PICOT Inhibits Cardiac Hypertrophy and Enhances Ventricular Function and Cardiomyocyte Contractility

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Abstract—Multiple signaling pathways involving protein kinase C (PKC) have been implicated in the development of cardiac hypertrophy. We observed that a putative PKC inhibitor, PICOT (PKC-Interacting Cousin Of Thioredoxin) was upregulated in response to hypertropic stimuli both in vitro and in vivo. This suggested that PICOT may act as an endogenous negative feedback regulator of cardiac hypertrophy through its ability to inhibit PKC activity, which is elevated during cardiac hypertrophy. Adenovirus-mediated gene transfer of PICOT completely blocked the hypertrophic response of neonatal rat cardiomyocytes to endothelin-1 and phenylephrine, as demonstrated by cell size, sarcomere rearrangement, atrial natriuretic factor expression, and rates of protein synthesis. Transgenic mice with cardiac-specific overexpression of PICOT showed that PICOT is a potent inhibitor of cardiac hypertrophy induced by pressure overload. In addition, PICOT overexpression dramatically increased the ventricular function and cardiomyocyte contractility as measured by ejection fraction and end-systolic pressure of transgenic hearts and peak shortening of isolated cardiomyocytes, respectively. Intracellular Ca\(^{2+}\) handling analysis revealed that increases in myofilament Ca\(^{2+}\) responsiveness, together with increased rate of sarcoplasmic reticulum Ca\(^{2+}\) reuptake, are associated with the enhanced contractility in PICOT-overexpressing cardiomyocytes. The inhibition of cardiac remodeling by overexpression of PICOT suggests that PICOT may provide an efficient strategy for treatment of cardiac hypertrophy and heart failure. (Circ Res. 2006;99:307-314.)

Key Words: cardiac hypertrophy ■ protein kinase C ■ PICOT ■ contractility

The myocardium undergoes adaptive hypertrophic growth to augment cardiac output in response to a variety of pathological insults. Although this response initially appears to be beneficial, sustained cardiac hypertrophy leads to an increased risk of sudden death or progression to heart failure.\(^1\) Therefore, therapies directed at inhibiting or reversing cardiac hypertrophy could be of significant clinical value. Intensive investigations in the past decade have revealed that multiple parallel intracellular signaling pathways can induce cardiac hypertrophy (reviewed previously\(^2\)–\(^4\)). No single pathway seems to regulate cardiac hypertrophy alone. Rather, it appears more likely that each pathway operates as a component of an orchestrated hypertrophic network.

In recent years, potential antihypertrophic and inhibitory feedback signaling pathways have been discovered. Their integration into the hypertrophic signaling pathway network adds considerable complexity. Atrial natriuretic factor (ANF), long considered to be a typical molecular marker for cardiac hypertrophy, has been shown to be antihypertrophic by activating cGMP-dependent protein kinase (PKG)\(^5\)\(^6\). In addition, a calcineurin inhibitory protein, myocyte-enriched calcineurin-interacting protein 1 (MCIP1), has been shown to participate in a negative feedback loop that limits the potentially deleterious effect of unrestrained calcineurin activity during cardiac hypertrophy\(^7\)\(^8\). Moreover, SOCS3 (a Suppressor Of Cytokine Signaling 3) binds to janus kinase (JAK) and acts as a negative feedback regulator of gp130-mediated cardiac hypertrophy\(^9\). Recently, the transcriptional repressor Nab1 has been shown to intervene in cardiac hypertrophy by repressing early-growth response (Egr) transcription factors\(^10\). These findings suggest that hypertrophic stimuli evoke antihypertrophic or negative feedback pathways, as well as positive hypertrophic signaling pathways. Augmenting these negative regulators, rather than inhibiting the positive regulators, may be a viable antihypertrophic strategy\(^11\).

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Protein kinase C (PKC) is a ubiquitously expressed serine/threonine kinase and has been implicated in the development of cardiac hypertrophy. The rat heart contains at least 5 PKC isozymes (PKCα, PKCβ1, PKCδ, PKCε, and PKCζ) with distinct expression patterns. The relative importance of these PKC isozymes in hypertrophic signaling remains controversial and crosstalks between the PKC isozymes may complicate data interpretation. In this study, we show that a putative PKC inhibitor, PICOT (PKC-Interacting Cousin Of Thioredoxin), functions as a negative regulator of cardiac hypertrophy. Interestingly, PICOT overexpression dramatically increased ventricular function and cardiomyocyte contractility.

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

Cell Culture and Hypertrophic Stimulation
Primary cultures of cardiomyocytes from 2-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 either collagen-coated culture dishes or cover slips and cultured in cardiomyocyte culture medium (DMEM supplemented with 10% FBS and 2 mmol/L glutamine; GIBCO BRL). To induce hypertrophy, cardiomyocytes were cultured in serum-free medium for at least 24 hours and then treated with 100 nmol/L endothelin-1 (ET-1) or 100 μmol/L phenylephrine PE (Figure 1B). In both cases, the extent of PICOT expression was examined (Figure 1C). The induction of ANF began 2 days after TAC, peaked after 2 weeks, and declined sharply thereafter. This ANF-induction pattern previously had been shown to correlate well with the onset of cardiac hypertrophy. In contrast, the induction of PICOT by TAC was rather slow, beginning approximately 1 week after the induction of ANF and continuing to increase when ANF expression was completely diminished at 3 weeks after banding (Figure 1C). Thus it appeared possible that ANF constituted an acute cardiac hypertrophy inhibitory mechanism, whereas PICOT had a later-onset inhibitory function. Fibroblasts and cardiomyocytes were separately isolated from the sham-operated and hypertrophied hearts (3 weeks after TAC), respectively, and the PICOT level in the protein extracts were determined by immunoblotting (Figure 1D). Approximately 2-fold increase in the PICOT level was detected in the hypertrophied cardiomyocytes but not in the fibroblasts. This indicates that PICOT functioned as an endogenous inhibitor of PKC.

Results
PICOT Is Upregulated During Cardiac Hypertrophy
To identify negative regulators of cardiac hypertrophy, a PCR-based differential screening technique was performed, based on the assumption that antihypertrophic or negative regulators were likely to be induced during cardiac hypertrophy, along with positive hypertrophic regulators. Transverse aortic constriction (TAC) was used to induce cardiac hypertrophy in adult rat hearts. Some novel genes, as well as numerous genes previously found to be associated with cardiac hypertrophy, were isolated (see the Table in the online data supplement). Among the genes that were expressed preferentially in the hypertrophied hearts, as compared with the sham-operated normal hearts, PICOT was of particular interest. PICOT was first identified as PKCθ-interacting protein by a yeast 2-hybrid screen. When transiently overexpressed in T cells, PICOT inhibited the activation of c-jun N-terminal kinase (JNK), activator protein-1 (AP-1), and nuclear factor κB (NF-κB), suggesting that PICOT functioned as an endogenous inhibitor of PKC. Considering that PKC isozymes play important roles in the development of cardiac hypertrophy, the elevated expression of PICOT in hypertrophied hearts suggested a novel negative feedback circuit in cardiac hypertrophy.

Northern blot analysis was used to confirm the induction of PICOT by hypertrophic stimuli in vivo and in vitro. The expression level of PICOT was approximately 3 times higher in hypertrophied hearts than in sham-operated hearts (Figure 1A). PICOT also was induced in neonatal rat cardiomyocytes after exposure to the hypertrophic agonists ET-1 or phenylephrine PE (Figure 1B). In both cases, the extent of PICOT induction was comparable to that of ANF. The induction of PICOT during the progression of cardiac hypertrophy also was examined (Figure 1C). The induction of ANF began 2 days after TAC, peaked after 2 weeks, and declined sharply thereafter. This ANF-induction pattern previously had been shown to correlate well with the onset of cardiac hypertrophy. In contrast, the induction of PICOT by TAC was rather slow, beginning approximately 1 week after the induction of ANF and continuing to increase when ANF expression was completely diminished at 3 weeks after banding (Figure 1C). Thus it appeared possible that ANF constituted an acute cardiac hypertrophy inhibitory mechanism, whereas PICOT had a later-onset inhibitory function. Fibroblasts and cardiomyocytes were separately isolated from the sham-operated and hypertrophied hearts (3 weeks after TAC), respectively, and the PICOT level in the protein extracts were determined by immunoblotting (Figure 1D). Approximately 2-fold increase in the PICOT level was detected in the hypertrophied cardiomyocytes but not in the fibroblasts. This indicates that...
the inhibitory activity of PICOT on the development of cardiac hypertrophy is cell autonomous.

**PICOT Inhibits the ET-1– or PE-Induced Hypertrophic Response**

To evaluate the ability of PICOT to inhibit cardiac hypertrophy, AdPICOT (PICOT-expressing recombinant adenovirus) was generated. Adenoviral infection routinely resulted in infection of more than 90% of the cultured neonatal rat cardiomyocytes (data not shown). Western blot analyses revealed a protein band in the cardiomyocytes transfected with AdPICOT but not in nontransfected cardiomyocytes or in cardiomyocytes transfected with AdLacZ (β-galactosidase-expressing adenovirus) (Figure 2A). The apparent molecular weight of the PICOT band was 40 to 42 kDa, which was slightly higher than the calculated molecular mass (38 kDa), suggesting that PICOT might be posttranslationally modified. At 24 hours after infection, the cardiomyocyte cultures were further stimulated with ET-1 or PE for 24 hours. These treatments significantly increased the size of the AdLacZ-transfected or untransfected cardiomyocytes, as assessed by measuring the surface area of the cells under a microscope. In contrast, no significant ET-1– or PE-induced increase in cell size was observed with the AdPICOT-transfected cardiomyocytes (Figure 2B).

Another feature of the hypertrophic response of cardiomyocytes is a pronounced sarcomeric rearrangement that can be detected by immunostaining with α-actinin antibody. Unlike the nontransfected or AdLacZ-transfected cardiomyocytes, the cardiomyocytes transfected with AdPICOT lacked prominent sarcomeric structures after ET-1 or PE treatment (Figure 2C). ANF expression also is a typical marker for cardiac hypertrophy. Treatment with ET-1 or PE significantly increased ANF immunoreactivity in nontransfected or AdLacZ-transfected cardiomyocytes. However, PICOT transfection blocked this elevated ANF immunoreactivity (Figure 2D). Lastly, the increased protein synthesis induced by ET-1 or PE treatment, as determined by 3H-leucine incorporation, was abrogated completely by PICOT transfection (Figure 2E). Taken together, these results indicated that the forced expression of PICOT inhibited agonist-induced cardiac hypertrophy in neonatal rat cardiomyocytes.

**PICOT Abrogates Pressure-Overload–Induced Cardiac Hypertrophy**

To investigate the role of PICOT as a negative regulator of cardiac hypertrophy in vivo, transgenic mice were generated that expressed PICOT in the heart, under the control of the α-MHC promoter (Figure 3A). Two independent lines (TG17 and TG60) were obtained with the α-MHC–PICOT transgene. Transgenic lines did not exhibit any obvious gross abnormalities. Western blot analysis revealed that cardiac PICOT expression in both lines was approximately 60% to 80% higher than in the wild-type littermates (Figure 3B). We observed that endogenous PICOT was present both in the cardiomyocytes and fibroblasts of hearts, and the transgene drove PICOT expression in the cardiomyocytes (data not shown).
To determine whether PICOT overexpression antagonized the hypertrophic response to physiologically relevant pressure overload, wild-type littermates and TG17 mice were subjected to TAC. The induction of pressure overload stimulated a 33% increase in the heart weight to body weight ratio in wild type over 14 days. In contrast, hypertrophic growth was blunted significantly ($P<0.05$) in transgenic mice, with approximately a 15% increase in the heart weight to body weight ratio (Figure 3C). Furthermore, microscopic analysis of histological sections revealed that the increase in the ventricular cross-sectional area was reduced significantly ($P<0.01$) in transgenic mice (a 25% increase in TAC versus sham) compared with wild type (an 82% increase in TAC versus sham) (Figure 3D). The induction of several fetal genes, including ANF and skeletal actin (SKA), is associated with cardiac hypertrophy. Northern blot analysis showed that the induction of these hypertrophic marker genes was reduced significantly in transgenic mice as compared with wild type (Figure 3E). These results indicated that PICOT abrogated the development of left ventricular hypertrophy induced by pressure overload in vivo. Similar results were obtained with TG60 (data not shown).

**PICOT Enhances Ventricular Function and Cardiomyocyte Contractility**

Previous reports showed that PKC activation was associated with a reduction of cardiac contractility via the dephosphorylation of phospholamban (PLB)\(^{19,20}\) or the phosphorylation of native thin filaments\(^{21}\) and troponin I (TnI)\(^{22,23}\). Because PICOT has been thought to inhibit PKC, its effects on cardiac contractility were examined. Adult cardiomyocytes were isolated from wild-type and transgenic hearts, and their contractility was measured. An increase in contractility of approximately 89% was observed in transgenic cardiomyocytes (WT, 5.8%; TG, 5.8%, $P<0.01$) as compared with wild type (WT, 1.1%; TG, 1.1%, $P<0.05$). To determine the in vivo functional consequences of PICOT overexpression, hemodynamic parameters were measured under basal conditions. PICOT transgenic mice showed a 27% increase in ejection fraction and an 18% increase in end-systolic pressure.
PICOT and Cardiac Hypertrophy

Figure 4. Cardiomyocyte contractility and Ca\(^{2+}\) transient measurement. A. Average parameters of cardiomyocyte contraction was determined (AdLacZ, n=38; AdPICOT, n=45). *P<0.05. B. Average parameters of Ca\(^{2+}\) transient properties were determined using fura2/acetoxymethyl ester (AdLacZ, n=38; AdPICOT, n=45). *P<0.05. C. Hysteresis loops of cytosolic Ca\(^{2+}\) content during steady-state contraction (Figure 4C). Upward and leftward shift of the loop in AdPICOT-transfected cardiomyocytes indicated increased cell shortening for any given cytosolic Ca\(^{2+}\) concentration, implying an increase in Ca\(^{2+}\) sensitivity of the myofilaments, compared with AdLacZ-transfected cardiomyocytes. These results indicate that increases in myofilament Ca\(^{2+}\) responsiveness, together with increased rate of SR Ca\(^{2+}\) reuptake, promote an enhanced contractile function in AdPICOT-transfected cardiomyocytes.

We observed that phosphorylation at Ser16 of pentameric PLB was significantly increased, whereas total pentameric PLB protein level was significantly reduced in AdPICOT-transfected cardiomyocytes. Therefore, a >2-fold increase in the ratio of phosphorylated PLB to total PLB was observed on PICOT overexpression (Figure 4D). Without a change in expression of SERCA-2 and calsequestrin (data not shown), the hyperphosphorylation of PLB is thought to render SERCA-2 more active, resulting in the enhanced SR Ca\(^{2+}\) reuptake in AdPICOT-transfected cardiomyocytes.

**PICOT Suppresses the Activation of PKC and Mitogen-Activated Protein Kinases**

PKC activation has been associated with the development of cardiac hypertrophy, and PICOT has been thought to function as an intrinsic inhibitor of PKC. Therefore, the activation of PKC in cardiomyocytes stimulated with ET-1 or PE was tested under the forced expression of PICOT. Using a PKC-specific phosphorylation assay, 2.5- and 4.5-fold increases were observed in the total PKC activity in cardiomyocytes treated with ET-1 or PE, respectively. These increases were abrogated completely by transfection with AdPICOT but not with AdLacZ (Figure 5A). PKC\(\alpha\), PKC\(\delta\), and PKC\(\varepsilon\) are the most highly expressed isoforms in the adult rat heart. In addition to these isozymes, PKC\(\zeta\) also is expressed abundantly in neonatal rat cardiomyocytes and weakly in the adult rat heart (data not shown). The near-complete suppression of total PKC activity by PICOT suggested that the activation of all these PKC isozymes might be abrogated by PICOT transfection. Thus the effect of PICOT overexpression on the specific activation of PKC\(\alpha\), PKC\(\varepsilon\), and PKC\(\zeta\) was evaluated using phospho-PKC, isozyme-specific antibodies. PKC\(\alpha\), PKC\(\varepsilon\), and PKC\(\zeta\) were activated significantly by stimulation with ET-1 or PE, and concomitant PICOT transfection prevented this activation (Figure 5B).

Because mitogen-activated protein (MAP) kinase pathways appear to be a merging point for divergent hypertrophic signaling pathways involving PKC, PICOT inhibition of MAP kinase activation was investigated using antibodies against phospho–extracellular signal-regulated kinase 1/2...
Figure 5. Effects of PICOT on PKC, ERK, JNK, and p38 activation in cardiomyocytes. After AdPICOT infection, neonatal cardiomyocytes were treated with 100 nmol/L ET-1 or 100 μmol/L PE or left untreated (no drug). A, Total PKC kinase activity was measured by assessing the PKC-specific phosphorylation of substrate proteins. The increase in total PKC activity induced by hypertrophic agonists was diminished significantly by PICOT transfection. B and C, Activation of PKCc, PKCe, PKCζ, ERK, JNK, and p38 was evaluated by Western blotting. The phosphorylated forms are the activated forms of these enzymes. Representative blots from 3 independent experiments are shown.

Discussion

As is common in most biological systems, it is evident that the hypertrophic signaling pathways are counteracted by a number of antihypertrophic or negative-feedback mechanisms.11 Strategies to stimulate the latter, as well as inhibit the former, may be of significant therapeutic value. Relatively well-characterized negative-feedback regulators of cardiac hypertrophy includes ANF,6 MCIP1,7,8 SOCS3,9 and Nab1.10

In this study, we report that PICOT is upregulated in neonatal cardiomyocytes stimulated with hypertrophic agonists, as well as in pressure-overloaded hearts. Because adenovirus-mediated expression of PICOT in cardiomyocytes or transgenic expression of PICOT in hearts abrogates the hypertrophic responses, it appears that PICOT constitutes a novel negative-feedback circuit for cardiac hypertrophy.

PICOT was first identified as a PKCθ-interacting protein in a yeast 2-hybrid screen.15 It has an amino-terminal thioredoxin homology (TH) domain that has a 29% amino acid identity and an additional 11% similarity with the thioredoxin family of proteins. Thioredoxin plays a critical role in regulating cardiac oxidative stress and recently has been shown to prevent the development of cardiac hypertrophy through a redox-sensitive mechanism.32 However, in a contrasting report, thioredoxin appears to promote cardiac hypertrophy by serving as a transcriptional cofactor, with its activity regulated by an endogenous inhibitor, thioredoxin-interacting protein (Txnip).33 Thus, it remains to be determined whether thioredoxin function in cardiomyocytes is pro- or antihypertrophic or both. PICOT is less likely 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, it contains an Ala-Pro-Gln-Cys sequence. Rather, the TH motif of PICOT appears to serve as a structural platform for a specific interaction with PKC isozymes. This notion is supported by the fact that thioredoxin directly interacts with PKCc, PKCd, PKCe, and PKCζ and inhibits their catalytic activities in vitro.34 However, the possibility that the TH domain of PICOT serves as a dominant negative form of thioredoxin cannot be ruled out.

The carboxy-terminal domain of PICOT contains 2 tandem repeats of an evolutionarily conserved domain of unknown function, referred to as the PICOT homology (PH) domain. We observed that this domain interacts with a number of sarcomeric proteins including MLP and crystallin αB (CryαB) (D.J., R.J.H., and W.J.P., unpublished data, 2006). Interestingly, these 2 molecules have been implicated in the development of dilated cardiomyopathy.35–37 It is possible that these interactions might be responsible for increased Ca2+ responsiveness of myofilaments. Vigorous genetic and biochemical studies are underway to elucidate a functional link associated with the interaction of PICOT and MLP or CryαB.

Deterioration in cardiac contractility is presumed to be a prerequisite for the transition from cardiac hypertrophy to heart failure. PKC-mediated phosphorylation of native thin filaments in failing human hearts is associated with reduced contractility,21 and PKCc triggers a chain of interactions that leads to decreased cardiac contractility.19,20 Moreover, inhibition of PKCe-mediated phosphorylation of TnI improves cardiac function in vivo.22,23 Therefore, it seems reasonable to speculate that augmentation of PICOT activity in hypertrophied or failing hearts may not only inhibit or reverse hypertrophic conditions but may also help restore cardiac contractility. Indeed, we found that transgenic or adenoviral mediated-overexpression of PICOT dramatically increases the ventricular function of hearts and the contractility of isolated cardiomyocytes. Detailed mechanisms underlying this positive inotropic effect of PICOT remain to be elucidated. Increased phosphorylation of PLB in the PICOT
overexpressing cardiomyocytes may reflect a part of the mechanisms. Other mechanisms might include changing phosphorylation status of myofilament proteins such as TnI and modulating function of other sarcomeric proteins such as MLP and CryαB.

In conclusion, we have elucidated a novel feedback inhibitory mechanism of cardiac hypertrophy signaling. PICOT, the central molecule in this feedback loop, blocks the development of cardiac hypertrophy in both in vitro and in vivo. PICOT has appeal as a potential therapeutic modality for preventing cardiac hypertrophy and heart failure because it not only blocks hypertrophic signaling but also enhances the inotropic property of cardiomyocytes.

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Disclosures
None.

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Materials and Methods

PCR-based subtraction and differential screening
RNA was isolated from normal and hypertrophied mice hearts using TRI reagent (Sigma) and an Oligotex mRNA Purification Kit (Qiagen, USA). Subtraction was performed using a PCR-Select cDNA Subtraction Kit (Clonetech, USA). The enriched PCR products were subcloned into pGEM-T Easy vector (Promega, USA).

RNA isolation and Northern blot analysis
Total RNA was isolated using TRI reagent (Sigma, USA). Equal amounts of total RNA (20 µg) were size-fractionated by electrophoresis through 1%-agarose gels and transferred to a nylon membrane (Schleicher & Schuell, Germany). The membrane was incubated in hybridization solution for 1 hr at 42°C and then further incubated overnight in the presence of cDNA probes radioactively labeled with [α-32P] dCTP (NEN, USA) using a Rediprime™II Kit (Amersham Pharmacia, USA). The membrane was washed and exposed to X-ray film (Kodak) for several days at -70°C. A probe for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Cell culture and hypertrophic stimulation
Primary cultures of cardiomyocytes from 2-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, USA) step gradients. Cells were plated onto either collagen-coated culture dishes or cover slips and cultured in cardiomyocyte culture medium (DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine; GIBCO BRL, USA). To induce hypertrophy, cardiomyocytes were cultured in serum-free medium for at least 24 hrs and then treated with 100 nM ET-1 or 100 µM PE for 24 hrs.

Generation of recombinant adenovirus AdPICOT
The Adeno-X Expression System Kit (Clontech, USA) was used to generate recombinant adenoviruses. Full-length rat PICOT cDNA tagged with HA at its carboxy terminus was subcloned into the pShuttle vector. The entire expression cassette from the vector was
excised and inserted into the subcloning site of the Adeno-X viral vector. This recombinant adenoviral plasmid was transfected into HEK293 cells to generate the infectious viral particles, AdPICOT. Cardiomyocytes were infected with recombinant adenoviruses for 2 hrs at a multiplicity of infection (M.O.I) of 50-100 particles/cell and incubated for an additional 24-48 hrs to ensure transgene expression. For in vivo experiments, AdPICOT was modified to express the green fluorescent protein (GFP). PICOT-HA-encoding DNA was subcloned into XbaI and NheI sites of the adenoviral shuttle vector pAdTrack, containing GFP under the control of the CMV promoter. The linearized shuttle vector was recombined in E. coli strain BJ5183 (Clontech, USA) with a serotype-5 first-generation adenoviral backbone denoted as AdEasy-1 (Clontech, USA). Successfully recombined viral backbones were transfected into HEK293 cells and grown in large quantities. Adenovirus was purified by standard CsCl ultracentrifugation and desalting. Viral titers were determined by the plaque assay. The absence of replication-competent adenovirus was confirmed by polymerase chain reaction (PCR) to assess the wild-type E1 region.

**Western blot analysis**

Cell lysates were obtained by solubilizing cardiomyocytes with SDS-lysis buffer (1% SDS, 10 mM Tris-Cl (pH 7.4), protease inhibitor cocktail; Boeringer Manheim, Germany). A total of 50 µg of cell lysate were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany). The membrane was blocked with 5% non-fat dry milk and incubated with antibodies against the HA tag (Boeringer Manheim, Germany), PKCα, PKCε, PKCζ, phospho-PKCα, ε, and ζ, JNK, phospho-JNK, ERK, phospho-ERK, p38, and phosphor-p38 (Cell Signaling, USA). Incubation usually occurred overnight in a cold room. The membrane was incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) (Boeringer Manheim, Germany) and developed using chemiluminescent substrates (Pierce, USA).

**Immunocytochemistry and cell-size measurements**

Cardiomyocytes grown on collagen-coated cover slips were infected with the recombinant adenoviruses for 24-36 hrs and then fixed with 3.5% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and blocked by incubation in 5% BSA solution for 1 hr at room temperature. The cells were incubated with anti-HA antibody
(Boeringer Manheim, Germany) or polyclonal anti-ANF antibody, further incubated with TRITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, USA), and mounted with Vectashield mounting medium (VECTA, USA). Immunofluorescence was analyzed under a microscope equipped with a 100X objective lens and filters for epifluorescence (Olympus, Japan). For cell-surface-area measurements, fixed and permeablized (0.1% Triton X-100 for 10min) cardiomyocytes were visualized by staining with FITC-conjugated phalloidin (Molecular Probes, USA) and the surface areas were measured using NIH image software.

Protein synthesis rate measurements
The transfected cardiomyocytes were cultured in serum-free DMEM medium for 12 hrs. The cells then were stimulated with an agonist for 12 hrs and incubated in the same medium containing 1.5 µCi/ml \[^{3}H\]leucine (NEN, USA) for an additional 20 hrs. Total proteins were precipitated by the addition of 10% TCA for 45 min on ice, resolubilized in 0.4 N NaOH for 1hr at 37°C, and subjected to liquid scintillation counting.

Generation of transgenic mice
A full-length mouse PICOT cDNA with human growth hormone 3’ UTR was subcloned Into a 5.5-Kb segment of the a-myosin-heavy-chain (a-MHC) promoter. The DNA construct was microinjected into FVB fertilized eggs and transgenic integration was confirmed by Southern blotting (Macrogen, Korea). Transgenic mice and wild-type littermates were analyzed at 8-10 weeks of age.

Histological analysis of hearts
Animals were sacrificed 14 days after TAC or sham surgery. Hearts were arrested at end-diastole and the left ventricle was freed from the right ventricle and weighed. Paraffin-embedded hearts and glycol methacrylate-embedded (Technovit 8100, Germany) hearts were cut into 4 µm and 1.5 µm slices, respectively. In addition, the ribbons were stained with hematoxylin-eosin solution. To measure the surface area of cardiomyocytes, suitable cross-sections with nearly circular capillary profiles and nuclei were selected. Sections were observed under an Axiophot microscope (Carl Zeiss, Germany) and analyzed with AnalysisSIS3.2 software (Soft-Imaging System, Germany).
Left ventricle hemodynamics
Male mice of 8-10 weeks of age (25-32 g) were used for the study. The animals were anesthetized with a cocktail of ketamine (100 mg/kg), xylazine (5 mg/kg) by intraperitoneal injection. Mice were placed on the controlled heating pads, and the temperature measured via rectal probe was maintained at 37°C. The animals were ventilated with a tidal volume of 500μl at 120 cycles/min, and underwent open-chest surgery. A microtip pressure-volume catheter (SPR-839; Millar Instruments, Houston, TX) was inserted into apex of left ventricle. After stabilization for 15 min, the signals were analyzed with PVAN3.5 software (Millar Instruments, Houston, TX).

Cell contractility measurements.
Mechanical properties of ventricular myocytes were assessed using a video-based edge-detection system (IonOptix; Milton, MA) as previously described. In brief, coverslips with cells attached were placed in a chamber mounted on the stage of an inverted microscope (Nikon Eclipse TE-100F) and superfused (about 1 ml/min at 25°C) with a Tyrode buffer (137 mM NaCl, 5.4 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 0.5 mM Taurin and 10 mM HEPES (pH 7.4)). The cells were field stimulated at a frequency of 0.5 Hz, 30 V using STIM-AT stimulator/thermostat placed on HLD-CS culture chamber/stim holder (Cell MicroControls, VA). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera, which rapidly scans the image area every 8.3 ms such that the amplitude and velocity of shortening or relengthening are recorded with good fidelity. Changes in cell length during shortening and relengthening were captured and analyzed using soft edge software (IonOptix).

Intracellular Ca²⁺ transient measurement
A same or separate group of myocytes were loaded with the 0.5μM fura2-AM (Molecular Probes, USA), a Ca²⁺-sensitive indicator, for 15 min at 25°C. Fluorescence measurements were recorded with a dual-excitation single-emission fluorescence photomultiplier system (IonOptix). Myocytes were placed on an inverted microscope and imaged through an Olympus Fluor ×40 oil objective, and were exposed to light emitted by a 75-W halogen lamp through either a 360- or 380-nm filter while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube.
after initial illumination at 360 nm for 0.5 s and then at 380 nm for the duration of the recording protocol. The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in the intracellular Ca\(^{2+}\) concentration were inferred from the ratio of the fura fluorescence intensity (FFI) at both wavelengths.

**PKC activity assay**

Neonatal cardiomyocytes were lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.01% SDS, 1 mM DTT, 50 mM NaF, 1 mM Na\(_3\)VO\(_4\), protease inhibitor cocktail) and spun at 14,000 X rpm for 20 min at 4°C. The supernatant fraction was collected and used for the PKC assay. Total PKC kinase activity was determined with the SignaTECT PKC Assay System (Promega, USA) that utilizes the biotinylated peptide neurogranin, which is the most specific PKC substrate. The biotinylated ³²P-labeled substrate was captured on a membrane and the radioactivity was measured with a scintillation counter. The specific activation of PKC\(\alpha\), PKC\(\varepsilon\), and PKC\(\zeta\) was evaluated by Western blotting using antibodies specific for each of the PKC isozymes and their phosphorylated forms.

**Statistics**

Where appropriate, the data were expressed as means ± SDs. Comparisons of the group means were made with a Student’s t test or one-way ANOVA with a Bonferroni post-test analysis (Statview V5.0, SAS). P<0.05 was considered statistically significant.

**References**


## Supplementary Table

Genes enriched from hypertrophied hearts

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