

## PI 3-kinase-Akt-p70 S6 Kinase in Hypertrophic Responses to Leukemia Inhibitory Factor in Cardiac Myocytes

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**Leukemia inhibitory factor (LIF) is a member of interleukin-6 related cytokines, which induces cardiac hypertrophy through glycoprotein (gp) 130. In this study, the role of phosphatidylinositol (PI) 3-kinase, Akt/protein kinase B (Akt/PKB), and p70 S6 kinase activation in LIF-induced hypertrophic responses such as stimulation of protein synthesis, atrial natriuretic peptide (ANP) gene expression, and reorganization of actin filaments into sarcomeric units was investigated in cultured cardiac myocytes. Treatment of cells with LIF resulted in sequential activation of PI 3-kinase, Akt/PKB, and p70 S6 kinase. Using inhibitors for PI 3-kinase and p70 S6 kinase activation, and adenovirus-mediated expression of dominant negative mutants of PI 3-kinase and Akt/PKB, we showed that PI 3-kinase activation was essential for stimulation of protein synthesis, ANP gene expression, and sarcomeric reorganization induced by LIF, while Akt/PKB activation was indispensable for ANP expression and stimulation of protein synthesis, but not for sarcomeric reorganization. Activation of p70 S6 kinase was necessary for stimulation of protein synthesis, but not for ANP gene expression or sarcomeric reorganization. These results indicated the essential role of PI 3-kinase-Akt/PKB-p70 S6 kinase pathway in the LIF-induced hypertrophic responses in cardiac myocytes.**

Myocardial hypertrophy is a part of the compensatory mechanisms that allows the myocardium to adapt at least in the short term to hemodynamic overload. In the longer term, however, adaptive myocardial hypertrophy may decay into maladaptive hypertrophy and heart failure. Although the mechanisms responsible for the transition between these two stages of hypertrophy are poorly defined, it has been reported that cardiac hypertrophy is an independent risk factor for cardiac morbidity, and results in an approximately two fold increase in the relative risk of mortality from cardiovascular disease (17).

Cardiac myocytes undergo cellular hypertrophy in response to diverse stimuli. It has been identified that cardiac myocyte hypertrophy is induced by mechanical stress (37), various growth factors acting through G protein-coupled receptors such as phenylephrine (27), angiotensin II (1, 24), and endothelin-1 (2). In addition, recent findings indicate that activation of the glycoprotein (gp) 130 cytokine receptor by cardiotrophin-1 (21, 36) and leukemia inhibitory factor (LIF) (18), cytokines of the interleukin (IL)-6 family, can lead to cardiac hypertrophy. Further, transgenic mice overexpressing both IL-6 and IL-6 receptor, in which gp130 protein is continuously activated, demonstrate myocardial hypertrophy (14). Hypertrophic responses induced by these agonists are characterized by reactivation of fetal

gene expression such as atrial natriuretic peptide (ANP) (3, 16), morphological changes including an increase in cell size and a reorganization of actin filaments into sarcomeric units (22, 36), and stimulation of protein synthesis in cultured cardiac myocytes (24, 36).

Recently, it has been reported that LIF induces the activation of phosphatidylinositol (PI) 3-kinase in cultured cardiac myocytes (20). It was also shown that a PI 3-kinase inhibitor wortmannin inhibited Akt/protein kinase B (PKB) and p70 S6 kinase activation induced by LIF, and that wortmannin and a p70 S6 kinase inhibitor rapamycin inhibited LIF-induced stimulation of protein synthesis. However, the involvement of Akt/PKB in LIF-induced p70 S6 kinase activation and the role of these kinases in the other features of hypertrophy remained to be examined.

In the present study, we investigated first if LIF induced sequential activation of PI 3-kinase, Akt/PKB, and p70 S6 kinase in cultured neonatal cardiac myocytes. Then, we examined how PI 3-kinase, Akt/PKB, and p70 S6 kinase were involved in LIF-induced hypertrophic responses such as stimulation of protein synthesis, ANP gene expression, and reorganization of actin filaments into sarcomeric units.

## **MATERIALS AND METHODS**

### *Cell Culture*

Primary cultured ventricular myocytes were prepared from neonatal Sprague-Dawley rat hearts as described previously (12, 32, 33). The culture medium was Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 supplemented with 5% calf serum and penicillin-streptomycin (0.02 U/ml and 0.02 mg/ml, respectively). 5-bromodeoxyuridine (100 mM) was added during the first 24 h to prevent proliferation of nonmyocytes. The medium was changed 24 h after seeding the cells to serum-free DMEM/F-12 containing 0.1% bovine serum albumin and ITS (10 µg/ml insulin, 10 µg/ml transferrin, and 10 ng/ml selenious acid; Becton Dickinson Labware).

### *Recombinant Adenovirus Vectors*

Adenovirus vectors encoding a dominant negative mutant of PI 3-kinase (AxCAΔp85), a dominant negative mutant of Akt (AxCAAkt-AA), a dominant active mutant of Akt (AxCAMyr-Akt) or bacterial β-galactosidase (AxCALacZ) were prepared as described previously (12, 15, 25, 30). Cardiac myocytes were infected with adenovirus vectors at the indicated multiplicity of infection (MOI) 24 h after seeding the cells. The cells were subjected to experiments 48 h after infection. More than 95% of cardiac myocytes were positive for β-galactosidase expression, when cells were infected with AxCALacZ under the experimental condition used in this study.

### *PI 3-kinase, Akt/PKB, and p70 S6 kinase Assay*

Kinase activities of PI 3-kinase, Akt/PKB, and p70 S6 kinase were measured as described previously (24, 25, 29). The cell lysates (500 µg protein) were immunoprecipitated with monoclonal anti-phosphotyrosine antibody (PY-20 clone; Transduction Laboratories), sheep polyclonal anti-Akt/PKB antibody, or rabbit polyclonal anti-p70 S6 kinase antibody (Upstate Biotechnology), and were examined by *in vitro* kinase assay using sonicated PI (Avanti Polar Lipids) for PI 3-kinase, histone H2B for Akt/PKB, or S6 peptide for p70 S6 kinase as the substrate, respectively. The radioactivities of incorporated <sup>32</sup>P into substrates were determined using a Fujix bio-imaging analyzer BAS 2000 or scintillation spectrometry.

### *Immunoblot Analysis*

Immunoblot analysis with phospho-specific or total Akt/PKB antibodies (New England Bio Labs) was carried out as described previously (29). Samples were subjected to 10 %

## PI 3K-AKT-P70 S6K PATHWAY IN CARDIAC HYPERTROPHY

SDS-polyacrylamide gel electrophoresis and the separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). Blots were incubated with rabbit polyclonal phospho-specific Akt/PKB antibody or total Akt/PKB antibody, and the primary antibodies were detected using horseradish peroxidase-labeled donkey anti-rabbit IgG, followed by enhanced chemiluminescence (Amersham Pharmacia).

### *Protein Synthesis Assay*

Protein synthesis was measured by [<sup>3</sup>H] leucine incorporation as described previously (28). Cardiac myocytes cultured on 24-well plates were stimulated with LIF (1000 U/ml) (Pepro Tech) for 24 h. 0.5 μCi/ml [<sup>3</sup>H] leucine was added 4 h before harvest. The radioactivities incorporated into trichloroacetic acid-precipitable materials were measured by liquid scintillation spectrometry.

### *Filamentous Actin Staining*

Cardiac myocytes cultured on collagen-coated glass cover slips were fixed with 4.0 % formaldehyde in PBS for 30 min, permeabilized with 0.2 % Triton X-100 for 4 min, and blocked in PBS containing 1 % BSA for 1 h. The cells were stained with fluorescein isothiocyanate-conjugated phalloidin (Sigma) for 1 h. After coverslips were mounted, sarcomeric actin reorganization was examined and photographed under a fluorescent microscopy.

### *Northern Blot Analysis*

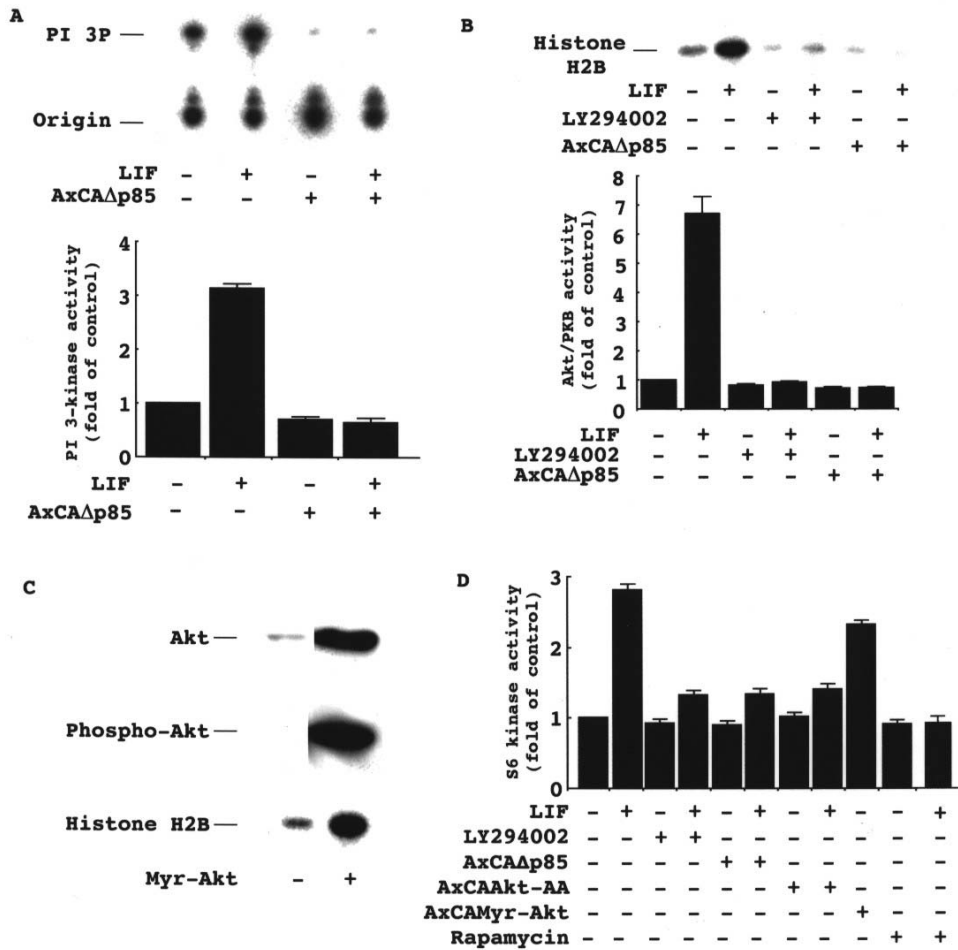
Total RNA (15 μg) prepared using ISOGEN (Nippon Gene) was subjected to electrophoresis on 1 % agarose gel containing formaldehyde, and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia). The membrane was hybridized with murine ANP cDNA probe labeled with [<sup>32</sup>P] dCTP. After washing, radioactivities were determined using a Fujix BAS 2000 bio-imaging analyzer.

## RESULTS

### *LIF Stimulates Sequential Activation of PI 3-kinase, Akt/PKB, p70 S6 Kinase in Cardiac Myocytes.*

To examine the role of PI 3-kinase, Akt/PKB, and p70 S6 kinase on the LIF-induced hypertrophic responses, we first analyzed if LIF induced sequential activation of these kinases in cultured cardiac myocytes. As shown in Fig. 1A, LIF induced a marked increase in the lipid kinase activity of PI 3-kinase, which was maximal at around 5-10 min. When a PI 3-kinase specific inhibitor LY294002 was added to the anti-phosphotyrosine immunoprecipitates, the spot corresponding to PI 3-monophosphate was completely disappeared (data not shown), confirming the specificity of this activity to PI 3-kinase.

Next, we examined the effect of LIF on kinase activity of Akt/PKB, and observed that the effect of LIF on kinase activity of Akt/PKB was time-dependent, which reached a maximum at 10 min (Fig. 1B). To examine the role of PI 3-kinase activation in LIF-induced Akt/PKB activation, we tested the effect of a PI 3-kinase specific inhibitor LY294002, and the expression of a dominant negative mutant of PI 3-kinase (Δp85) on LIF-induced Akt/PKB activity. LY294002 is a specific inhibitor of PI 3-kinase but has no inhibitory effect against PI 3-kinase nor a number of intracellular serine/threonine or tyrosine kinases at 50 μM (26, 34). LY294002 (50 μM) completely inhibited the LIF-induced activation of Akt/PKB (Fig. 1B). Δp85 is a mutant of a regulatory subunit p85 of PI 3-kinase which lacks a binding site for a catalytic subunit p110 of PI 3-kinase and has been widely used as a dominant negative mutant of PI 3-kinase (9, 15, 25, 31). In cells infected with AxCAΔp85, LIF-induced activation of Akt/PKB was inhibited (Fig. 1B), which was associated with the



**Fig. 1. LIF Stimulates Sequential Activation of PI 3-kinase-Akt/PKB-p70 S6 Kinase in Cardiac Myocytes.**

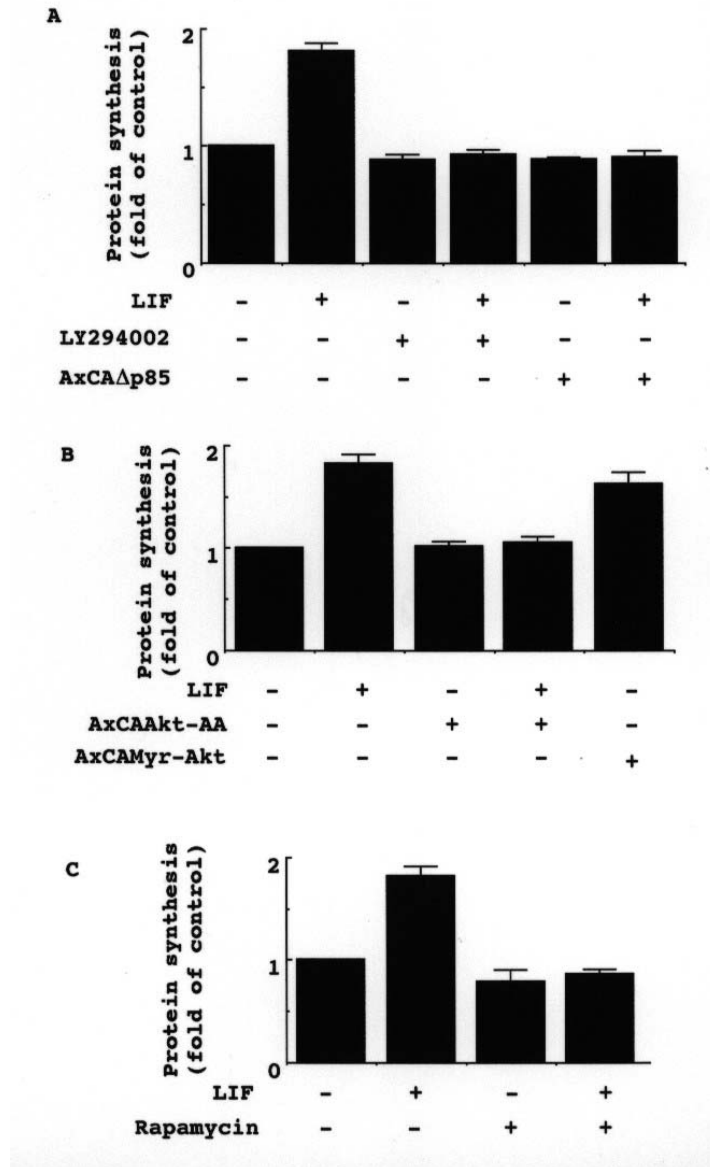
A, Cardiac myocytes were infected with or without a dominant negative mutant of PI 3-kinase (AxCAΔp85) at 5 MOI, and stimulated with 1000 U/ml of LIF for 10 min. PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates was analyzed by *in vitro* kinase assay using PI as a substrate.

B, Cardiac myocytes were treated with 50 μM of LY294002 for 10 min, or infected with a dominant negative mutant of PI 3-kinase at 5 MOI, and stimulated with 1000 U/ml of LIF for 10 min. Kinase activity of Akt/PKB was analyzed by *in vitro* kinase assay using histone H2B as a substrate.

C, Cardiac myocytes were infected with or without a constitutive active mutant of Akt/PKB (Myr-Akt) at 30 MOI. Cell lysate was subjected to immunoblot analysis with total or phospho-specific Akt/PKB antibody (upper and middle panels), and *in vitro* kinase assay for Akt/PKB (lower panel). D, Cultured cardiac myocytes were pretreated with or without 50 μM of LY294002 for 10 min or 30 μM of rapamycin for 30 min, or infected with AxCAΔp85 at 5 MOI, AxCAAkt-AA at 200 MOI or AxCAMyr-Akt at 30 MOI. And then cells were stimulated with 1000 U/ml of LIF for 15 min. p70 S6 kinase activity was analyzed by *in vitro* kinase assay using S6 peptide as a substrate.

Values shown are means ± standard error (S. E.) of at least three independent trials, and expressed as fold of the unstimulated levels.

PI 3K-AKT-P70 S6K PATHWAY IN CARDIAC HYPERTROPHY



**Fig. 2. PI 3-kinase-Akt/PKB-p70 S6 kinase Pathway Mediates Protein Synthesis Stimulated by LIF in Cardiac Myocytes.**

- A, Cardiac myocytes were incubated with or without 50  $\mu$ M of LY294002 for 10 min, or infected with AxCΔp85 at 5 MOI. And then, cells were stimulated with 1000 U/ml of LIF for 24 h. Protein synthesis was measured by [ $^3$ H] leucine incorporation into TCA-precipitable materials.
- B, Cardiac myocytes infected with AxCΔAkt-AA at 5 MOI or AxCAMyr-Akt at 30 MOI were stimulated with or without 1000 U/ml of LIF for 24 h. Protein synthesis was measured by [ $^3$ H] leucine incorporation.
- C, Cardiac myocytes were incubated with or without 30  $\mu$ M of rapamycin for 30 min prior to stimulation with 1000 U/ml of LIF for 24 h. Values shown are means  $\pm$  S. E. of at least three independent trials, and expressed as fold of control.

inhibition of PI 3-kinase activity by this mutant (Fig. 1A). The infection with AxCALacZ did not alter the LIF-induced PI 3-kinase or Akt/PKB activation (data not shown). These results clearly indicated that LIF-induced activation of Akt/PKB was mediated by PI 3-kinase in cardiac myocytes.

As has been reported (20), treatment with LIF resulted in marked activation of p70 S6 kinase (Fig. 1D), which peaked at around 15 min after addition of LIF. To assess the role of PI 3-kinase-Akt/PKB pathway in p70 S6 kinase activation by LIF, the effects of LY294002, and expression of dominant negative mutants of PI 3-kinase ( $\Delta p85$ ) and Akt/PKB (Akt-AA) on p70 S6 kinase activation were tested. Akt-AA is a mutant of Akt/PKB in which Thr308 and Ser473 are replaced by alanine, and dominantly interferes with endogenous Akt/PKB activity (15, 30). LY294002 inhibited the LIF-induced p70 S6 kinase activation significantly (Fig. 3B). Moreover, infection of cells with AxCA $\Delta p85$  or AxCAAkt-AA significantly suppressed LIF-induced p70 S6 kinase activation (Fig. 1D), whereas the infection with AxCALacZ did not affect it (data not shown). These data indicated that the activation of p70 S6 kinase by LIF was mediated by the PI 3-kinase-Akt/PKB pathway in cardiac myocytes. Further, we also examined the effect of expression of Myr-Akt, an active mutant of Akt/PKB on p70 S6 kinase activity in cardiac myocytes. Myr-Akt comprised of the entire coding sequence of c-Akt fused in-frame to the Src myristoylation signal is membrane targeted and constitutively active (8). Infection of AxCAMyr-Akt increased expression of Akt/PKB and phosphorylated Akt/PKB, and augmented the kinase activity of Akt (Fig. 1C). The expression of Myr-Akt induced activation of p70 S6 kinase (Fig. 1D), suggesting that Akt/PKB activation is necessary and sufficient for p70 S6 kinase activation by LIF.

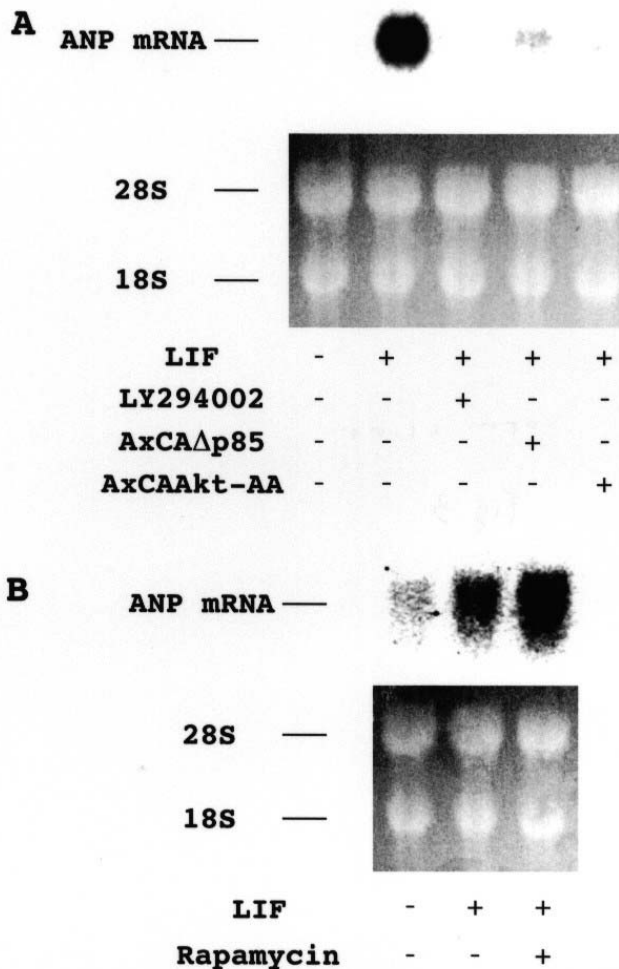
*PI 3-kinase-Akt/PKB-p70 S6 kinase Pathway Mediates Protein Synthesis Stimulated by LIF in Cardiac Myocytes.*

To examine the role of PI 3-kinase-Akt/PKB-p70 S6 kinase pathway in LIF-induced stimulation of protein synthesis, the effects of LY294002, rapamycin, and expression of  $\Delta p85$  and Akt-AA on [<sup>3</sup>H] leucine incorporation were analyzed. Rapamycin, a p70 S6 kinase inhibitor indeed completely inhibited the activation of p70 S6 kinase by LIF (Fig. 1D). Consistent with previous reports (20), treatment with LIF induced a 1.8-fold increase in [<sup>3</sup>H] leucine incorporation (Fig. 2). Treatment with LY294002 and expression of  $\Delta p85$  completely inhibited the LIF-induced protein synthesis (Fig. 2A), while the infection with AxCALacZ did not alter LIF-induced protein synthesis (data not shown), indicating that PI 3-kinase activity is necessary for LIF-induced protein synthesis in cardiac myocytes. Infection of cells with AxCAAkt-AA also inhibited LIF-induced protein synthesis completely (Fig. 2B), and infection of cells with AxCAMyr-Akt increased protein synthesis by approximately 1.6-folds (Fig. 2B). These data indicated that the activation of Akt/PKB is necessary and sufficient for protein synthesis stimulated by LIF. Further, as shown in Fig. 2C, rapamycin also inhibited protein synthesis stimulated by LIF in cardiac myocytes. These results indicated that PI 3-kinase-Akt/PKB-p70 S6 kinase pathway mediated LIF-induced stimulation of protein synthesis.

*PI 3-kinase and Akt/PKB, but not p70 S6 Kinase are Indispensable for ANP Gene Expression Induced by LIF in Cardiac Myocytes.*

ANP mRNA expression is one of the hypertrophic responses in cardiac myocytes. As LIF has been reported to induce ANP gene expression in cultured cardiac myocytes (36), we investigated whether the activation of PI 3-kinase-Akt/PKB-p70 S6 kinase pathway was required for ANP gene expression stimulated by LIF in cardiac myocytes. In cells treated with LIF, the expression of ANP mRNA was markedly enhanced (Fig. 3). Pretreatment with

PI 3K-AKT-P70 S6K PATHWAY IN CARDIAC HYPERTROPHY

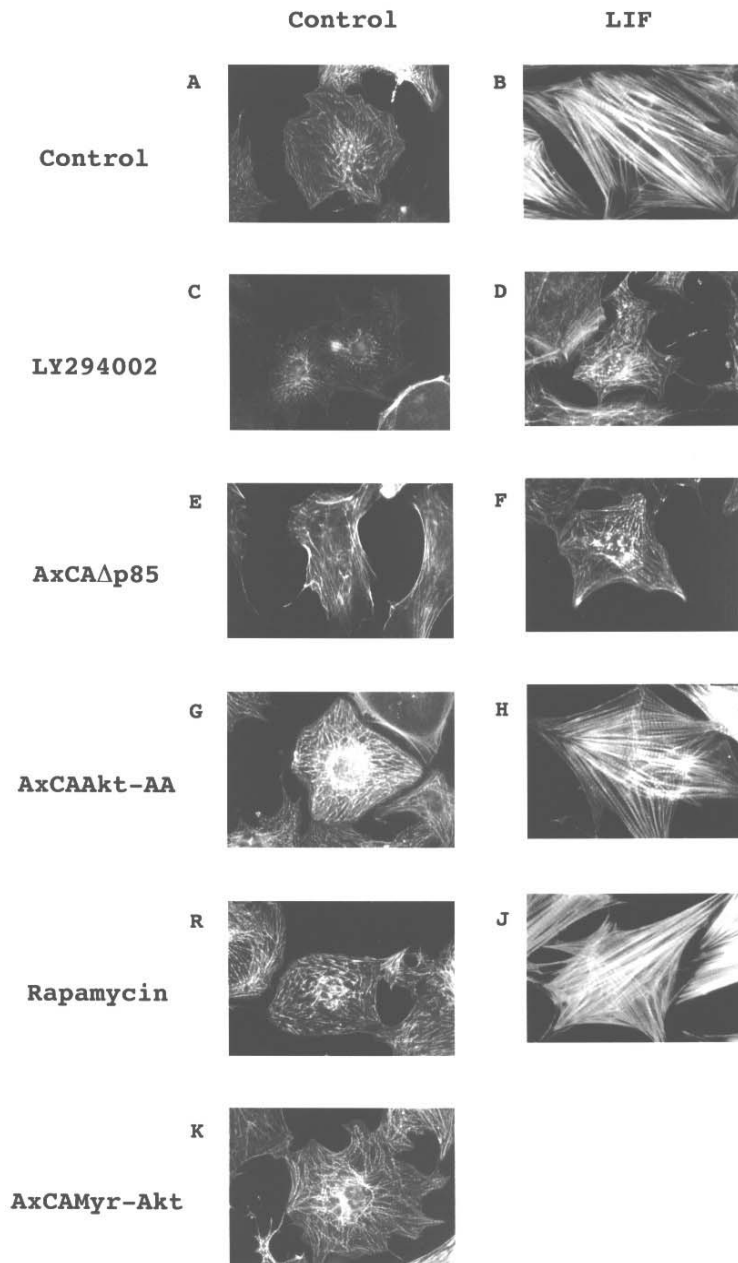


**Fig. 3. PI 3-kinase and Akt/PKB, but not p70 S6 Kinase are Indispensable for ANP Gene Expression Induced by LIF in Cardiac Myocytes.**

A, Cardiac myocytes were incubated with 50  $\mu$ M of LY294002 for 10 min, or infected with AxCA $\Delta$ p85 at 5 MOI or AxCAAkt-AA at 200 MOI, and then stimulated with 1000 U/ml of LIF for 24 hr. Total RNA was isolated, and Northern blot analysis was performed with <sup>32</sup>P-labeled ANP cDNA fragment as a probe.

B, Cells were pretreated with or without 30  $\mu$ M of rapamycin for 30 min, and then stimulated with 1000 U/ml of LIF for 24 hr. The results shown were representative of three independent trials.

LY294002, or the expression of  $\Delta$ p85 or Akt-AA completely inhibited the gene expression of ANP stimulated by LIF (Fig. 3A). However, rapamycin did not alter gene expression of ANP (Fig. 3B). These data indicated that PI 3-kinase and Akt/PKB activation was necessary for ANP gene expression induced by LIF, whereas p70 S6 kinase activation was dispensable for this induction.



**Fig. 4. PI 3-kinase, but not Akt/PKB or p70 S6 kinase is Necessary for Reorganization of Actin Filaments into Sarcomeric Units Induced by LIF in Cardiac Myocytes.**

Cardiac myocytes were incubated with 50  $\mu$ M of LY294002 for 10 min (C, D) or 30  $\mu$ M of rapamycin for 30 min (R, J), or infected with AxCA $\Delta$ p85 at 5 MOI (E, F), AxCAAkt-AA (G, H) at 200 MOI, or AxCAMyr-Akt at 30 MOI (K). And then, cells were stimulated with (B, D, F, H, J) or without (A, C, E, G, R, K) 1000 U/ml of LIF for 24 hr. Sarcomeric reorganization was examined by the staining with fluorescein isothiocyanate-conjugated phalloidin, and representative fluorescent photographs were shown.



## PI 3K-AKT-P70 S6K PATHWAY IN CARDIAC HYPERTROPHY

### *PI 3-kinase, but not Akt/PKB or p70 S6 kinase is Necessary for Reorganization of Actin Filaments into Sarcomeric Units Induced by LIF in Cardiac Myocytes.*

As LIF has been reported to induce reorganization of actin filaments into sarcomeric units in cultured cardiac myocytes (36), we next investigated the role of PI 3-kinase, Akt/PKB, p70 S6 kinase in sarcomeric reorganization stimulated by LIF in cardiac myocytes. LIF induced sarcomeric reorganization, as assessed by staining with fluorescein isothiocyanate-conjugated phalloidin (Fig. 4A, B). Treatment with LY294002 completely inhibited the LIF-induced sarcomeric reorganization (Fig. 4C, D). In cells infected with AxCA $\Delta$ p85, LIF-induced sarcomeric reorganization was almost completely abolished (Fig. 4E, F), but the infection with AxCALacZ did not alter LIF-induced sarcomeric reorganization (data not shown). These findings indicated the essential role of PI 3-kinase activation in LIF-induced sarcomeric reorganization. However, expression of Akt-AA did not inhibit LIF-induced sarcomeric reorganization at all (Fig. 4G, H). Further, expression of Myr-Akt did not induce sarcomeric reorganization (Fig. 4K). p70 S6 kinase inhibitor rapamycin also did not inhibit LIF-induced sarcomeric reorganization (Fig. 4R, J). These data implied that the activation of PI 3-kinase was essential for reorganization of actin filaments into sarcomeric units stimulated by LIF in cardiac myocytes, but the activation of Akt/PKB or p70 S6 kinase was not necessary.

## DISCUSSION

In the present study, the role of PI 3-kinase, Akt/PKB, and p70 S6 kinase in LIF-induced hypertrophic responses in cultured cardiac myocytes was examined. We found that LIF sequentially activates PI 3-kinase-Akt/PKB-p70 S6 kinase pathway. Further, stimulation of protein synthesis was inhibited by inhibition of each three components of this kinase cascade. In contrast, ANP mRNA expression was attenuated by inhibition of PI 3-kinase or Akt/PKB, but not by inhibition of p70 S6 kinase, and sarcomeric reorganization was repressed by PI 3-kinase inhibition, but not by Akt/PKB or p70 S6 kinase inhibition. These results indicated that PI 3-kinase-Akt/PKB-p70 S6 kinase pathway is critically involved in the hypertrophic responses induced by LIF in cardiac myocytes, and that each component of this pathway differentially participates in these responses to LIF.

A number of enzymes possessing PI 3-kinase activity have been identified and are divided into four classes; class 1a, 1b, 2, and 3 (26). Among them, the most characterized is class 1a PI 3-kinase which is a heterodimer composed of a regulatory subunit p85 and a catalytic subunit p110, and its lipid kinase activity is regulated by the binding of regulatory subunit SH2 domains to tyrosine phosphorylated proteins (5, 26). Recently, it has been reported that LIF induces activation of PI 3-kinase pathway in cultured cardiac myocytes (20). They showed that wortmannin, a PI 3-kinase inhibitor inhibited Akt/PKB and p70 S6 kinase activation by LIF. In this study, we also demonstrated that another PI 3-kinase inhibitor LY294002 suppressed the activation of Akt/PKB and p70 S6 kinase induced by LIF in cardiac myocytes. Activation of PI 3-kinase activity in anti-phosphotyrosine immunoprecipitate suggested that class 1a PI 3-kinase is involved in LIF-induced activation of these kinases. However, it is possible that other classes of PI 3-kinase are also involved in the LIF-induced signal transduction pathways, since wortmannin and LY294002 inhibit not only class 1a PI 3-kinases but also other classes of PI 3-kinases to various extents (26). Therefore, we used a more specific molecular tool, the adenovirus expressing a dominant negative mutant of PI 3-kinase ( $\Delta$ p85), which is a mutant of a regulatory subunit p85 lacking a binding site for a catalytic subunit p110 of PI 3-kinase. Our results with this mutant clearly indicated that Akt/PKB and p70 S6 kinase activation by LIF is dependent on PI 3-kinase

activity in cardiac myocytes, and suggested that class 1a PI 3-kinase plays an important role in the signal transduction pathways of LIF in cardiac myocytes. p70 S6 kinase activation is shown to be mediated by redundant signaling pathways (23), and it had not been examined if p70 S6 kinase activation induced by LIF was dependent on Akt/PKB activity. Therefore, we tested the effect of adenovirus-mediated expression of dominant negative mutant of Akt/PKB (Akt-AA) on p70 S6 kinase activation by LIF, since pharmacological inhibitor specific for Akt/PKB has not yet been reported. Akt-AA inhibited the activation of p70 S6 kinase by LIF. These data clearly indicated that LIF sequentially activates PI 3-kinase-Akt/PKB-p70 S6 kinase pathway in cardiac myocytes.

IL-6-related cytokines, such as LIF and cardiotrophin-1, induce hypertrophy in cardiac myocytes through gp130 which is a common  $\beta$ -receptor of the IL-6-related cytokine family (18, 21, 36). However, the role of PI 3-kinase-Akt/PKB-p70 S6 kinase pathway in the phenotypic changes associated with cardiac hypertrophy has not been elucidated yet, except that LIF induces protein synthesis through PI 3-kinase and p70 S6 kinase activation (20). Using a PI 3-kinase inhibitor LY294002 and the dominant negative mutant of PI 3-kinase, we showed the requirement of PI 3-kinase activity in LIF-stimulated protein synthesis, ANP gene expression, and sarcomeric reorganization in cardiac myocytes. The downstream effectors of PI 3-kinase, both Akt/PKB and p70 S6 kinase were also required for stimulation of protein synthesis induced by LIF. However, sarcomeric reorganization did not involve Akt/PKB and p70 S6 kinase activation, suggesting other downstream effector(s) of PI 3-kinase than Akt/PKB mediates the sarcomeric reorganization induced by LIF. PI 3-kinase has been reported to transduce its signal to multiple effectors including Akt/PKB and certain isoforms of protein kinase Cs. Among them, Rac1 GTPase is shown to be regulated by PI 3-kinase activity in other cell types (10, 35), and involved in actin filament reorganization in cardiac myocytes (22). Therefore, it is possible that Rac1 is another separate downstream target of LIF-induced PI 3-kinase activation, which mediates PI 3-kinase-dependent sarcomeric reorganization in cardiac myocytes. Further, we revealed that LIF-induced ANP expression required PI 3-kinase and Akt/PKB activation, but not p70 S6 kinase activation. This observation was in accordance with a very recent report in which isoproterenol-induced ANP induction is shown to be mediated by activation of Akt/PKB (19). As it was also shown that phosphorylation and inhibition of glycogen synthase kinase 3 $\beta$  by Akt/PKB is involved in isoproterenol induction of ANP, glycogen synthase kinase 3 $\beta$  might be a downstream effector of LIF-induced Akt/PKB activation in ANP mRNA induction in cardiac myocytes. Further studies are needed for identifying the effector molecules acting downstream of PI 3-kinase or Akt/PKB, which mediate LIF-induced sarcomeric reorganization and ANP expression.

PI 3-kinase-mediated activation of Akt/PKB has been implicated in anti-apoptotic effect of growth factors (4, 6, 7, 11), and pressure overload was shown to induce massive myocyte apoptosis in ventricle-restricted gp130 knockout mice (13). Therefore, there is any probability that the inhibition of PI 3-kinase-Akt/PKB pathway induces cell death and inhibits cell reaction to LIF in cardiac myocytes. However, the expression of the dominant negative mutants of PI 3-kinase or Akt/PKB did not alter the viable cell number as assessed by WST-1 assay, and the activation of extracellular signal-regulated kinase by LIF (data not shown). Moreover, although Akt-AA inhibited protein synthesis and ANP gene expression, it did not repress sarcomeric reorganization induced by LIF. These results indicated that the inhibition of PI 3-kinase-Akt/PKB pathway by the procedures we used did not induce cell death of cardiac myocytes nor suppressed all responses to LIF, confirming the specificity of our results.

## PI 3K-AKT-P70 S6K PATHWAY IN CARDIAC HYPERTROPHY

In summary, the results of the present study demonstrated that LIF induces sequential activation of PI 3-kinase, Akt/PKB, and p70 S6 kinase, and that this PI 3-kinase-Akt/PKB-p70 S6 kinase pathway is essential for LIF-induced hypertrophic responses in cardiac myocytes.

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