PICOT Attenuates Cardiac Hypertrophy by Disrupting Calcineurin–NFAT Signaling

Dongtak Jeong,* Ji Myoung Kim,* Hyeseon Cha, Jae Gyun Oh, Jaeho Park, Soo-Hyeon Yun, Eun-Seon Ju, Eun-Seok Jeon, Roger J. Hajjar, Woo Jin Park

Abstract—PICOT (protein kinase C–interacting cousin of thioredoxin) was previously shown to inhibit pressure overload-induced cardiac hypertrophy, concomitant with an increase in ventricular function and cardiomyocyte contractility. The combined analyses of glutathione S-transferase pull-down experiments and mass spectrometry enabled us to determine that PICOT directly interacts with muscle LIM protein (MLP) via its carboxyl-terminal half (PICOT-C). It was also shown that PICOT colocalizes with MLP in the Z-disc. MLP is known to play a role in anchoring calcineurin to the Z-disc in the sarcomere, which is critical for calcineurin–NFAT (nuclear factor of activated T cells) signaling. We, therefore, suggested that PICOT may affect calcineurin–NFAT signaling through its interaction with MLP. Consistent with this hypothesis, PICOT, or more specifically PICOT-C, abrogated phenylephrine-induced increases in calcineurin phosphatase activity, NFAT dephosphorylation/nuclear translocation, and NFAT-dependent transcriptional activation in neonatal cardiomyocytes. In addition, pressure overload–induced upregulation of NFAT target genes was significantly diminished in the hearts of PICOT-overexpressing transgenic mice. PICOT interfered with MLP–calcineurin interactions in a dose-dependent manner. Moreover, calcineurin was displaced from the Z-disc, concomitant with an abrogated interaction between calcineurin and MLP, in the hearts of PICOT transgenic mice. Replenishment of MLP restored the hypertrophic responses and the increase in calcineurin phosphatase activity that was inhibited by PICOT in phenylephrine-treated cardiomyocytes. Finally, PICOT-C inhibited cardiac hypertrophy to an extent that was comparable to that of full-length PICOT. Taken together, these data suggest that PICOT inhibits cardiac hypertrophy largely by negatively regulating calcineurin–NFAT signaling via disruption of the MLP–calcineurin interaction. (Circ Res. 2008;102:0-0.)

Key Words: cardiac hypertrophy ■ PICOT ■ muscle LIM protein ■ calcineurin ■ NFAT

The myocardium undergoes hypertrophic growth in response to a variety of pathological insults, including hypertension, ischemic heart disease, valvular insufficiency, and cardiomyopathy. At the single-cell level, cardiac hypertrophy is characterized by increases in cell diameter and length and by alterations in gene expression.1,2 Although cardiac hypertrophy is thought to be initially beneficial by maintaining and/or augmenting cardiac output, a sustained hypertrophic state leads to deleterious alterations in left ventricular architecture and is a leading predictor for the development of arrhythmias, heart failure, and sudden death.3–5 Therapies that are directed at inhibiting or reversing cardiac hypertrophy are therefore of significant clinical value. Intensive investigations in the past have revealed crosstalk between multiple parallel prohypertrophic signaling pathways that comprise a complex hypertrophy signaling network (reviewed previously1–5). In addition, potential antihypertrophic and inhibitory feedback signaling pathways have been discovered, adding further complexity to the regulatory network.6 Augmenting these negative signaling pathways may be a viable antihypertrophic strategy.7

We have previously shown that PICOT (protein kinase [PKC]–interacting cousin of thioredoxin) activity constitutes a negative feedback loop for cardiac hypertrophy.8 PICOT expression is upregulated on hypertrophic stimulation and, in turn, abrogates the development of cardiac hypertrophy. In addition, PICOT overexpression significantly enhances ventricular function and cardiomyocyte contractility. With these unusual characteristics, antihypertrophic and positive inotropic activities in the same molecule, PICOT appears to be a potential modality for preventing cardiac hypertrophy and heart failure. However, the detailed molecular mechanisms...

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underlying the 2 distinct activities of PICOT have not been determined.

In this study, we examined the signaling pathways associated with PICOT by isolating PICOT binding partners. Our results reveal that PICOT inhibits cardiac hypertrophy largely by blocking the calcineurin–NFAT signaling pathway via competitive binding to muscle LIM protein (MLP).

**Materials and Methods**

An expanded Materials and Methods section is in the online data supplement, available at http://circres.ahajournals.org.

**Cell Culture and Hypertrophic Stimulation**

Primary cultures of cardiomyocytes from 1- to 3-day-old Sprague–Dawley rats were prepared as described. Briefly, ventricular tissue was enzymatically dissociated, and the resulting cell suspension was enriched for cardiomyocytes using Percoll (Amersham Pharmacia) step gradients. Cells were plated onto collagen-coated culture dishes or coverslips and cultured in cardiomyocyte culture medium (DMEM supplemented with 10% FBS, 2 mmol/L-glutamate, and 0.1% Triton X-100, 50 μmol/L 5-bromodeoxyuridine; Gibco-BRL). To induce hypertrophy, cardiomyocytes were cultured in serum-free medium for at least 24 hours and then treated with 100 μmol/L PE for 40 hours. Luciferase activity was measured using a Lumat LB 9501 luminometer (Berthold).

**Quantitative RT-PCR**

Total RNA was isolated with TRI reagent (Sigma). Reverse-transcription was performed using ImProm II reverse-transcriptase (Promega) with oligo-dT priming. PCR was performed using an ABI PRISM Sequence Detector System 7500 (Applied Biosystems) with SYBR Green (Takara) as fluorescent and ROX (Takara) as reference dyes. PCR primers used were: atria natriuretic factor, 5'-AGG TAG ACC ACC TGG AGG AG-3' and 5'-CCT TGG CTG TTA TCT TCG GTA CCG-3', brain natriuretic peptide, 5'-GCT GCT TGG GGC ACA AGA TAG-3' and 5'-GGT CTI CCT ACA ACA ACT TCA-3', myocyte-enriched calcineurin interacting protein (MCIP1), 5'-TCC AGT GGC TTC TTG ACT GAG-3' and 5'-ACT GGA AGG TGG TGT CCT TGT C-3'.

**Contractility Measurements**

Adult rat ventricular myocytes were isolated via enzymatic dissociation, as described previously, and infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C for 12 to 15 hours. Only green fluorescent protein–positive cells were examined. Contraction amplitudes and rates of contraction and relaxation were recorded using a dual excitation spectrofluorometer and a video-edge detection system (IonOptix), as described previously.

**Statistics**

Where appropriate, the data are expressed as means±SDs. Comparisons of the group means were made using a Student t test or 1-way ANOVA with a Bonferroni post test analysis (Statview version 5.0, SAS). P<0.05 was considered to be statistically significant.

**Results**

**PICOT Binds to the MLP via Its Carboxyl-Terminal PICOT Homology Domain**

The amino-terminal region of PICOT (residues 1 to 143) is highly homologous to thioredoxin and is thus referred to as the thioredoxin homology (TH) domain, whereas the carboxyl-terminal region (residues 145 to 335) contains 2 tandem repeats provisionally termed the PICOT homology (PH) domain (Figure 1A). We generated GST-PICOT fusion proteins, designated GST-PICOT, GST-PICOT-N, and GST-PICOT-C, which contain the full-length, amino-terminal, and carboxyl-terminal regions of PICOT, respectively. Similarly, we generated recombinant adenoviruses that express HA-tagged full-length, amino-terminal, and carboxyl-terminal PICOT, designated AdPICOT, AdPICOT-N, and AdPICOT-C, respectively (Figure I in the online data supplement and Figure 1A). GST pull-down assays revealed that PICOT directly binds to PKCζ, but not to PKCα or PKCe, via...
its amino-terminal TH domain; this result is consistent with previous reports (data not shown).12

To identify proteins that interact with the carboxyl-terminal half of PICOT, GST pull-down experiments coupled with mass spectrometric analysis were performed. Purified GST-PICOT-C was incubated with extracts from rat hearts, and proteins that coprecipitated with PICOT-C were separated by SDS-PAGE and stained with Coomassie blue. Mass spectrometric analysis of the distinct protein bands revealed that MLP (also known as CRP3) was associated with PICOT (data not shown). MLP was particularly of interest because it was previously shown to function as a component of the stretch sensor machinery and defects in MLP lead to dilated cardiomyopathy and heart failure.13,14 To verify that PICOT directly interacts with MLP, a series of pull-down assays was conducted. Purified GST-PICOT was incubated with extracts from NIH-3T3 cells that were transfected with HA-tagged CRP1, CRP2, and MLP (CRP3) expression vectors. The resulting precipitates were separated by SDS-PAGE, transferred to poly(vinylidene difluoride), and blotted with an anti-HA antibody. The results showed that PICOT specifically interacted with CRP2 and MLP, but not with CRP1 (Figure 1B). A similar pull-down experiment showed that MLP coprecipitated with PICOT-C but not with PICOT-N (Figure 1C). Conversely, purified GST-MLP was incubated with extracts from NIH-3T3 cells that were infected with AdPICOT, AdPICOT-N, and AdPICOT-C. Western blotting of the resulting precipitates revealed that PICOT and PICOT-C, but not PICOT-N, coprecipitated with MLP (Figure 1D). GST-MLP fusion proteins containing full-length MLP, the amino-terminal, or carboxyl-terminal halves of MLP were generated and designated as GST-MLP, GST-MLP-N, and GST-MLP-C, respectively. A pull-down experiment performed with these fusion proteins revealed that MLP-N, but not MLP-C, binds to PICOT (Figure 1E). Notably, PICOT and MLP were coimmunoprecipitated from lysates of adult rat left ventricle and neonatal cardiomyocytes, implying that the interaction between PICOT and MLP is physiologically relevant (Figure 1F). Finally, PICOT and MLP colocalized in the Z-disc when adult heart sections were immunostained with anti-PICOT and anti-MLP antibodies. PICOT colocalized also with a known Z-disc protein, α-actinin (Figure 1G). These data indicate that PICOT binds to MLP through a direct interaction between the carboxyl-terminal PH domain of PICOT and the amino-terminal half of MLP.

PICOT Inhibits PE-Induced Activation of Calcineurin–NFAT Signaling

MLP is required for calcineurin–NFAT signaling at the sarcomeric Z-disc.15 Our finding that PICOT directly interacts with MLP raised the possibility that PICOT may affect calcineurin–NFAT signaling. To test this hypothesis, calcineurin phosphatase activity was measured in cardiomyocytes that were stimulated with PE, an agonist for cardiac hypertrophy, and infected with either AdLacZ or AdPICOT. Adenoviral infection preceded PE stimulation by 24 hours to allow the adequate expression of β-galactosidase (control) or PICOT. Calcineurin phosphatase activity was measured in cardiomyocytes that were infected with AdLacZ and AdPICOT. Calcineurin phosphatase activity significantly increased in cardiomyocytes that were infected with AdLacZ (P < 0.05) in response to PE treatment. However, AdPICOT infection blunted the increase in calcineurin activity induced by PE (Figure 2A). Activated calcineurin directly binds to NFAT transcription factors, resulting in NFAT dephosphor-
NFAT was significantly dephosphorylated by PE in cardiomyocytes infected with AdLacZ. However, NFAT dephosphorylation was unaltered by PE in cardiomyocytes that were infected with AdPICOT, implying that PICOT inhibits the PE-induced increase in calcineurin activity (Figure 2B). The subcellular localization of NFAT in neonatal cardiomyocytes was examined by immunostaining (supplemental Figure II). Nuclear localization of NFAT was observed in approximately 40% of unstimulated cardiomyocytes. However, treatment with PE increased the percentage of cardiomyocytes with nuclear NFAT staining to approximately 70%. Infection of cardiomyocytes with AdPICOT completely abrogated the PE-induced NFAT nuclear translocation, whereas the control AdLacZ had no effect. Infection with AdPICOT-C, but not AdPICOT-N, was effective in blocking NFAT nuclear translocation to a level comparable to that observed with AdPICOT (Figure 2C). To examine whether the alterations in NFAT nuclear translocation affected NFAT transcriptional activity, cardiomyocytes were transfected with a luciferase reporter plasmid driven by 3 NFAT binding sites.9 Treatment with PE significantly increased NFAT transcriptional activity. Infections with AdPICOT and AdPICOT-C completely inhibited PE-induced NFAT activation, whereas infection with AdLacZ or AdPICOT-N did not (Figure 2D). Hypertrophic marker genes encoding brain natriuretic peptide and MCIP1 are known to be direct transcriptional targets of NFAT in cardiomyocytes.15,16 Pressure overload markedly elevated the expression level of brain natriuretic peptide and MCIP1, which was significantly blunted in the hearts of PICOT transgenic (TG) mice (Figure 2E and 2F). These data indicate that PICOT inhibits PE-induced increases in calcineurin activity and NFAT nuclear translocation and transcriptional activation and that the carboxyl-terminal PH domain is sufficient for this inhibition.

**PICOT Interferes With the MLP–Calcineurin Interaction**

MLP directly associates with calcineurin and this interaction is reported to be critical for calcineurin–NFAT signaling.15 To further characterize the mechanism underlying the inhibitory action of PICOT on the agonist-induced activation of the calcineurin–NFAT signaling pathway, we examined whether PICOT affects the physical interaction between MLP.
and calcineurin. GST-PICOT or GST-MLP was incubated with extracts from NIH-3T3 cells that had been transfected with calcineurin expression constructs (left). GST-MLP, GST-MLP-N, and GST-MLP-C were used for similar pull-down assays (right). Precipitates were separated by SDS-PAGE and immunoblotted with anti-calcineurin antibody. B, GST-MLP was incubated with extracts (100 µg) from NIH-3T3 cells that had been transfected with a calcineurin expression construct in the presence of varying amounts of extracts (0 to 400 µg) from NIH-3T3 cells transfected with a PICOT expression construct. Precipitates were separated by SDS-PAGE and immunoblotted with anti-calcineurin and anti-PICOT antibodies. C, NIH-3T3 cells were transfected with HA-MLP expression vector (0.5 µg), calcineurin expression vector (4 µg), and varying amounts of PICOT expression vector (0 to 2 µg). Cell lysates were prepared and subjected to immunoprecipitation with anti-HA antibody. Precipitates were separated by SDS-PAGE and blotted with anti-calcineurin and anti-PICOT antibodies. D, Whole cell lysates from left ventricles of WT and PICOT TG mice were immunoprecipitated with anti-MLP antibody. Precipitates were separated by SDS-PAGE and blotted with anti-calcineurin antibody (left) or with anti-PICOT antibody (right). E, Left ventricular tissue sections from WT and TG mice were coimmunostained with anti-calcineurin and anti-α-actinin antibodies and then analyzed by confocal microscopy.

Figure 3. Competitive binding of PICOT and calcineurin to MLP. A, GST-PICOT and GST-MLP fusion proteins were incubated with extracts from NIH-3T3 cells that had been transfected with calcineurin expression constructs (left). GST-MLP, GST-MLP-N, and GST-MLP-C were used for similar pull-down assays (right). Precipitates were separated by SDS-PAGE and immunoblotted with anti-calcineurin antibody. B, GST-MLP was incubated with extracts (100 µg) from NIH-3T3 cells that had been transfected with a calcineurin expression construct in the presence of varying amounts of extracts (0 to 400 µg) from NIH-3T3 cells transfected with a PICOT expression construct. Precipitates were separated by SDS-PAGE and immunoblotted with anti-calcineurin and anti-PICOT antibodies. C, NIH-3T3 cells were transfected with HA-MLP expression vector (0.5 µg), calcineurin expression vector (4 µg), and varying amounts of PICOT expression vector (0 to 2 µg). Cell lysates were prepared and subjected to immunoprecipitation with anti-HA antibody. Precipitates were separated by SDS-PAGE and blotted with anti-calcineurin and anti-PICOT antibodies. D, Whole cell lysates from left ventricles of WT and PICOT TG mice were immunoprecipitated with anti-MLP antibody. Precipitates were separated by SDS-PAGE and blotted with anti-calcineurin antibody (left) or with anti-PICOT antibody (right). E, Left ventricular tissue sections from WT and TG mice were coimmunostained with anti-calcineurin and anti-α-actinin antibodies and then analyzed by confocal microscopy.
Anchorage of calcineurin at the Z-disc is essential for the coordinated dephosphorylation of NFAT.\textsuperscript{20,21} It has also been shown that calcineurin is dislocated from the Z-disc in MLP\textsuperscript{+/−} mice.\textsuperscript{15} We, therefore, assessed the localization of calcineurin in the hearts of WT and PICOT TG mice by immunohistochemistry. The expression pattern of calcineurin indicated that calcineurin colocalizes with MLP and α-actinin in WT hearts. However, the prominent striated expression pattern of calcineurin was significantly lost in PICOT TG hearts (Figure 3E), indicating that PICOT induces the dislocation of calcineurin from the Z-disc. Immunostaining with anti-MLP and anti-α-actinin revealed prominent striation in both WT and PICOT TG mice, excluding gross Z-disc abnormalities in PICOT TG mice (Figure 3E).

If our model is correct, replenishment of MLP should nullify the inhibitory activity of PICOT in calcineurin–NFAT signaling. To test this hypothesis, cardiomyocytes were infected with AdPICOT or coinfected with AdPICOT and AdMLP in the presence or absence of PE. The expression of PICOT and MLP was confirmed by Western blotting (Figure 4A). Whereas infection with AdPICOT alone inhibited PE-induced hypertrophy, coinfection with AdMLP and AdPICOT completely restored the AdPICOT-mediated repressed hypertrophy, as assessed by increased cell size, enhanced sarcomeric rearrangement, and upregulation of a hypertrophic marker gene, atria natriuretic factor (Figure 4B through 4D). The calcineurin phosphatase activity that was inhibited by infection with AdPICOT was also restored by coinfec tion with AdMLP (Figure 4E). Calcineurin expression levels were essentially unaltered (Figure 4F).

The Carboxyl-Terminal PH Domain of PICOT Is Sufficient in Inhibiting Cardiomyocyte Hypertrophy

The above data indicate that PICOT inhibits activation of the calcineurin–NFAT signaling pathway by competitively binding to MLP through its carboxyl-terminal PH domain. We therefore examined whether inhibition of calcineurin–NFAT signaling by the PH domain correlated with the inhibition of cardiomyocyte hypertrophy. Neonatal cardiomyocytes were infected with AdPICOT, AdPICOT-N, AdPICOT-C, or Ad-LacZ and were then further stimulated with PE. PE treatment significantly increased the size of AdLacZ-infected, as well as uninfected, cardiomyocytes. In contrast, the increase in cell size was completely abrogated by AdPICOT or AdPICOT-C infection and partially abrogated by AdPICOT-N infection.

**Table 1.**

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**Figure 4.** The antihypertrophic activity of PICOT nullified by replenishment of MLP. Neonatal cardiomyocytes were infected with AdPICOT alone or coinfected with AdPICOT and AdMLP and further stimulated with PE (100 μmol/L) for 24 hours. A, Cell lysates (50 μg) were separated by SDS-PAGE and blotted with anti-PICOT or anti-MLP antibodies. B, The cell surface area of cardiomyocytes was measured using i-solution software (n=100 cells each group). \( \cdot P < 0.05 \) against controls. C, Sarcomeric reorganization of cardiomyocytes was visualized by phalloidin staining (pictures are not shown). The morphology of myofibrils was semiquantitatively scored based on the area occupied by the reorganized sarcomere relative to the total cell area as follows: less than one-third of the cell area (white bar); more than one-third but less than two-thirds of the cell area (gray bar); more than two-thirds of the cell area (black bar) (n=50 for each group). \( \cdot P < 0.05 \) against controls. D, The relative expression levels of atria natriuretic factor (ANF) were determined by quantitative RT-PCR (n=3). \( \cdot P < 0.01 \) against controls. E, Cell lysates (3 μg) were subjected to calcineurin phosphatase activity assays (n=3). \( \cdot P < 0.05 \) against controls. F, Cell lysates (50 μg) were separated by SDS-PAGE and blotted with anti-calcineurin antibodies.
PICOT-C inhibits cardiac hypertrophy as efficiently as full-length cardiomyocytes (Figure 5B). These data indicate that cardiomyocytes, respectively, and 40% in AdPICOT-N infected cardiomyocytes, whereas the increases were only increased from 8% to 88% on PE treatment in AdLacZ-infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C and the extent of cell shortening was determined (AdLacZ, n=25; AdPICOT, n=27; AdPICOT-N, n=37; AdPICOT-C, n=14). *P<0.05 against controls.

Figure 5. Dissection of the antihypertrophic and inotropic effects of PICOT. A, Neonatal cardiomyocytes that had been infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C were treated for 24 hours in the presence or absence of PE (100 μmol/L). The cell surface area of cardiomyocytes was measured using i-solution software (n=100 cells each group). *P<0.05 against controls. B, Sarcomeric reorganization of cardiomyocytes was visualized by phalloidin staining. The morphology of myofibrils was semiquantitatively scored based on the area occupied by the reorganized sarcomere relative to the total cell area as indicated in Figure 4C (n=50 for each group). *P<0.05 against controls. C, Isolated adult cardiomyocytes were infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C and the extent of cell shortening was determined (AdLacZ, n=25; AdPICOT, n=27; AdPICOT-N, n=37; AdPICOT-C, n=14). *P<0.05 against controls.

We have previously shown that PICOT enhances cardiomyocyte contractility. Isolated adult rat cardiomyocytes were infected with AdPICOT, AdPICOT-N, and AdPICOT-C, and the consequential mechanical properties were determined using a dual-excitation spectrofluorometer equipped with a video-edge detection system. Whereas AdPICOT increased cell shortening by ~25%, neither AdPICOT-N nor AdPICOT-C significantly increased cell shortening (Figure 5C). These results indicate the presence of a separate mechanism for enhancement of contractility by PICOT.

**Discussion**

PICOT was first identified as a PKCδ-interacting protein in a yeast 2-hybrid screen. It has an amino-terminal TH domain that is highly homologous to the thioredoxin family proteins. PICOT is unlikely to be involved in intracellular redox regulation because the TH domain of PICOT lacks the conserved Cys-Gly-Pro-Cys motif that is essential for catalytic activity. Instead, the TH domain may serve as a structural motif for specific interactions with PKC. In our pull-down experiments using GST-PICOT and GST-PICOT-N fusion proteins, we confirmed that the TH domain binds to PKC (data not shown). The carboxyl-terminal region of PICOT contains 2 tandem repeats of an evolutionarily conserved domain of unknown function, referred to as the PH domain. In this study, we found that the PH domain directly binds to MLP. MLP interacts with telethonin, a titin binding protein, at the Z-disc and is critical for cardiac cytoarchitectural organization. MLP-deficiency leads to a selective defect in the mechanical stretch response and eventually to dilated cardiomyopathy and heart failure. Moreover, a human MLP mutation (W4R) that is associated with dilated cardiomyopathy results in a marked defect in MLP–telethonin interactions. It was, therefore, suggested that MLP functions as a pivot for the reception and transduction of mechanical stress signals in the Z-disc. Recently, MLP was found to play an essential role in calcineurin–NFAT signaling pathways, perhaps by anchoring calcineurin to the Z-disc. Calcineurin, a Ca\(^{2+}\)/calmodulin-dependent phosphatase, plays an essential role in cardiomyocyte growth and gene expression by promoting dephosphorylation and nuclear translocation of NFAT. A number of proteins bind to the catalytic subunit of calcineurin, calcineurin A, and regulate its enzymatic activity, including calcineurin B homologous protein, FKBP38, Cabin/Cain, MCIPI1, and a F-box adaptor protein atrogin-1. This study has demonstrated that PICOT competes with calcineurin for binding to MLP and, thus, interferes with the MLP–calcineurin interaction in vitro and in vivo.

We have also shown that disruption of the MLP–calcineurin interaction by PICOT inhibited increases in calcineurin phosphatase activity on PE treatment and also resulted in dislocation of calcineurin from the Z-disc; this result is consistent with a previous report showing that calcineurin is dislocated from the Z-disc in MLP-deficient mice. Therefore, sufficient MLP appears to be required both for PE-induced calcineurin phosphatase activation and anchoring hypertrophy largely by abrogating calcineurin–NFAT signaling through its carboxyl-terminal PH domain.

![Diagram](http://circres.ahajournals.org/Download/3111359296.s9203c0.png)
calcineurin to the Z-disc. This notion is partially supported by our data showing that replenishment of MLP suppressed the function of PICOT in inhibiting PE-induced calcineurin phosphatase activation and hypertrophy in cardiomyocytes. It is currently unclear whether anchoring calcineurin to the Z-disc via an interaction with MLP is prerequisite for the induction of calcineurin phosphatase activity or vice versa. It is possible that anchoring calcineurin at the Z-disc may facilitate its access to specific substrates such as NFAT, which is also localized at the Z-disc.20 The extent to which anchoring calcineurin at the Z-disc contributes to activation of downstream NFAT signaling remains to be determined. In this sense, it is intriguing to note that calsarcin-1, a sarcoplasmic negative regulator of calcineurin, does not affect the localization of calcineurin at the Z-disc.10

We do not rule out another interesting possibility that the molecular events associated with PICOT, MLP, and calcineurin shown in this study are also occurring in nucleus because all 3 of these proteins are known to localize to the nucleus as well as the Z-disc. PICOT was shown to be phosphorylated and translocated to the nucleus in response to oxidative stress in human T cells.29 MLP was shown to be exclusively nuclear in nonmyogenic cells and in differentiating myogenic cells, whereas it is associated with F-actin in mature myotubes and myocytes.30 Moreover, MLP was shown to be relocated to the nucleus in hypertrophied and failing hearts.31,32 Interestingly, calcineurin is not only capable of dephosphorylating NFAT, but the presence of calcineurin in the nucleus is also important for full NFAT transcriptional activity.33 The colocalization of PICOT, MLP, and calcineurin in the nucleus, especially in stressed conditions, suggests a scenario that MLP-associated calcineurin, which can be intervened by PICOT, may act as a transcriptional coactivator that is critical for eliciting hypertrophic responses. This hypothesis needs to be tested further. In conclusion, our study has revealed a novel regulatory point for modulating calcineurin–NFAT signaling which may provide a new therapeutic strategy for the treatment of cardiac hypertrophy and heart failure.

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Disclosures

None.

References


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**Immunoprecipitation**

Protein extracts (500 µg) from adult rat left ventricle, neonatal cardiomyocytes, or HEK293 cells were incubated with control IgG (Zymed), anti-PICOT, or anti-HA tag (Boehringer Mannheim) antibodies overnight at 4°C, and precipitated using protein A agarose (Invitrogen). The precipitates were washed with the GST binding buffer, subjected to SDS-PAGE, and blotted onto PVDF membranes (Bio-Rad).

**Western blot analysis**

Cardiomyocyte or HEK293 cell lysates were obtained by solubilizing cells in RIPA buffer (1% NP-40, 50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate, 150 mM NaCl, 10 mM NaF) containing a protease inhibitor cocktail (Boehringer Mannheim). Protein precipitates or cell lysates (50 µg) were separated by SDS-PAGE and transferred onto PVDF membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk and incubated overnight with antibodies against the HA tag (Boehringer Mannheim), α-tubulin (Santa Cruz), MLP (Abcam), calcineurin A (Chemicon), NFATc4 (Santa Cruz), phosphor-NFATc4 (Santa Cruz), or PICOT. The membranes were then incubated with a secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch) and developed using chemiluminescent substrates (Pierce).

**Immunohistochemistry**

The apical parts of the hearts were stored in 25% sucrose solution at 4 °C for 24 h, embedded in OCT compound, and sectioned (6 µm). The sections were fixed with acetone at -20°C for 5 min and dried in the air at room temperature (RT). After washing twice with phosphate buffered saline (PBS), the sections were blocked in PBS
containing 5% normal horse serum, 0.1% NaN3, 1% Triton X-100 and 0.2% bovine horse serum for 30 min at 37°C. The sections were subsequently incubated at 4 °C overnight with antibodies of PICOT, calcineurin (Chemicon), MLP (Abcam), and α-actinin (Sigma). After intensive washing with PBS containing 0.2% BSA, the sections were immunolabeled with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) or with Rhodamine-labeled goat anti-rabbit IgG (Jackson ImmunoResearch). After sections were washed, mounted with glycerol, and examined by confocal laser image microscopy (Nikon) at wavelength 460 nm for FITC, and 570 nm for Rhodamine.
Supplementary Figure 1.

(A) GST-PICOT fusion proteins were purified, separated by SDS-PAGE and stained with Coomassie blue.  (B) Protein extracts from neonatal cardiomyocytes that had been infected with adenoviruses expressing HA-PICOT fusion proteins were separated by SDS-PAGE and blotted with anti-HA antibody.
Supplementary Figure 2.

Neonatal cardiomyocytes that had been infected with AdLacZ, AdPICOT, AdPICOT-N, or AdPICOT-C were treated for 24 h in the presence or absence of PE (100 µM). Nuclear localization of NFAT was assessed by immunostaining with anti-NFATc4 antibody. Representative cytoplasmic and nuclear staining of NFATc4 is shown. DAPI was used to stain the nucleus.