent in cells depleted of PC-1 (Figure 3C). The steady-state levels of the α2δ did not change in NRVMs depleted of PC-1 or stimulated with HS (Supplemental Figure 1 E). These data, then, are consistent with a model in which increases in α1C protein contribute to HS-induced cardiomyocyte hypertrophy and point to PC-1 as an upstream regulator.

To quantify LTCC abundance on the cardiomyocyte cell surface more directly, we selectively biotinylated surface proteins under conditions of HS stress. We isolated biotinylated proteins and analyzed equivalent amounts of protein by SDS/PAGE. In control cells, HS treatment resulted in an increase in plasma membrane-associated α1C, but that increase was absent in PC-1 KD cells (Figure 3D). These findings suggest that PC-1 is required for mechanical stress-induced increases in LTCC at the cell surface.

Increases in steady-state levels of α1C could result from increased expression or decreased turnover of the protein. To assess the role of protein expression, we measured the change in steady-state levels of α1C after HS (2 hours) in the presence of the protein translation inhibitor cycloheximide (Cx, 35 μmol/L), 20 minutes before and during the HS stimulus. Inhibition of new protein synthesis had no impact on HS-induced increases in α1C protein (Figure 3E), suggesting that α1C protein degradation is a major mechanism of PC-1-dependent control. Plasma membrane levels of LTCC are regulated in part by proteasomal degradation of α1C subunit.[29] To assess the role of the ubiquitin-proteasome system in HS-induced increases in α1C subunit protein, we treated NRVMs with the proteasome inhibitor, MG132 (1 μmol/L). Exposure to MG132 (2 hours) provoked increases in α1C levels similar to those observed in HS-treated cells. Importantly, addition of MG132 to HS-treated cells did not further increase α1C levels. Also, MG132 treatment restored α1C levels in cells silenced for PC-1 (Figure 3F). These data suggest that declines in α1C in HS-treated cells subjected to PC-1 knockdown derive from increased proteasomal turnover. Further, they suggest that increases in LTCC levels triggered by HS-induced mechanical stress are driven by decreased proteasomal turnover. Having uncovered PC-1-dependent stabilization of LTCC in response to HS, we next examined a different model of mechanical stress elicited by cyclic mechanical stretch (MS).[20] Exposure of NRVMs to MS for 2 hours resulted in induction of the calcineurin/NFAT pathway as evidenced by increases in RCAN1.4 protein levels (Supplemental Figure 1 F) and ERK1/2 activation (Supplemental Figure 1 G). siRNA knockdown of PC-1 inhibited the induction of these signaling pathways by MS. Importantly, steady-state levels of α1C manifested stretch-induced increases in a manner dependent on the presence of PC-1 (Supplemental Figure 1 H). Additionally, SKF 96365 (10 μM), a specific SOCE inhibitor, did not prevent the increase in α1C protein levels or RCAN1.4 after MS stimulation (Supplemental Figure 1 I–J), in agreement with our observation that SOCE is not involved in stretch-induced hypertrophy. Not surprisingly, whereas nifedipine, a LTCC inhibitor, blocked MS-induced stimulation of RCAN1.4, it did not affect MS-induced increases in α1C (Supplemental Figures 1 I–J). Together, these data confirm our findings with HS-induced mechanical stretch and lend further support to a model of PC-1-dependent mechanosensation and LTCC protein stabilization in the development of hypertrophy.

Mechanical stress-induced ROS accumulation triggers increases in Ca^{2+} spark frequency[30]. Given this, we tested whether ROS are involved in LTCC stabilization. Depleting ROS using the scavenger compound trolox, however, had no effect, suggesting that ROS accumulation is not a major mechanism governing stabilization of LTCC by PC-1 (Supplemental Figure 1 K).

PC-1 C-terminus is sufficient to promote LTCC stabilization and cardiomyocyte hypertrophy

The 200 amino acid, cytoplasmic C-terminal tail of PC-1 has been implicated in a number of downstream signaling events; further, it can be cleaved and localized to the nucleus via a nuclear localization signal present within its sequence.[31] To test for a potential role in the development of cardiac hypertrophy, we infected cells with lentivirus expressing the C-terminal tail (p200) (Supplemental Figure 1 L).[32] Over-expression of p200 in NRVMs

resulted in a nearly two-fold increase in [^{3}H]Leucine incorporation as compared with control infected cells (Figure 4A). This cardiomyocyte growth was abolished by treatment with nifedipine (Figure 4A). The p200-induced increase in [^{3}H]Leucine incorporation was also associated with an increase in β-MHC and RCAN1.4 protein and RCAN1.4 mRNA levels, markers of cardiac hypertrophy (Figure 4B). Our earlier data suggested that the PC-1-dependent effects on hypertrophy were independent of the ability of PC-1 to activate a transcriptional response (Figure 3E). This would suggest that the ability of p200 to induce hypertrophy is independent of p200 nuclear localization. To test this, we engineered a lentivirus harboring p200 protein lacking the nuclear localization signal (p200Δ). Expression of p200Δ triggered similar increases in [^{3}H]Leucine incorporation as observed with p200 (Figure 4A, Supplemental Figure 1 L).

Our findings reveal that PC-1 is required for a stretch-induced hypertrophic response in NRVMs in a manner that requires both LTCC function and increases in LTCC surface localization. Furthermore, over-expression of the C-terminal domain of PC-1 was sufficient to recapitulate the effects of full-length PC-1. To examine whether the p200-induced hypertrophic response results in an increase of LTCC biosynthesis, we performed both Western analysis and immunoprecipitation experiments. As observed with HS treatment, protein but not mRNA levels of α1C were significantly increased after 48 hours of p200 expression (Figure 4C).

Furthermore, increased association of α1C and β2 LTCC subunits was observed in reciprocal immunoprecipitation assays (Figure 4D). These data are consistent with a model in which the C-terminus of PC-1 acts to trigger hypertrophic growth in response to mechanical stress.

PC-1 interacts with LTCC

Having confirmed a functional link between PC-1 and α1C, we next tested for evidence of a physical interaction between the proteins. As standard IP experiments with membrane channel proteins can be problematic, we employed a proximity ligation assay (PLA) to test for regulated physical proximity between PC-1 and α1C. NRVM's grown on cover slips were fixed and incubated with primary antibodies targeting α1C (mouse antibody) and PC-1 (rabbit antibody). Cells were next exposed to corresponding DNA-linked secondary antibodies, and the slides were processed according to the manufacturer's instructions (Duolink, Sigma). Probing the cells using only the mouse α1C primary antibody (and not the PC-1 antibody) resulted in minimal background signal. In contrast, probing with both rabbit PC-1 and mouse α1C primary antibodies resulted in the formation of numerous distinct puncta, indicating that the two secondary antibodies are in close proximity (<40 nm), (Figure 4E). Similar results were obtained independently using a rabbit α1C primary antibody and mouse PC-1 primary antibody (Supplemental Figure 1 M). These findings, then, suggest that PC-1 and LTCC proteins interact physically under basal conditions.

PC-1 is required for cardiac hypertrophy and normal heart function

To define the role of PC-1 in adult heart, we engineered a line of mice with cardiomyocyte-restricted silencing of PC-1 (α-MHC-Cre; *Pkd1$^{f/f}$* henceforth referred to as PC-1 KO).

Recombination in these hearts was confirmed by PCR, and Western blot analysis of protein extracts from these hearts confirmed cardiomyocyte-specific loss of PC-1 (Supplemental Figure 2 A, Figure 5A).

PC-1 KO animals developed normally, manifesting no apparent phenotype at birth. However, by 8 weeks of age, echocardiographic examination revealed a distinct defect in systolic function (Supplemental Figure 2 B). Moreover, our results show that ventricular function in these animals remained persistently depressed at 15 weeks of age (Supplemental Figure 2 C). As it has been reported that mice haploinsufficient for α1C subunit manifest a similar phenotype[33], we compared the levels of α1C protein in control (α-MHC cre) and PC-1 KO hearts. Interestingly, Western analysis revealed a significant decrease in α1C LTCC protein abundance in PC-1 KO hearts (Supplemental Figure 2 D), consistent with a role for PC-1 in regulating LTCC levels and in the normal functioning of adult heart.

Next, we subjected mice (9–11 weeks old) to increased afterload by TAC. Both control (α-MHC cre) and PC-1 KO mice were subjected to TAC or sham surgery, and structural, functional, and molecular events were evaluated at three weeks post-surgery. As expected, TAC triggered an increase in the mass of the α-MHC cre transgenic hearts as compared with sham-operated controls (heart weight/body weight, HW/BW, increased 80% from 4.2 ± 0.2 to 7.6 ± 1.4 mg/g, $p<0.001$) (Figures 5B–C). Similar findings were observed when heart weight was normalized to tibia length (HW/TL) (Figure 5C). In contrast, TAC surgery elicited no significant heart growth in PC-1 KO hearts (HW/BW; Sham 4.3 ± 0.4, TAC 5.4 ± 1.0 mg/g, p=NS) (Figures 5B–C). This blunting of load-induced growth was confirmed by HW/TL measurements (Figure 5C). These data support a model in which PC-1 is essential for development of pressure overload-induced cardiac hypertrophy.

Examination of myocardial structure revealed the expected increases in cardiomyocyte cross-sectional area in TAC-stressed control heart, consistent with cardiomyocyte hypertrophy (Figure 5D). Those increases, however, were absent in TAC-exposed PC-1 KO heart. TAC elicited significant fibrosis in control heart, which was significantly ($p<0.05$) attenuated in PC-1 KO heart (Figure 5E). These data provide further evidence for a requirement of PC-1, functioning as a mechanosensor, in stress-induced cardiac growth.

In control animals, TAC surgery triggered an increase in the steady-state levels of RCAN1.4 protein and mRNA levels of hypertrophic markers (βMHC, BNP, ANF, RCAN1.4). These effects were blunted in the PC-1 KO animals (Figures 5F–G). No changes in PC-2 mRNA levels were detected in PC-1 KO mouse cardiac tissue under baseline conditions. Interestingly, both *Pkd-1* and *Pkd-2* mRNA levels were increased after TAC in control mice, and TAC-induced increases in PC-2 transcript were blunted in PC-1 KO mice (Supplemental Figures 2 E–F). However, PC-2 protein levels manifested no significant change under these conditions (Supplemental Figure 2 G–H).

Finally, we tested whether blunting of load-induced cardiac hypertrophy in PC-1 KO mice led to protection against cardiac dysfunction. PC-1 KO mice manifested baseline contractile dysfunction (%FS 40 ± 3 PC-1 KO versus 65 ± 4 WT, $p<0.0001$). TAC-triggered ventricular dilation in control heart at 3 weeks was significantly exacerbated in PC-1 KO heart (Figure

5H). Ventricular function 3 weeks post-TAC surgery decreased significantly in both control and PC-1 KO animals, although the extent of decline was significantly greater in the PC-1 KO animals (%FS 33%±15 PC-1 KO versus 65%±4 WT, $p<0.0001$) (Figure 5H).

Discussion

Mechanisms governing disease-related mechanotransduction in heart remain elusive, despite the fact that it is a proximal trigger of cellular events culminating in prevalent forms of disease. Here, we evaluated the role of PC-1, a molecule expressed in cardiomyocytes whose function was unknown but yet is capable of serving as a mechanical sensor in other cell types. We report that PC-1 is required for stretch-induced cardiomyocyte hypertrophy *in vitro* and pressure overload-induced hypertrophy *in vivo*. PC-1 is required for normal contractile function at baseline. Mechanistically, PC-1 functions to stabilize LTCC protein, thereby promoting LTCC sarcolemmal localization and function. We go on to map this protein-stabilizing activity to the cytoplasmic C-terminus of PC-1. Together, these data uncover a novel mechanism governing load-induced hypertrophic remodeling in heart.

Mechanotransduction and PC-1

Conversion of extracellular cues into intracellular biochemical responses is critical to a wide range of biological events. The heart is subject to stimuli, both physiological and pathological, which elicit a robust plasticity response.[3] In particular, mechanical forces impinging on the cardiomyocyte trigger a host of biochemical, signaling, electrical, metabolic and transcriptional events.[34] Whereas much is known about the intracellular events elicited by mechanical stress[35], relatively little is known about how that stress is sensed and how it triggers intracellular events. PC-1 is a large integral membrane glycoprotein with a long N-terminal extracellular domain, eleven transmembrane domains, and a short intracellular C-terminal tail. This 200 amino acid intracellular C-terminus participates in signaling with other proteins, or after proteolytic cleavage it can modulate pathways such as mTOR, calcineurin-NFAT, Wnt, AP-1, and STAT6.[5, 31, 36, 37] PC-1 also interacts with PC-2, a Ca^{2+}-regulated non-selective cation channel.[7, 38] PC-1 can also modulate levels of cytoplasmic Ca^{2+} through PC-2, IP_3R or STIM1 regulation.[31, 39]

Originally identified by positional cloning[4], PC-1 functions to transduce extracellular mechanical events in a number of cell types.[5, 7, 9, 31] In zebrafish, PC-2 loss-of-function results in cardiac dysfunction and atrio-ventricular conduction block.[40] Interestingly, the subcellular localization of PC-1 and PC-2 in cardiomyocytes remains uncertain, with some evidence pointing to both plasmalemmal and sarcoplasmic reticulum localization.[41]

Mechanical stress is one of the primary stimuli to induce cardiac hypertrophy.[42] Integrins, angiotensin-1 receptor (AT1R), and stretch-activated ion (SA) channels have all been characterized as mechanosensors in cardiomyocytes,[43] but these findings remain conflicting, suggesting that other mechanosensors may also be present. PC-1 is a membrane protein best described as part of the primary cilium in renal epithelial cells, serving to sense and transduce shear stress to modulate a variety of downstream pathways.[5, 31, 36, 37] However, PC-1 has also been suggested to work as a mechanosensor via other mechanisms.[9–11]

We employed two distinct *in vitro* models of mechanical stretch: hypo-osmotic stress and cyclic mechanical stretch. Hypo-osmotic stress has been used previously to impose static mechanical stretch, homologous to the stress of cyclic mechanical stretch[44]. In the latter, the degree of stretch employed (20%) likely represents a pathological, as opposed to physiological, trigger[30]. Importantly, all of our observations in HS conditions were reproduced in the cyclic stretch model. Thus, our data point to an important role of PC-1 in mechanotransduction within cardiomyocytes.

Ca^{2+}-dependent control of cardiomyocyte growth

Perturbations of intracellular Ca^{2+} signaling[45, 46] accompany many forms of heart disease and contribute to the pathogenesis of cardiac hypertrophy and failure. The LTCC is the major mediator of Ca^{2+} influx into cardiomyocytes and an important determinant of action potential morphology. The role of the LTCC, the proximal element in Ca^{2+} signaling, in the pathophysiology of cardiac hypertrophy remains incompletely characterized.

Intracellular Ca^{2+} metabolism and signaling are altered in both hypertrophic and failing hearts, contributing to disease pathogenesis.[47] Some of these changes have common features between the two phenotypes, while others are contrasting. In either case, altered Ca^{2+} handling contributes to the activation of several kinase and phosphatase cascades, including those involving MAP kinases, protein kinase C, and calcineurin.[48, 49] Together, such abnormal profiles of signaling lead to disturbances in gene regulation, which may promote disease progression. Entry of a small amount of Ca^{2+} via LTCC triggers the release of much larger amounts of Ca^{2+} from intracellular stores.[26] As a result, PC-1-dependent governance of LTCC protein stability is likely to have an amplified impact on intracellular Ca^{2+} handling events. Also, membrane impedance is relatively high during phase 2 of the action potential in many species, so changes in LTCC abundance have important effects on action potential morphology and duration. The LTCC in heart comprises a large pore-forming subunit α1c (also known as $\alpha_1$1.2) plus auxiliary subunits α2-δ and β2. In failing human ventricular myocytes, LTCC activity often exceeds that predicted from constant levels of α1c transcript and protein, suggesting post-translational regulation of the channel complex, possibly via phosphorylation.[50] This is the first report to uncover regulated control of LTCC subunit protein catabolic events.

Silencing the gene encoding α1C results in embryonic lethality.[51] Deletion of one copy of the α1C subunit gene (Cacna1c) results in decreased steady-state levels of α1C protein and a decrease in LTCC activity in adult cardiomyocytes.[33] Animals harboring this heterozygous genotype display cardiac dysfunction by 3–10 weeks of age with increased systolic chamber size. Interestingly, we report here that mice harboring cardiomyocyte-specific loss of PC-1 manifest a similar phenotype with a significant decrease in ventricular contractile function by 8–10 weeks, driven by an increase in LVESD. Steady-state levels of α1C in these mice are significantly low, suggesting that the phenotype derives, at least in part, from a decrease in LTCC activity.

LTCC-dependent control of cardiomyocyte hypertrophy

A multitude of studies have linked Ca^{2+} influx from LTCCs with the intracellular signaling and gene regulatory events that trigger cardiac hypertrophy and disease.[35] Consistent with this idea, LTCC blockers can inhibit pressure overload-induced hypertrophy.[33] Here, we report that PC-1 suppresses proteasome-dependent degradation of α1C subunit protein. Interestingly, this decrease had no effect on PE-induced hypertrophy, consistent with the notion that β-adrenergic stimulation of LTCC activity stems mainly from increases in the open state of the channel rather than channel abundance.[52] In contrast, PC-1 knockdown blunted the hypertrophy induced mechanically by HS or MS treatment. HS treatment led to an increase in α1C steady state levels within an hour of treatment, as well as LTCC formation as evidenced by increased association with the β-subunit and surface localization of α1C protein. We also tested for a role of ROS in the stabilization of LTCC protein after stretch, but in fact, antioxidant (trolox) had no effect. These data suggest that the stabilization of α1C is a proximal trigger of the hypertrophic response. Further, they uncover a previously unrecognized role for regulated proteasome-dependent LTCC degradation in the hypertrophic response.

Cardiomyocyte-specific deletion of PC-1 *in vivo* resulted in attenuation of the hypertrophic response to pressure overload. Consistent with our *in vitro* findings, PC-1 KO resulted in lower steady-state α1C levels *in vivo*. These findings fit well with earlier studies that reported blunting of pressure overload-induced hypertrophy by pharmacological inhibition of LTCC activity or genetic down-regulation of the β2-subunit.[53, 54]

Goonasekera *et al* reported that mice haploinsufficient for α1C manifest a slightly exaggerated hypertrophic response to TAC or other stressors.[33] It remains unclear how these mice mount a hypertrophic response, whereas PC-1 KO animals (with a similar decreases in α1C abundance) do not. One possibility is that $\alpha1C^{+/-}$ mice retain the ability to stabilize the remaining α1C protein, resulting in an increase in LTCC activity relative to sham-operated controls. Alternatively, PC-1 may function through additional pathways to influence the response to pressure-overload stress.

While hypertrophy has been associated in a number of studies with increases in LTCC activity, increased protein levels of LTCC have not been observed.[55–57] In our hands, 3 weeks post-TAC, α1C protein levels show no increase from baseline in either control or PC-1 KO hearts (Supplemental Figure 3A). Our *in vitro* data suggest a model of PC-1-dependent increases in LTCC steady-state levels which correlate with the hypertrophic response. Interestingly, protein levels of β2 increase post-TAC only in PC-1 KO mice, perhaps as a compensatory response to diminished LTCC levels (Supplemental Figure 3B and 3C).

The specific mechanism whereby PC-1 stabilizes α1C protein remains unclear. PC-1 KO hearts manifest a decrease in α1C levels consistent with a role for PC-1 in regulating protein stability. The effect is rapid, occurring within 1 hour of stress induction, and appears independent of a direct role for PC-1 in transcription. Localization of PC-1 to the plasma membrane and the evidence of physical proximity from the PLA assay suggest that the mechanism could be direct; however PC-1 has also been implicated in a number of

downstream pathways including AKT activation,[58] which could represent an indirect mechanism for LTCC stabilization. Interestingly, one report documented existence of an endogenous, 100 kDa PC-1 cleavage product which functions to reduce SOCE via direct inhibition of STIM1 translocation.[59]

Conclusions

We report here that PC-1 serves as a mechanosensor in cardiomyocytes and governs a novel mechanism of regulated degradation of LTCC protein. This cascade participates importantly in stress-induced hypertrophic growth triggered by hypotonic stress, mechanical stress, and increased afterload. These findings uncover a previously unrecognized mechanism of stress-induced mechanotransduction, opening the potential prospect for therapeutic manipulation. Going forward, future studies will define mechanisms whereby the C-terminal domain of PC-1 regulates proteasome-dependent LTCC catabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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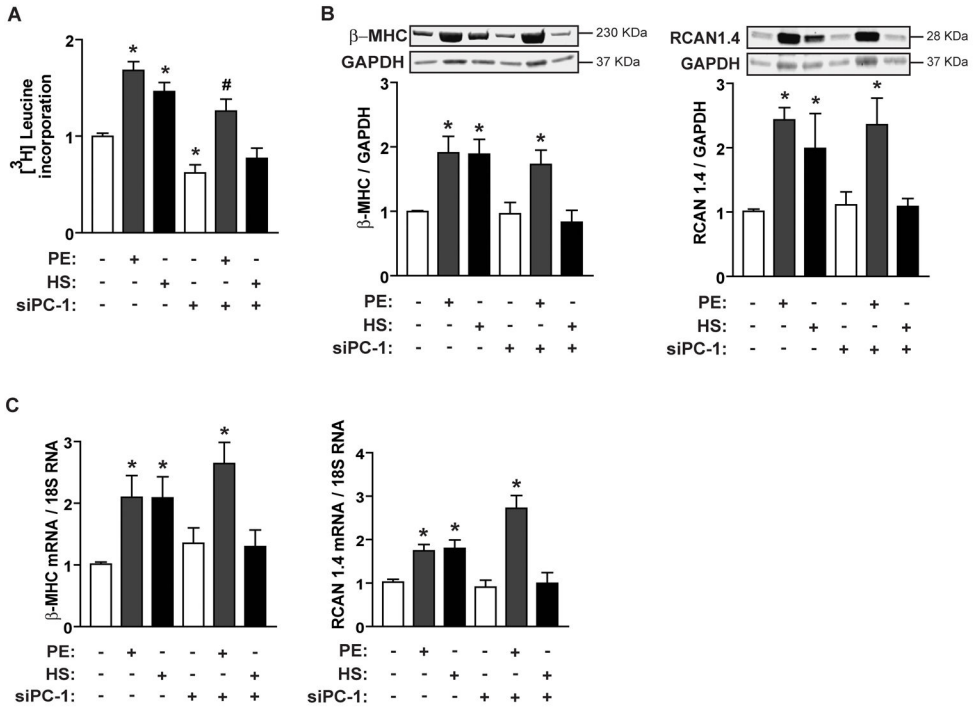

Figure 1.

PC-1 mediates *in vitro* cardiomyocyte hypertrophy. A, [^{3}H]Leucine incorporation in cardiomyocytes stimulated with PE or HS and exposed to PC-1 siRNA (siPC-1) or transfected with unrelated sequences. B, Representative Western blot of β-MHC (left) and RCAN1.4 (right). Mean data of β-MHC/GAPDH and RCAN1.4/GAPDH calculated from densitometric analysis after hypertrophic stimuli. C, mRNA levels of β-MHC and RCAN1.4 are depicted as β-MHC/18S (left) and RCAN1.4/18S (right), respectively. Values are mean ± SEM, analyzed by Welch's test, one-way ANOVA followed by Tukey's test (n=5–8). **P*<0.05 vs. control, $^{\#}$*P*<0.05 vs. siPC-1.

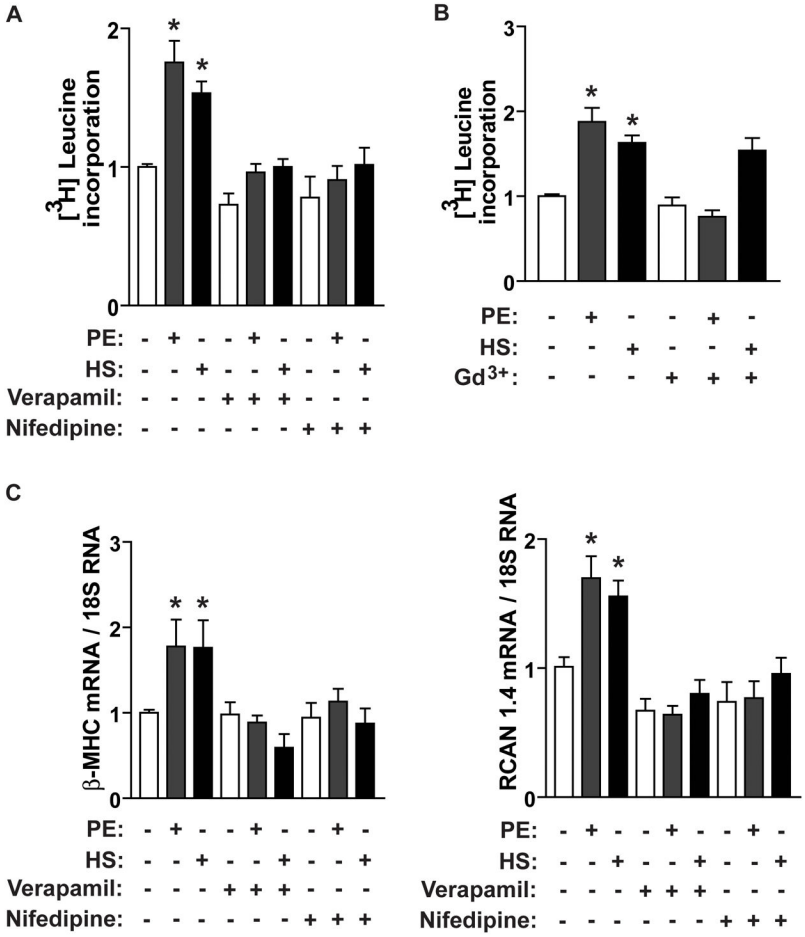

Figure 2.

L-type calcium channel activity is required for mechanical stretch-induced cardiomyocyte hypertrophy. A–B, [^{3}H]Leucine incorporation in cardiomyocytes stimulated with PE or HS in presence of verapamil or nifedipine, or Gd^{3+}. C, Ratios of β-MHC/18S (left) and RCAN1.4/18S (right) transcripts. Values are mean ± SEM analyzed by Wech's test, one-way ANOVA followed by Tukey's test (n=4–6). *P<0.05 vs. control.

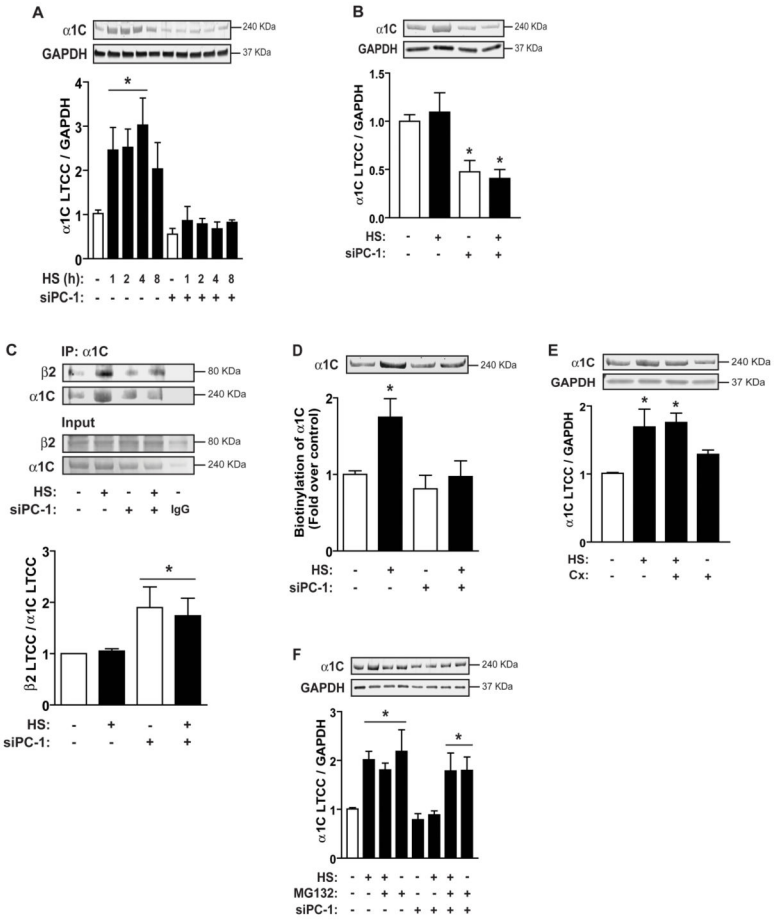

Figure 3.

PC-1 stabilizes α1C LTCC subunit protein. A, Time course of changes α1C/GAPDH ratios from Western blot densitometric analysis. B, Representative Western blot for α1C and GAPDH, and α1C/GAPDH ratio in cardiomyocytes stimulated with HS (48 h) in the setting of PC-1 knockdown (siPC-1). C, Immunoprecipitation of α1C subunit and Western blot for β2 and α1C subunit from siPC-1 and control cells stimulated with HS and their densitometric quantification. D, Plasmalemmal surface protein biotinylated after HS stimulation (2 h) in siPC-1 and control cells. Representative Western blots and densitometric analysis for α1C. E, α1C/GAPDH ratios from immunoblot analysis of cells stimulated with HS (2 h) in presence of cycloheximide (Cx). F, α1C and GAPDH protein levels by Western blot, as well as quantified ratios of α1C/GAPDH, from siPC-1 and control cardiomyocytes stimulated with HS (2 h) in the presence or absence of MG132. Values are mean ± SEM analyzed by Welch's test, one-way ANOVA followed by Tukey's test (n=3–5). **P*<0.05 vs. control.

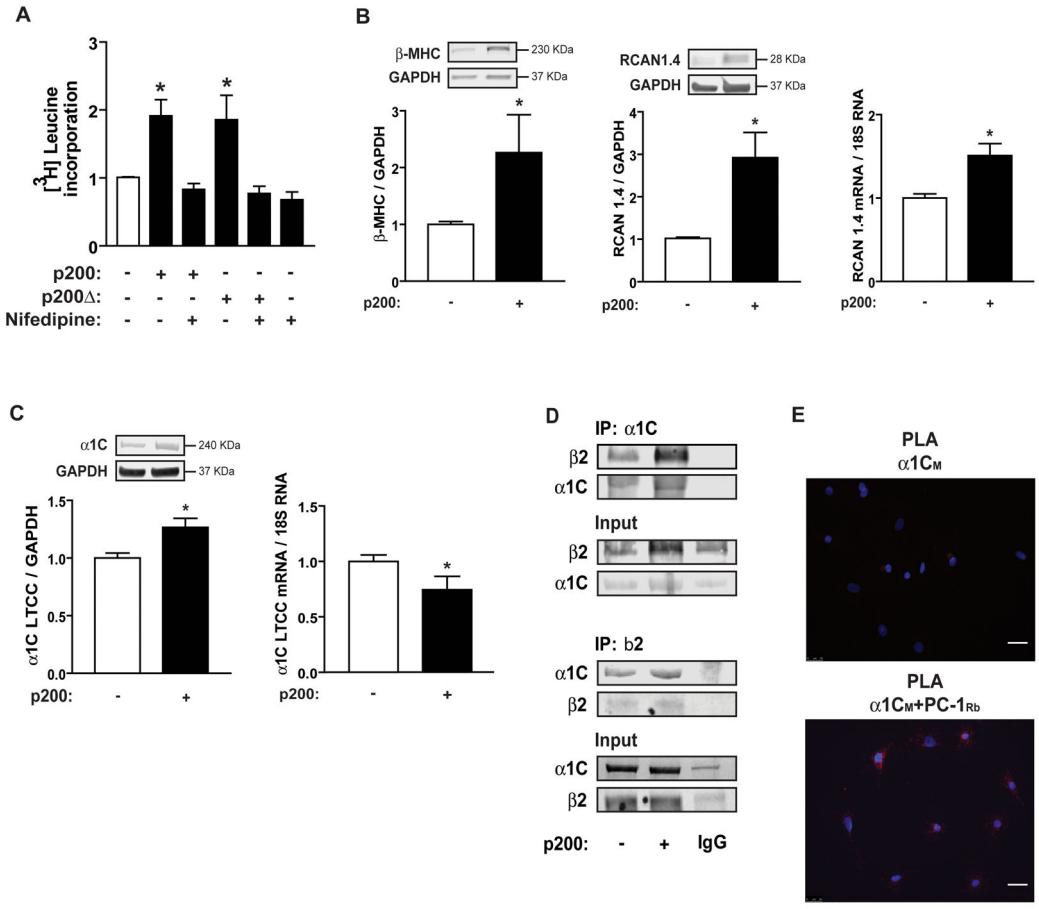

Figure 4.

Over-expression of PC-1 C-terminus induces cardiomyocyte hypertrophy. A, [^{3}H]Leucine incorporation in cardiomyocytes transfected with a C-terminal PC-1 peptide (p200) or the C-terminus lacking the nuclear localization signal (p200Δ). Nifedipine was used as LTCC blocker. B, Representative Western blots of β-MHC (left) and RCAN1.4 (middle) and RCAN1.4 mRNA (right) in cardiomyocytes transfected (48 h) with p200, as well as quantified data of β-MHC/GAPDH, RCAN1.4/GAPDH and RCAN1.4/18S. C, Left, quantification of protein levels and representative Western blot of α1C subunit in cells transfected with p200 (48 h); right, α1C mRNA levels depicted as α1C/18S ratio in identical experiments. D, Immunoprecipitation of α1C and β2 subunit from cardiomyocytes transfected with p200. Representative Western blots of α1C and β2 are shown for each of the immunoprecipitations. E Representative image of NRVMs fixed and probed with anti-α1C antibody (mouse, M) and anti-PC-1 antibody (rabbit, Rb) for Proximity Ligation Assay (PLA). Upper Panel: Control using only the α1C primary antibody (and not the PC-1 antibody) followed by both secondary antibodies. Lower Panel: Cells were incubated with both PC-1 and α1C primary antibodies resulting in formation of distinct puncta, indicating that the two secondary antibodies are in close proximity. Values are mean ± SEM analyzed by Student's unpaired *t*-test or Welch's test, one-way ANOVA followed by Tukey's test (n=4–6). *P<0.05 vs. control.

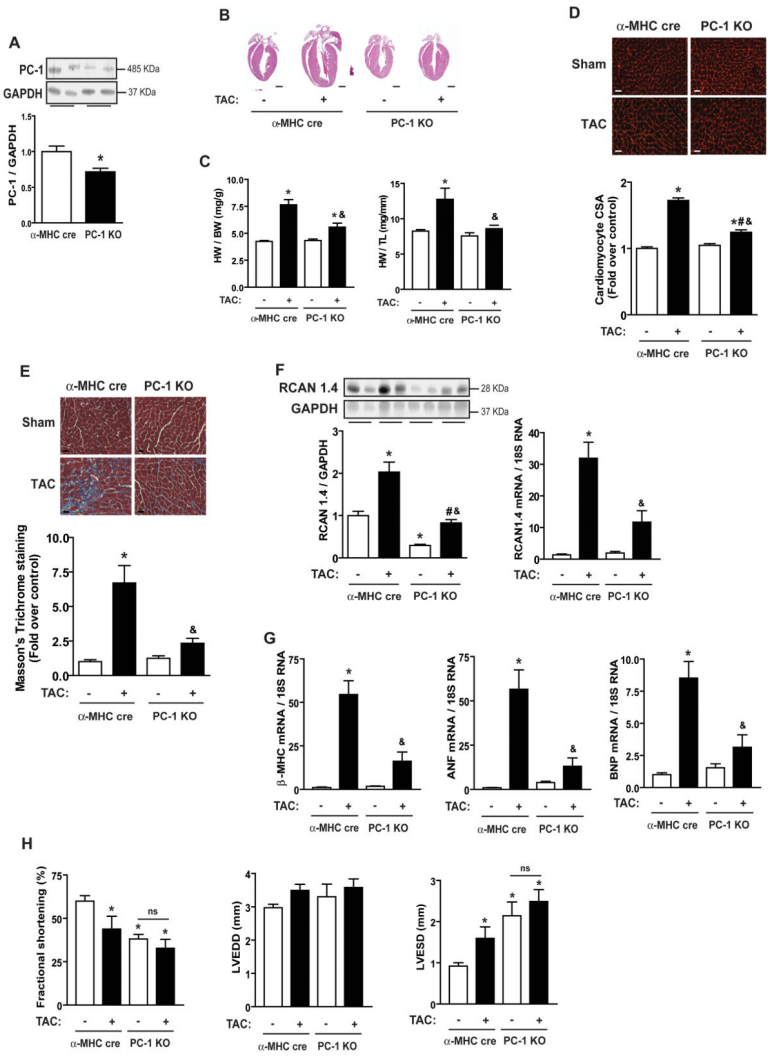

Figure 5.

Cardiac hypertrophy is attenuated in PC-1 KO mice. A, Representative PC-1 Western blot from the ventricular tissue of PC-1 KO mice and its densitometric quantification. B, Hematoxylin and eosin (H&E) staining in four-chamber heart sections after TAC. C, Heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) ratios are depicted. Representative images of left ventricular cardiomyocytes (transverse section of left ventricle) stained with: D, Wheat germ agglutinin (red) and cross-sectional area (CSA) of cardiomyocytes quantified from at least 25 cells per section and 2 sections per group and E, Masson's trichrome (blue) and densitometric analysis of the tissue sections. F, Representative Western blots of RCAN1.4 and GAPDH (above) with RCAN1.4/GAPDH depicted (below left). mRNA levels of RCAN1.4 are presented as RCAN1.4/18S ratio (below right). G, mRNA quantification of hypertrophic markers (β-MHC, ANF, BNP) are depicted. H, Ventricular function, quantified as percent fractional shortening (%FS, left) and ventricular dimensions, LVEDD (center) and LVESD (right), are depicted for hearts 3-weeks post Sham or TAC surgery (α-MHC-cre, PC-1 KO mice). Scale bar: 150 μm (B) and 20 μm (D–E). Values are mean ± SEM analyzed by Welch's test, one-way ANOVA

followed by Tukey's test (n=6–8). *P<0.05 vs. α-MHC-cre, $^{\#}P$<0.05 vs. PC-1 KO and $^{\&}P$<0.05 vs. α-MHC-cre after TAC.