Regulation of Cardiac Hypertrophic Signaling by Prolyl Isomerase Pin1

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Rationale: **Cardiac hypertrophy results from the complex interplay of differentially regulated cascades based on the phosphorylation status of involved signaling molecules. Although numerous critical regulatory kinases and phosphatases have been identified in the myocardium, the intracellular mechanism for temporal regulation of signaling duration and intensity remains obscure. In the nonmyocyte context, control of folding, activity, and stability of proteins is mediated by the prolyl isomerase Pin1, but the role of Pin1 in the heart is unknown.**

Objective: **To establish the role of Pin1 in the heart.**

Methods and Results: **Here, we show that either genetic deletion or cardiac overexpression of Pin1 blunts hypertrophic responses induced by transaortic constriction and consequent cardiac failure in vivo. Mechanistically, we find that Pin1 directly binds to Akt, mitogen activated protein kinase (MEK), and Raf-1 in cultured cardiomyocytes after hypertrophic stimulation. Furthermore, loss of Pin1 leads to diminished hypertrophic signaling of Akt and MEK, whereas overexpression of Pin1 increases Raf-1 phosphorylation on the autoinhibitory site Ser259, leading to reduced MEK activation.**

Conclusions: **Collectively, these data support a role for Pin1 as a central modulator of the intensity and duration of 2 major hypertrophic signaling pathways, thereby providing a novel target for regulation and control of cardiac hypertrophy. (*Circ Res*. 2013;112:1244-1252.)**

Key Words: Akt ■ cardiomyocyte ■ heart failure ■ hypertrophy ■ Pin1 ■ Raf-MEK-ERK ■ signal transduction

Molecular signaling cascades are characteristically based on interwoven networks held in regulated balance. Tuning the network by selectively altering signal duration and intensity provides another layer of regulation above and beyond binary on/off switching mechanisms. This capacity to influence triggered signaling cascades characterizes the unique property of Pin-1. Almost 15 years ago, the loss of Pin1 in both yeast and human cells was shown to cause defects in cytokinesis.[1] From this seminal observation, hundreds of studies contributed to deciphering the mechanism, function, and biological consequences of Pin1 activity.[2,3] By way of analogy, Pin1 acts as a molecular orchestrator that does not choose the music but rather sets the amplitude and duration. Pin1 accomplishes this feat by posttranslational modification of protein structure based on specific recognition of a major regulatory phosphorylation motif (Ser/Thr-Pro) belonging to the family of proline (Pro)-directed protein kinases that include Akt, Pim-1, cyclin-dependent protein kinases, etc. The presence of a proline residue means the molecule can adopt either *cis* or *trans* conformations, often with widely divergent biological activities, depending on configuration. Pin1 is a highly conserved peptidyl-prolyl isomerase that lowers the activation energy necessary for isomerization around the Pro-directed neighboring phosphorylation site. Isomerization modification leads to changes in stabilization of proteins in active configurations, enhanced degradation, or even accessibility for further modifications by other enzymes. Clearly, Pin1 is a critical element of survival and proliferative signaling in the field of cancer, but the role of this critical regulatory molecule in the myocardium has not been previously examined.

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The consequences of Pin1 activation are multifaceted, including transcriptional reprogramming[4–7] and altered signaling cascades

Original received January 28, 2013; revision received March 12, 2013; accepted March 13, 2013. In February 2013, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 11.98 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.301084/-/DC1.

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Nonstandard Abbreviations and Acronyms	
AAV-Pin1	AAV9 harboring Pin1 downstream of the MLC2v promoter
EGFP	enhanced green fluorescent protein
NRCM	neonatal rat cardiomyocyte
TAC	transaortic constriction

involved in proliferation, cell survival, lineage commitment, and cellular aging. Elevation of Pin1 expression in cancer suggests a role in cellular proliferation,[8] and Pin1 has been touted as a potential therapeutic target for slowing proliferation[9,10] because inhibition of Pin1 leads to mitotic arrest and apoptosis.[11,12] These postulates are consistent with Pin1 induction by growth factors as well as the family of Pin1 target substrates responsible for regulation of mitosis.[1] Presumably, increased Pin1 expression correlates with mitosis to preserve the delicate rhythm of molecular signals leading to cell division, but Pin1 can be a double-edged sword for cell survival. Numerous mediators of apoptosis are regulated by Pin1 activity. However, because Pin1 does not choose the music but instead propagates the rhythm, the outcome can be either to enhance survival or to accentuate death signaling. Several targets of Pin1 regulate survival, including protein kinase B (Akt), which is stabilized by Pin1, resulting in prolonged and persistent activation.[13,14] Pin1 suppresses apoptosis mediated by cell death–associated proteins.[15] Pin1 also may promote survival via increasing vascular endothelial growth factor expression,[16] enhanced nuclear factor-κB signaling,[17] or tuning autophagy.[18] Clues are emerging to implicate Pin1 in the regulation of cell commitment to the differentiated state through 2 canonical signaling pathways mediated by Nanog and Notch.[7,19] One of the most intriguing aspects of Pin1 biology is the link to aging, particularly in the context of neurobiology. Genetic deletion of Pin1 in mice leads to an early-onset neurodegeneration syndrome resembling Alzheimer disease. Conversely, Pin1 overexpression decreases β-amyloid production, restores τ function, and promotes cell cycle reentry in neuronal cells,[20,21] whereas inhibition of Pin1 halts proliferation.[10,22] These observations have led to the speculation that Pin1 links neurodegenerative disease, cancer, and aging, with Pin1 playing a protective role to antagonize the aging phenotype in neurons.[20,23,24] In fact, the phenotype of Pin1 knockout mice recapitulates the premature aging observed in telomerase-deficient mice.[25]

We can extrapolate 2 essential points from the assembled Pin1 literature: (1) Pin1 is a facilitator of timing and intensity for multiple distinct signaling cascades, and (2) Pin1 potentiates the biological consequences of signal transduction without serving to initiate them. Targets of Pin1 action are well-known mediators of myocardial signaling, including the Akt and mitogen activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) cascades.[26,27] The impact of Pin1 in areas of proliferation, survival, aging, and cell fate determination places this molecule central to many of the most significant areas of current myocardial signal transduction under investigation.[2] Turning to the myocardium where the functions of Pin1 are yet to be determined, the present study documents differential regulated expression of Pin1 in the heart during development and after pressure overload. Furthermore, we elucidate the significance of Pin1 in cardiac hypertrophy.

Methods

Detailed methods can be found in the Online Data Supplement.

Mice

All experimental procedures were performed according to the guidelines established by San Diego State University for experiments in animals, and all protocols were approved by Institutional Animal Care and Use Committee. Animal model surgery and echocardiographic and hemodynamic analyses were performed as previously described.[28,29]

Histology and Staining

Hearts fixed in 10% formalin were embedded in paraffin, sectioned at 4 μm thickness, and used for immunohistochemistry.[30]

Immunoblot Analysis

Whole-cell lysates were resolved by SDS-PAGE. Immunoblot analyses were performed with Pin1, phosphorylated Akt, phosphorylated MEK, phosphorylated Raf-1, phosphorylated ERK, ERK (CST), GAPDH (Chemicon), Akt (Santa Cruz), and MEK (Transduction Laboratory). There are 2 closely migrating immunoreactive bands for Pin1 evident by immunoblot analysis. However, the relationship of these 2 immunoreactive bands relative to Pin1 biological function remains unclear. Intensity of the lower band remained consistently detectable but with varying intensity throughout our immunolabeling studies. Staining with a phosphospecific antibody to Ser71 did not correlate conclusively with either band in the immunoblot analysis (Online Figure IA). Alternatively to phosphorylation status, we speculated that mobility may correlate with subcellular localization, but subcellular fractionation findings were inconclusive. Further possible explanations include alternative splicing or posttranslational modification other than Ser71 phosphorylation. Delineation of the functional differences, if any, for these 2 related Pin1 bands remains the subject of future studies. Intensities of immunoblot bands were measured using ImageJ software (National Institutes of Health).

Cardiomyocyte Culture

Neonatal rat cardiomyocytes (NRCMs) were prepared from ventricles of 1-day-old Wistar rats and cultured by standard protocol.[30] NRCMs were treated with serum or phenylephrine for the indicated times.

Adenovirus and siRNA

Recombinant adenovirus strains harboring enhanced green fluorescent protein (EGFP) or human Pin1 with EGFP were generated as previously described.[31] NRCMs were transduced with the adenovirus at a multiplicity of infection of 25. NRCMs were transfected with siRNA to Pin1 (Invitrogen) using HiPerfect (Qiagen). Functional comparability for EGFP, Pin1, or EGFP-Pin1 was assessed in direct comparison studies in HeLa cells because HeLa cells have previously been used for demonstration of Pin1-mediated effects.[9,32] Impact of Pin1 on ERK activation was observed by phosphorylation status of the kinase. ERK activation is extended by Pin1 overexpression in HeLa cells treated with EGF. Similar to previously reported findings,[33] ERK phosphorylation was increased by EGF stimulation at the 1-minute time point. However, activation returned to basal levels in MOCK- or EGFP-expressing cells by the 30-minute time point, whereas, in comparison, overexpression of Pin1 or Pin1-EGFP delayed ERK dephosphorylation (Online Figure II). Therefore, with respect to potentiation of ERK phosphorylation, the function of Pin1-GFP fusion protein is comparable to Pin1 alone.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction

Total RNA was isolated from NRCMs with the Quick-RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. cDNA synthesis of RNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and quantitative real-time polymerase chain reaction was performed with the Quanti-Tect SYBR Green PCR kit (Qiagen). Relative levels of gene expressions were normalized to high-resolution picture transmissions expression using the $\Delta\Delta C_t$ method.

Adeno-associated Virus Serotype 9

Adeno-associated virus (AAV) serotype 9 harboring human Pin1 was generated. The virus (1×10^{11} total virus particles) was injected into

6-week-old mice via the tail vein, and mice were subjected to pressure overload at 6 weeks after AAV injection.

Proximity Ligation Assay

Proximity ligation assay was performed to detect protein–protein interaction according to the manufacturer's instruction.

Statistical Analysis

Data are shown as mean±SEM. Statistical analysis was performed with GraphPad Prism (Graphpad Software Inc). Multiple-group comparison was performed by 1- or 2-way ANOVA followed by the Bonferroni procedure for comparison of mean values. Comparisons between 2 groups were performed with the Student *t* test. Values of $P<0.05$ were considered statistically significant.

Results

Pin1 Expression Decreases With Age and Is Upregulated After Pressure Overload in the Heart

Pin1 was highly expressed in postnatal hearts at 1 week, decreasing by 46% at 3 months and 48% by 1 year of age (Figure 1A). Pin1 cellular localization shifted during development from predominantly nuclear in neonatal mouse hearts to increasingly cytosolic in adult mouse hearts (Figure 1B). In the pathologically challenged heart exposed to pressure overload through transaortic constriction (TAC), Pin1 was evident in perivascular areas (Figure 1D), and expression was increased (157% induction at 14 days; Figure 1C). Phosphorylation of Pin1 on Ser71, which inhibits its catalytic activity,[34] increased with age (Online Figure IA) but decreased after TAC (Online Figure IB). These results suggest that Pin1 is important for cardiac development and plays a role in the regulation of cardiac signal transduction after hypertrophic and mechanical stress because perivascular regions are hot spots for strain, remodeling, and increased cardioactive signaling in the heart.

Loss of Pin1 Attenuates Hypertrophy and Preserves Cardiac Function on Pressure Overload

Global Pin1 knockout mice[35] were shown to exhibit a multitude of age-associated diseases,[20,21,36] but cardiac function was not assessed. Echocardiographic analysis of hearts in Pin1 knockout mice up to 6 months of age (Figure 2A) showed a normal cardiac phenotype under basal conditions (Online Table). Pin1 knockout mice subjected to pathological challenge by TAC showed significantly lower mortality rate during 4 weeks after TAC (11%) relative to wild-type littermate controls (63%) as depicted by Kaplan–Meier analysis (Figure 2B). Pressure overload induced cardiac hypertrophy (127% increase) without affecting cardiac function in wild-type mice at 2 weeks after TAC, whereas increased wall thickness was attenuated in Pin1 knockout mice (Figure 2C). Posterior wall thickness and left ventricular diameter at diastole increased by 132% and 144%, respectively, in conjunction with a loss of fractional shortening by 33% in wild-type mice (Figure 2C) at 4 weeks after TAC. Increased left ventricular diameter at diastole was diminished and cardiac function was preserved in Pin1 knockout mice (Figure 2C). Equivalent systolic arterial blood pressure between wild-type and TAC hearts confirmed uniform constriction of the aorta in both groups (Figure 2D). Echocardiographic data were corroborated by terminal invasive hemodynamic assessment at 4 weeks after TAC, confirming that maximally and minimally developed pressure over time (max dP/dt and min dP/dt) were higher in Pin1 knockout mice compared with wild-type controls (Figure 2D). The heart weight/body weight ratios were also decreased in Pin1 knockout mice (Figure 2E). The cross-sectional area of myocytes increased in wild-type mice by 177% after TAC, but Pin1 knockout mice showed blunted cellular enlargement (Figure 2F). Collectively, these data indicate that deletion of Pin1 attenuates the hypertrophic response to pressure overload and preserves cardiac function.

Loss of Pin1 Dampens Cultured Cardiomyocyte Hypertrophy via Inhibition of Akt and MEK Activation

The molecular mechanism underlying attenuation of cardiac hypertophy by Pin1 deletion was determined using siRNA to deplete Pin1 in NRCMs followed by hypertrophic stimulation with serum or phenylephrine. Successful knockdown of Pin1 protein was confirmed by immunoblot (Figure 3A). Cell size increased by 153% and atrial natriuretic peptide levels rose by 556% on growth induction by serum stimulation in NRCM treated with scrambled RNA sequence as control (Figure 3B and 3C). In addition, phenylephrine stimulation increased cell area by 133% and atrial natriuretic peptide by 470% (Figure 3B and 3C). In contrast, increases in cell size and atrial natriuretic peptide expression on hypertrophic stimulation were mitigated by siRNA knockdown of Pin1 (siPin1; Figure 3B and 3C). Known binding partners of Pin1 in noncardiac cell types include Akt and MEK,[13,33] both of which are established mediators of hypertrophic signaling.[26,27] Thus, participation of Akt and MEK in Pin1-mediated regulation of hypertrophic remodeling was determined after Pin1 depletion. Direct interaction between Pin1 and Akt or MEK was demonstrated by proximity ligation assay (Figure 3D) and immunoprecipitation (Online Figure III) in NRCMs. Interestingly, the interaction of Pin1 with Akt and MEK was increased under hypertrophic growth conditions (Figure 3D). Pin1 binding modifies target protein conformation and activity after phosphorylation; therefore, phosphorylation of Akt and MEK after serum or phenylephrine treatment was measured in siRNA knockdown of Pin1-treated NRCMs. Sustained Akt phosphorylation up to 120 minutes after serum stimulation or MEK activation within 1 minute after phenylephrine exposure was attenuated by siRNA knockdown of Pin1 (Figure 3E and 3F). Therefore, attenuation of hypertrophy after siRNA knockdown of Pin1 treatment is likely, in part, because of combined interference with well-accepted Akt- and MEK-dependent prohypertrophic signaling pathways. Confirmation of MEK pathway activation is corroborated by phosphorylation of ERK, the direct downstream target of MEK (Online Figure IVA and IVB). Extrapolation to the in vivo context shows that Akt and ERK phosphorylation was increased in hearts of wild-type mice; however, activation of both enzymes was blunted in Pin1 knockout hearts measured after 4 days of TAC (Figure 3G). Collectively, these data show that loss of Pin1 compromises Akt and MEK activation, thereby attenuating cardiomyocyte hypertrophy in vitro and in vivo.

Pin1 Overexpression Preserves Cardiac Function After Pressure Overload

Cardiac-specific Pin1 overexpression was accomplished using an AAV serotype 9 harboring Pin1 downstream of

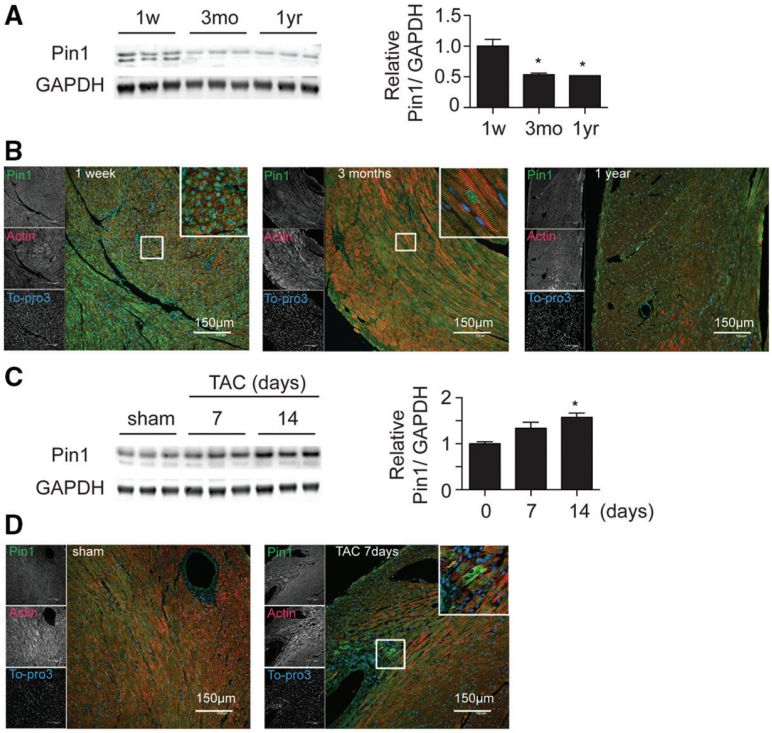

Figure 1. Pin1 decreases with aging and is induced after pressure overload in the murine heart. A, Immunoblot showing decline of Pin1 expression in the heart during development at 1 week, 3 months, and 1 year of age (**left**) with densitometric quantification on the **right**. **P*<0.05 vs 1 week (n=3). **B**, Paraffin-embedded sections from mouse hearts at the same time points as in **A** were stained for Pin1 (green), actin (red), and To-pro3 (blue). Boxed regions are shown at higher magnification in the **upper right corner**. **C**, Immunoblot showing induction of Pin1 expression in the heart 7 and 14 days after transaortic constriction (TAC; **left**) with densitometric quantification on the **right**. **P*<0.05 vs day 0 (n=3). **D**, Paraffin-embedded sections from mouse hearts 7 days after TAC were stained with Pin1 (green), actin (red), and To-pro3 (blue). Boxed regions are shown at higher magnification in the **upper right corner**.

cytomegalo virus–enhanced cardiac-specific myosin light chain promoter (AAV9 harboring Pin1 downstream of the MLC2v promoter [AAV-Pin1]).[37] Adult mice were infected with AAV-Pin1 and allowed to accumulate Pin1 protein in the heart for 6 weeks after viral exposure (Figure 4A). Basal cardiac function between mice receiving control virus (AAV-control) and AAV-Pin1 was comparable at 6 weeks after viral exposure (data not shown). Hearts of normal versus AAV-Pin1–treated mice were then challenged by TAC surgery at 12 weeks of age. Systolic blood pressure increased to the same extent in both the AAV-control and AAV-Pin1 groups (Figure 4C). Posterior wall thickness increased by

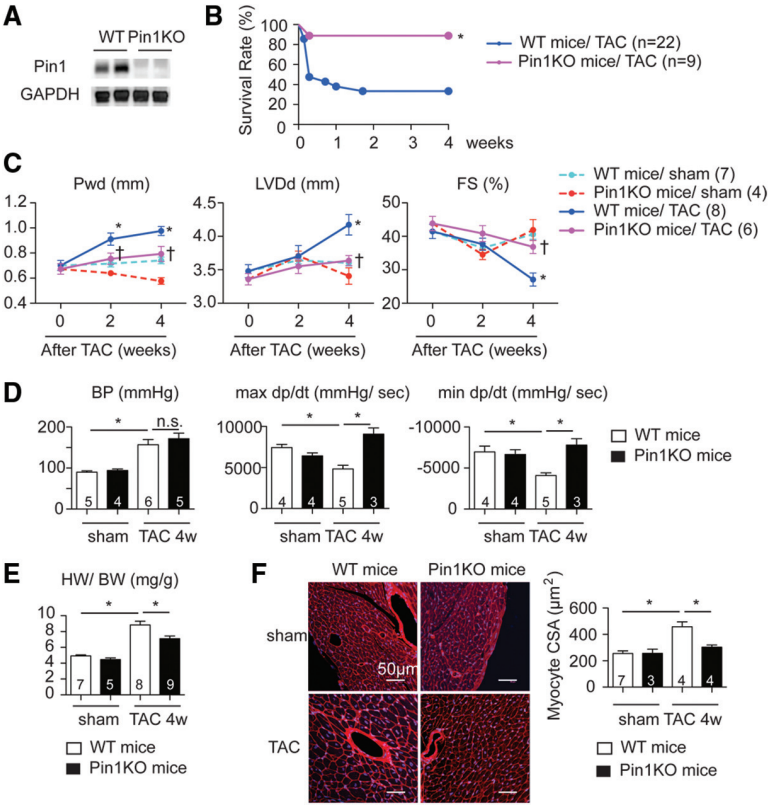

Figure 2. Loss of Pin1 attenuates hypertrophy and preserves cardiac function on pressure overload. A, Immunoblot showing Pin1 expression in the heart of wild-type (WT) and Pin1 knockout (Pin1 KO) mice. **B**, Kaplan–Meier curve after transaortic constriction (TAC) procedure. **P*<0.05 vs WT mice exposed with TAC. **C**, Echocardiographic analysis at the indicated time points after TAC procedure showing posterior wall thickness at diastole (Pwd), left ventricular dimension at diastole (LVDd), and fractional shortening (FS). **P*<0.05 vs WT sham, †*P*<0.05 vs WT TAC at the same time point. **D**, Invasive hemodynamic assessment 4 weeks after TAC showing arterial systolic blood pressure (BP), maximal (max dp/dt) and minimal (min dp/dt) changes of developed pressure over time (**P*<0.05). **E**, Heart weight/body weight ratio (HW/BW) at 4 weeks after TAC (**P*<0.05). **F**, Sections 4 weeks after TAC stained with wheat germ agglutinin (WGA; red) and To-pro3 (blue; **left**); quantification of cross-sectional area (CSA) of cardiomyocytes (**right**; **P*<0.05).

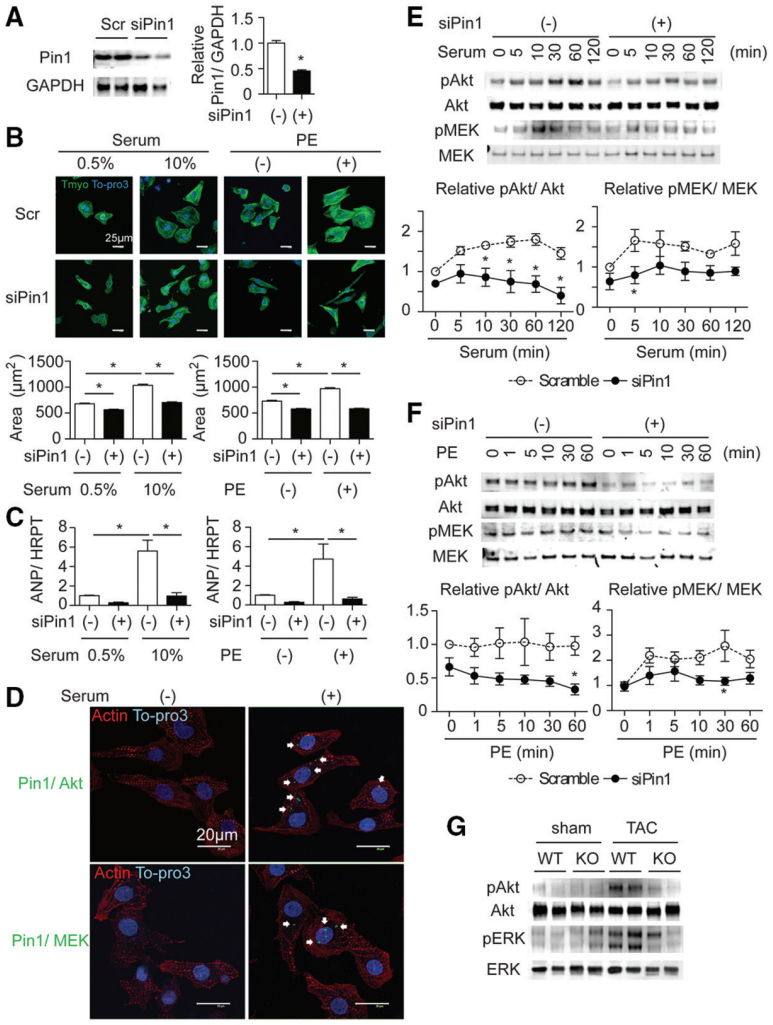

Figure 3. Loss Pin1 attenuates cardiomyocyte hypertrophy in vitro via regulating Akt and MEK pathway. A, Immunoblot showing Pin1 expression after scramble (Scr) or Pin1-specific siRNA treatment (siPin1) in neonatal rat cardiomyocytes (NRCMs; **left**) and densitometric quantification (**right**). *P<0.05 vs Scr (n=8). **B**, Immuncytochemistry of NRCMs treated with 10% serum or phenylephrine (PE) and stained for tropomyosin (Tmyo; green) and To-pro3 (blue) after Scr or siRNA treatment (**top**). Quantification of cardiomyocyte area on the **right**. *P<0.05 (n=3). **C**, Quantitative real-time polymerase chain reaction showing atrial natriuretic peptide (ANP) expression in NRCMs under the same conditions as in **B**. *P<0.05 (n=3). **D**, Proximity ligation assay showing direct interaction of Pin1 with Akt (**top**) or MEK (**bottom**) in cardiomyocytes (green dots, **white arrows**) with (**right**) or without (**left**) stimulation. Cardiomyocytes were also stained with Tmyo (red) and To-pro3 (blue). **E**, Immunoblots showing time course analysis for Akt and MEK phosphorylation after serum stimulation in Scr and siPin1-treated NRCMs (**top**). Densitometric quantification is shown in the **bottom** for Akt (**left**) and MEK (**right**; n=5). **F**, Immunoblot showing time course analysis for Akt and MEK phosphorylation after PE stimulation in Scr and siPin1-treated NRCMs (**top**). Densitometric quantification is shown in the **bottom** for Akt (**left**) and MEK (**right**; n=6). **G**, Immunoblot showing Akt and ERK phosphorylation in the heart of wild-type (WT) and Pin1 knockout (KO) mice at 4 days after transaortic constriction (TAC).

121% with a loss of fractional shortening by 19% in AAV-control mice (Figure 4B) 6 weeks after TAC. In comparison, cardiac function was preserved in AAV-Pin1 mice (Figure 4B) and subsequently confirmed by invasive hemodynamic assessments (Figure 4C). Comparable results were observed in AAV-control and AAV-Pin1–treated mice when either wall thickness (Figure 4B) or heart weight/body weight ratios were examined (Figure 4D). However, at the cellular level, cardiomyocyte size was increased by 167% in AAV-control mice after TAC, whereas AAV-Pin1 treatment blunted cellular hypertrophy (Figure 4E). Survival was comparable among all groups (6 of 9 for the AAV control versus 11 of 12 for the AAV-Pin1 group). In summary, cardiac-specific Pin1 overexpression preserves cardiac function and blunts cardiac hypertrophy at the cellular level in vivo on pressure overload.

Overexpression of Pin1 Attenuates Cardiomyocyte Hypertrophy In Vitro Through Inhibition of MEK but Not Akt

The molecular basis for Pin1-mediated attenuation of cardiac hypertrophy was delineated in further analyses involving NRCMs subjected to hypertrophic stimuli in vitro. Pin1 overexpression in NRCMs (Figure 5A) attenuated hypertrophic responses relative to the EGFP-expressing control cells where cell size increased by 183% and atrial natriuretic peptide expression by 396% after serum treatment (Figure 5B and 5C). Furthermore, in control NRCMs, cell area increased by 200% and atrial natriuretic peptide increased by 397% after phenylephrine stimulation, both of which are inhibited by Pin1 overexpression. Exogenously expressed Pin1 localized to both cytosol and nuclear compartments (Figure 5B), which was confirmed using cell fractionation samples (data not shown). The kinetics of Akt phosphorylation, indicative of activation in response to serum or phenylephrine stimulation, were similar in control and Pin1-overexpressing NRCMs, suggesting that Akt is not responsible for Pin1 overexpression–mediated decreases in the hypertrophic response (Figure 5D and 5E). In contrast, in NRCMs treated with serum or phenylephrine, MEK and ERK activation was significantly decreased by Pin1 overexpression (Figure 5D and 5E; Online Figure IV). Involvement of the MEK/ERK cascade in Pin1-overexpression signaling was corroborated in vivo by measurement of phosphorylated Akt and ERK in the hearts of mice treated with AAV-control or AAV-Pin1 and subjected to TAC. ERK but not Akt activation was blunted in AAV-Pin1 TAC hearts compared with controls (Figure 5F). Collectively, these data indicate that Pin1 overexpression blunts TAC-induced hypertrophy via inhibition of MEK but not Akt signaling.

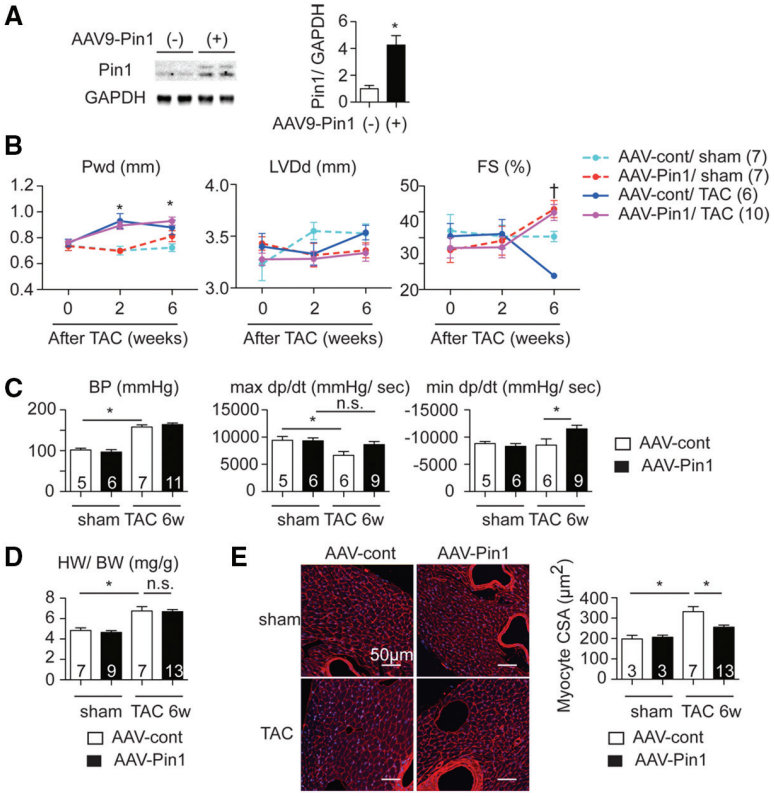

Figure 4. Adeno-associated virus serotype 9 (AAV9)-Pin1 attenuates hypertrophy and preserves cardiac function on pressure overload. **A**, Immunoblot showing Pin1 expression in the heart of mice injected with empty AAV9 vector (AAV-cont) and with AAV9 harboring Pin1 (AAV-Pin1). **Right**, Densitometric quantification of Pin1 expression (n=3–4). **B**, Echocardiographic analysis at the indicated time points after transaortic constriction (TAC) procedure showing posterior wall thickness at diastole (Pwd), left ventricular dimension at diastole (LVDd), and fractional shortening (FS). **P*<0.05 vs wild-type (WT) sham, †*P*<0.05 vs WT TAC at the same time point. **C**, Invasive hemodynamic assessment 6 weeks after TAC showing arterial systolic blood pressure (BP), maximal (max dp/dt) and minimal (min dp/dt) changes of developed pressure over time (**P*<0.05). **D**, Heart weight/ body weight ratio (HW/BW) at 6 weeks after TAC (**P*<0.05). **E**, Sections 6 weeks after TAC stained with wheat germ agglutinin (WGA; red) and To-pro3 (blue; **left**), quantification of cross-sectional area (CSA) of cardiomyocytes (**right**; **P*<0.05). n.s. indicates not significant.

The seemingly paradoxical finding that Pin1 inhibition and overexpression blunted MEK activation can be reconciled by the involvement of additional inhibitory signaling upstream of MEK, ultimately leading to reduced MEK activation. Specifically, Raf-1 is a direct activator of MEK and a target of Pin1, but phosphorylation of Raf-1 on Ser259 inhibits kinase activity, leading to reduced MEK phosphorylation.[38] Indeed, Pin1 binding to Raf-1 was confirmed by proximity ligation assay (Figure 6A), and compared with GFP-expressing controls, phosphorylation of the Raf^{Ser259} inhibitory site was induced by serum and phenylephrine in NRCMs overexpressing Pin1 (Figure 6B and 6C). Thus, Pin-1 overexpression blunts MEK activation through phosphorylation of the Raf^{Ser259} inhibitory site.

Discussion

Elevation of Pin1 in cancer cells suggests a role in regulation of cellular growth[8] consistent with Pin1 induction by growth factors, including insulin-like growth factor-1.[39] In comparison, unlike transformed proliferative cells, the postmitotic nature of mature cardiomyocytes underscores a distinct context-dependent nature for Pin1 effects. Extrapolating from Pin1 literature, neurons (like cardiomyocytes) are also notoriously resistant to growth stimuli, prompting comparisons between postmitotic neuronal cells and transformed cells. Comparing these 2 divergent cell types, Pin1 expression acts as molecular timer for signaling to maintain healthy aging in postmitotic cells such as neurons,[20,25] whereas dysregulation of Pin1 in proliferating cells alters regulators of cell cycle and commitment, leading to oncogenic transformation.[7,12,19,32] Mature cardiomyocytes respond to growth stimuli by remodeling,[40,41] and our findings support a role for Pin1 as a regulator of myocardial hypertrophy on both cellular and organ levels. The significance for Pin1 in the myocardial context is the novel finding that canonical hypertrophic signaling pathways are influenced by Pin1 activity, ultimately determining the adaptive cellular response and overall outcome of the heart when challenged by hypertrophic stimulation.

Pin1 increases proliferation and survival in tumor cells through maintenance of oncogenic proteins such as Akt.[13] In the cardiac context, Akt is a well-accepted mediator of cardiac hypertrophy.[27] Constitutive overexpression of Akt induces cardiomyocyte hypertrophy in vitro and in vivo, ultimately leading to heart failure,[42,43] whereas transient Akt activation also induces cardiac hypertrophy; however, cardiac function is preserved.[43] Therefore, the intensity and duration of Akt activation are critical for physiological versus pathological remodeling. Akt activation in cardiomyocytes was treated with serum peaks at 60 minutes, whereas Akt activation coupled with Pin1 silencing is weaker and returns to basal levels within 10 minutes after stimulation (Figure 3E). Conversely, Akt activation in the cardiomyocyte context is unaffected by Pin1 overexpression (Figure 5D and 5E). Although increased Pin1 expression can correspond to enhanced Akt activation, this correlation does not hold true for all cancer entities[13] and obviously does not apply for cardiomyocytes. Therefore, we posit that facilitation of Akt activation by Pin1 is likely to be cell type dependent, and because the heart possesses rather high Pin1 expression, endogenous Pin1 may be sufficient to saturate Akt regulation with additional Pin1 overexpression of no further consequence. However, loss-of-function studies confirm that Pin1 is a powerful modulator of Akt signal duration and intensity, implicating Pin1 in the regulation of cardiomyocyte hypertrophy.

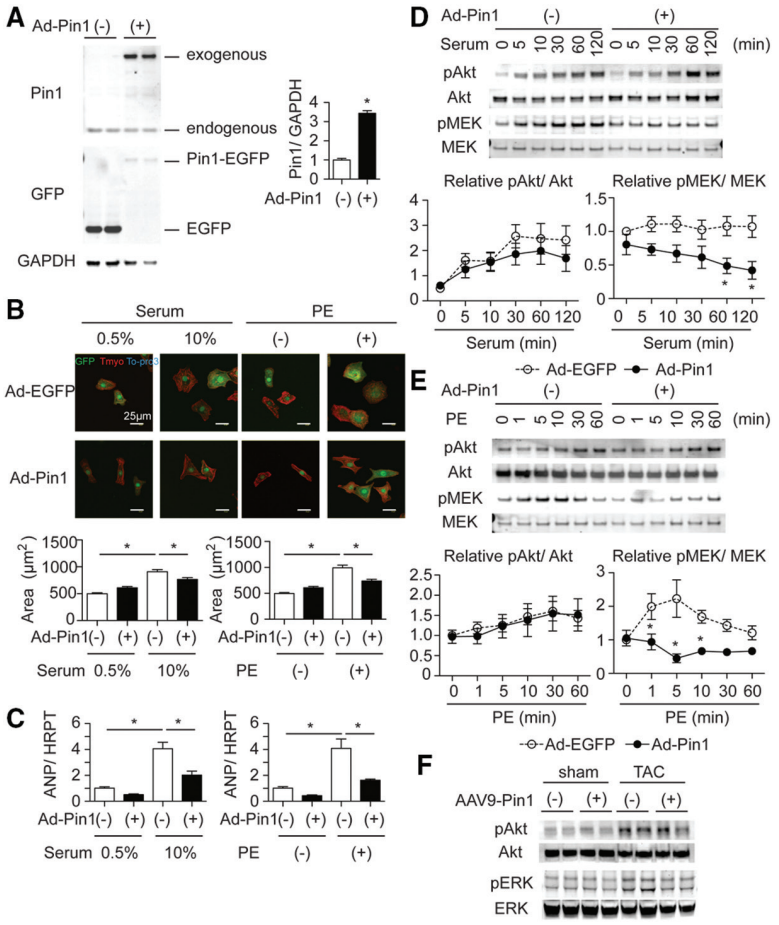

Figure 5. Overexpression of Pin1 attenuates cardiomyocyte hypertrophy in vitro via regulating MEK pathway. A, Immunoblot showing Pin1 and green fluorescent protein (GFP) expression in neonatal rat cardiomyocytes (NRCMs) transduced with adenovirus-expressing EGFP (Ad-EGFP) or Pin1-EGFP (Ad-Pin1). **Right**, Densitometric quantification of Pin1 expression. **P*<0.05 (n=6). **B**, Immuncytochemistry of NRCMs treated with 10% serum or phenylephrine (PE) and stained for GFP (green), tropomyosin (Tmyo; red), and To-pro3 (blue) after Ad-EGFP or Ad-Pin1 induction (**top**). **Bottom**, Cell size quantification only of cardiomyocytes expressing EGFP. **P*<0.05 (n=3). **C**, Quantitative real-time polymerase chain reaction showing atrial natriuretic peptide (ANP) expression in NRCMs under the same conditions as in **B**. **P*<0.05 (n=3). **D**, Immunoblot showing time course analysis for Akt and MEK phosphorylation after serum stimulation in Ad-EGFP– and Ad-Pin1–transduced NRCMs (**top**). Densitometric quantification is shown on the **bottom** for Akt (**left**) and MEK (**right**; n=7). **P*<0.05 vs control at the same time point. **E**, Immunoblot showing time course analysis for Akt and MEK phosphorylation after PE stimulation in Ad-EGFP– and Ad-Pin1–transduced NRCMs (**top**). Densitometric quantification is shown on the **bottom** for Akt (**left**) and MEK (**right**; n=6). **P*<0.05 vs control at the same time point. **F**, Immunoblot showing Akt and ERK phosphorylation in the heart of AAV-cont and AAV-Pin1 mice at 4 days after TAC.

The MEK-ERK cascade regulates fundamental cellular processes that govern cell transformation, differentiation, proliferation, and survival.[44] Like Akt in the cardiac context, the MEK-ERK cascade is another major inducer of hypertrophy.[26,45] In tumor cells, Pin1 modulates MEK activity through direct binding and maintenance of activation.[33] Similarly, Pin1 binds directly to MEK in cardiomyocytes after hypertrophic stimuli (Figure 3D), and Pin1 silencing attenuated MEK/ERK induction after stimulation independently of Akt (Figure 3E and 3F; Online Figure IVA and IVB). These data clearly show that Pin1 regulates the MEK-ERK pathway in cardiomyocytes and is needed for full hypertrophic response in vivo and in

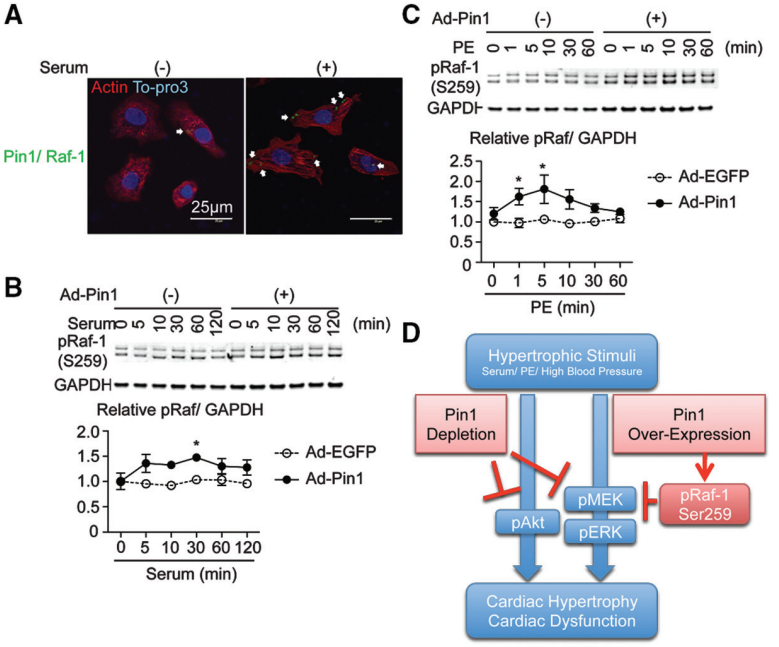

Figure 6. Raf-1 phosphorylation on an autoinhibitory site is increased in the cardiomyocyte transduced with Ad-Pin1. A, Proximity ligation assay showing direct interaction of Pin1 with Raf-1 in cardiomyocytes (green dots, **white arrows**) with (**right**) or without stimulation (**left**). Cardiomyocytes were also stained with tropomyosin (Tmyo; red) and To-pro3 (blue). **B**, Immunoblots showing time course analysis for Raf-1 phosphorylation on Ser259 (pRaf) after serum stimulation in enhanced green fluorescent protein (EGFP)– and Pin1-treated neonatal rat cardiomyocytes (**top**). Densitometric quantification is shown on the **bottom** for pRaf (n=5). **P*<0.05 vs control at the same time point. pRaf protein levels were normalized with GAPDH as a loading control. **C**, Immunoblots showing time course analysis for pRaf after phenylephrine (PE) stimulation (**top**). Densitometric quantification is shown in the **bottom** for pRaf (n=5). **P*<0.05 vs control at the same time point. pRaf protein levels were normalized with GAPDH as a loading control. **D**, Schematic showing the mechanistic impact of Pin1 depletion and overexpression on cardiac hypertrophy.

vitro. The overtly paradoxical finding that Pin1 overexpression reduced MEK activation prompted assessment of Raf-1 autoinhibition, revealing that MEK activation was blocked because of increased RafSer259 inhibitory site phosphorylation (Figures 5D, 5E, 6B, and 6C). The precise mechanism of how overexpression Pin1 increases Raf^{Ser259} inhibitory site phosphorylation remains unclear, and Pin1 has an opposite effect on Raf-1 activation by regulating dephosphorylation at alternative phosphorylation sites in NIH3T3 cells,[46] requiring further studies to clarify the role of Pin1 on Raf-1 activation in NRCMs. Mechanistically, the influence of Pin1 can be attributed, at least in part, to alterations in the intensity and duration of Akt- and MEK-dependent signaling cascades influenced by Raf-1–dependent feedback regulation of Pin1 activity.

Pin1 expression is likely to be tightly regulated within a narrow window to facilitate appropriate hypertrophic responses, as attenuation of cardiac growth was observed in response to both loss and overexpression of Pin1 after TAC (Figures 2 and 4) and as demonstrated by the ability to blunt MEK/ERK signaling through direct mechanisms in silencing studies or indirect feedback though the Raf-1 feedback cascade.

Groups receiving AAV treatment underwent surgery at postnatal 12 weeks that include a requisite 6-week incubation period for sufficient Pin1-AAV expression when delivered at 6 weeks of age. The 6-week postdelivery AAV incubation necessitated the use of a 26-gauge needle to allow survival of the 12-week-old animals at the time of TAC banding. Therefore, the appropriate control group for Pin1-AAV injections is the control-AAV cohort treated and operated on at the identical age. In comparison, the surgical protocol for mice not receiving AAV treatment consisted of TAC banding at 8 weeks of age because AAV incubation time for expression was not necessary. Because these young mice cope better with pressure overload, a 27-gauge needle was used for constriction surgery, resulting in greater constriction relative to the virus-treated groups at 12 weeks, leading to increased wild-type mouse mortality, although the measured developed systolic pressure was comparable at the time of euthanization as quantified by invasive hemodynamic assessment.

In the present study, we focused on 2 major well-established pro hypertrophic pathways, namely Akt- and the Raf-MEK-ERK cascade. Pin1 regulates activity or stability of phospho proteins only; therefore, activation per se of Akt and ERK is still possible in NRCMs treated with siRNA or hearts from Pin1 knockout mice. Signal duration and intensity are altered by modulation of Pin1, whereas in the Akt/ERK knockout mice, these pathways are deleted completely.[47–49] Other signaling pathways might be involved and contributing to the observed phenotype, which is noted as a limitataion of for the interpretation of our study.

Collectively, our results indicate a pivotal role for Pin1 as a regulator of the intensity and duration of the prohypertrophic signaling network in the heart. Previously documented effects of Pin1 in the noncardiac context will be the focus of future studies involving the multifaceted nature of Pin-1–mediated consequences for many significant areas of current myocardial signal transduction under investigation, including proliferation, survival, aging, and cell fate determination.

Acknowledgments

We thank the Sussman laboratory members for their critical review of the article; Dr Roger J. Hajjar, Mount Sinai School of Medicine, for the generous gift of pTRUF; and Dr Giannino Del Sal, Laboratorio Nazionale CIB, for the generous gift of human Pin1 cDNA.

Sources of Funding

This study was supported by the National Institutes of Health (R21HL102714, R01HL067245, R37HL091102, P01HL085577, RC1HL100891, R21HL102613, R21HL104544, and R01HL105759 to M.S.A. and RO1 HL75573, RO1 HL104535, and RO3 EB011698 to C.C.G.), American Heart Association (11POST7610164), The Uehara Memorial Foundation and Mochida Memorial Foundation for Medical and Pharmaceutical Research (H.T.), Deutsche Forschungsgemeinschaft DFG (1659/1–1 to M.V. and 3900/1–1 to M.H.K.), DZHK (German Centre for Cardiovascular Research) and BMBF (German Ministry of Education and Research to O.J.M.), and Rees-Stealy Research Foundation, San Diego Chapter of the Achievement Rewards for College Scientists Foundation, American Heart Association (Predoctoral Fellowship 10PRE3410005), and Inamori Foundation (to S.D.).

Disclosures

None.

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Novelty and Significance

What Is Known?

- Cardiac hypertrophy is regulated not only by activation of specific signal transduction pathways but also by the intensity and duration of the respective enzymatic activities.
- Pin1 regulates the duration and intensity of activity by enzymes responsible for cell growth and survival in cancer cells.

What New Information Does This Article Contribute?

- Pin1 binds directly to key mediators of cardiac remodeling after hypertrophic stimulation in cardiomyocytes in vitro.
- Loss of Pin1 attenuates hypertrophic signaling of Akt and MEK.
- Overexpression of Pin1 increases Raf-1 phosphorylation at the auto-inhibitory site, leading to impaired MEK activation.

The role of Pin1 as a regulator of cell growth and survival has been described in cancer cells. This is the first report elucidating the role of Pin1 as a modulator of Akt and MEK signaling during pressure overload in the heart. Our data show that Pin1 operates within a restricted range because both overexpression and downregulation of Pin1 attenuate cardiac hypertrophy. Our findings suggest that Pin1 represents a novel target for therapeutic intervention during pathological cardiac hypertrophy.