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# Significance of ERK cascade compared with JAK/STAT and PI3-K pathway in gp130-mediated cardiac hypertrophy

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**Kodama, Hiroaki, Keiichi Fukuda, Jing Pan, Motoaki Sano, Toshiyuki Takahashi, Takahiro Kato, Shinji Makino, Tomohiro Manabe, Mitsushige Murata, and Satoshi Ogawa.** Significance of ERK cascade compared with JAK/STAT and PI3-K pathway in gp130-mediated cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 279: H1635–H1644, 2000.—We compared the role of the Raf-1/mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MEK)/extracellular signal-regulated protein kinase (ERK)/p90<sup>RSK</sup> cascade in gp130-mediated cardiac hypertrophy with the contribution of the Janus kinase (JAK)/signal transduction and activation of transcription (STAT) and phosphatidylinositol 3-kinase (PI3-K) pathways. Primary cultured neonatal rat cardiomyocytes were stimulated with leukemia inhibitory factor (LIF). LIF sequentially activated Raf-1, MEK1/2, ERK1/2, and p90<sup>RSK</sup>. We used PD-98059 (a specific MEK inhibitor), AG-490 (a JAK2 inhibitor), and wortmannin (a PI3-K inhibitor) to confirm that this cascade was independent of the JAK/STAT and PI3-K/p70 S6 kinase (S6K) pathways. PD-98059, AG-490, and wortmannin suppressed the LIF-induced increase in [<sup>3</sup>H]phenylalanine uptake by 54.7, 21.5, and 25.6%, respectively, and inhibited the increase in cell area by 61.2, 42.8, and 39.2%, respectively. Reorganization of myofilaments was predominantly suppressed by AG-490. LIF-induced expression of *c-fos*, brain natriuretic peptide, and skeletal  $\alpha$ -actin mRNA was markedly suppressed by PD-98059 and moderately suppressed by wortmannin and AG-490. Atrial natriuretic peptide was significantly suppressed by AG-490. These findings indicate that this pathway is critically involved in protein synthesis, induction of *c-fos*, brain natriuretic peptide, and skeletal  $\alpha$ -actin expression and is partially involved in myofilament reorganization and atrial natriuretic peptide induction in gp130-mediated cardiac hypertrophy.

leukemia inhibitory factor; mitogen-activated protein kinase; cardiomyocyte

TARGETED DISRUPTION of the gp130 gene, a common signal transducer for the interleukin (IL)-6 family of cytokines [IL-6, IL-11, oncostatin M, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and cardiotrophin-1] (10, 24), led to failure of the myocardium to

mature (1, 42) and suggested that members of this cytokine family may be important cardiac hypertrophic growth factors. Transgenic mice expressing IL-6 and IL-6 receptor displayed constitutive tyrosine phosphorylation of gp130 in the myocardium and cardiac hypertrophy (9). Chen (4) and Hirota et al. (8) demonstrated that aortic pressure overload in ventricle-restricted gp130 knockout mice displays the rapid onset of dilated cardiomyopathy and massive induction of myocyte apoptosis compared with the control mice, which exhibit compensatory hypertrophy. Pennica et al. (23) recently cloned a novel IL-6 family cDNA for cardiotrophin-1 from an ES cell library that had a potent hypertrophic effect on cardiomyocytes. These findings indicated that a gp130-dependent signaling pathway might be critically involved in the hypertrophic response of cardiomyocytes.

We (13) and Kunisada et al. (16) previously demonstrated that LIF causes cardiac hypertrophy and activates the Janus kinase (JAK)/signal transduction and activation of transcription (STAT) pathway, and Kunisada et al. (17) reported that overexpression of constitutively active STAT3 augmented the LIF-induced increase in [<sup>3</sup>H]leucine uptake and hypertrophy marker gene expression, whereas overexpression of a dominant-negative STAT3 decreased these events. Oh et al. (22) reported that LIF activated phosphatidylinositol 3-kinase (PI3-K) and that PI3-K stimulated protein kinase B and p70 S6 kinase (S6K) in cardiomyocytes. PI3-K activates Akt kinase (15) and other serine-threonine kinases and plays an important role not only in activation of glucose transport and glycogenesis (5, 38) but also in protein synthesis via p70 S6K. Insulin-like growth factor-I (11) and ANG II, well-known hypertrophic growth factors for cardiomyocytes, have been shown to activate the PI3-K pathway (26, 27). These findings confirmed the significance of the JAK/STAT and the PI3-K pathways in gp130-mediated cardiac hypertrophy.

Kunisada et al. (16) reported that the signaling pathway downstream of gp130 also activated an extracel-

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lular signal-regulated protein kinase (ERK) in cardiomyocytes. However, they did not address the significance of this cascade and upstream signaling of ERK in gp130-mediated cardiac hypertrophy. ERK, or mitogen-activated protein kinase (MAPK), is one of a family of serine/threonine kinases thought to play a central role in the signaling events leading to cell proliferation or differentiation in a variety of cell types (21, 31). The ERK cascade may transduce signals from diverse receptor types, including receptor protein tyrosine kinases, G protein-coupled receptors, and cytokine receptors, to produce growth responses. In cultured cardiomyocytes, the well-known cardiac hypertrophic growth factors phenylephrine, endothelin-1, ANG II, and fibroblast growth factor activate p42 and p44 isoforms of ERK (2, 5, 29). However, the contribution of the ERK cascade to the induction of cardiac hypertrophy remains controversial. Glennon et al. (7) demonstrated that antisense oligodeoxynucleotides against the ERK isoforms p42 and p44 inhibited the morphological changes of hypertrophy in cardiomyocytes exposed to phenylephrine. Post et al. (25) demonstrated that dominant-interfering mutants of ERK p42 and p44 as well as the use of PD-98059, an inhibitor of MAPK/ERK (MEK), failed to block phenylephrine-induced atrial natriuretic peptide (ANP) expression. Interestingly, neither carbachol nor ATP, which activate ERK, can induce cardiac hypertrophy (25). Thus it would be of interest to characterize the role of this pathway in gp130-mediated cardiac hypertrophy.

To address the significance of this cascade in gp130-mediated cardiac hypertrophy, we compared the role of this pathway with that of the JAK/STAT and PI3-K/p70 S6K pathways by analyzing their contribution to protein synthesis, gene expression, and myofilament reorganization.

## METHODS

**Cell culture.** Primary cultures of cardiomyocytes were prepared from the ventricles of 1-day-old neonate Wistar rats, as described previously (13, 14). Cells were seeded at a density of  $1-5 \times 10^5$  cells/cm<sup>2</sup> on gelatin-coated dishes. The nonmyocyte population was <5%, as determined by immunofluorescence staining with monoclonal antisarcomeric myosin antibody (MF20). Recombinant murine LIF was purchased from Genzyme. After 24 h of serum depletion, cardiomyocytes were stimulated with LIF (1,000 U/ml) in the presence or absence of MEK inhibitor (PD-98059, 30  $\mu$ M), PI3-K inhibitor (wortmannin, 10 nM), and JAK2 inhibitor (AG-490, 20  $\mu$ M) (20).

**Immunoprecipitation and Western blot analysis.** Cell lysates were prepared with lysis buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 0.25% sodium deoxycholate, 50 mM NaF, 10 mM Na<sub>3</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin. Lysates were immunoprecipitated using anti-STAT3, Raf-1, ERK1/2, p70 S6K, and p90<sup>RSK</sup> polyclonal antibody (Santa Cruz) at 4°C for 12 h and then protein G-Sepharose (Sigma Chemical) for 1 h. Proteins were separated on 7.5–12.5% SDS-polyacrylamide gels. Western blot analysis was performed as described previously (13, 14). Anti-phospho-ERK, phospho-MEK polyclonal antibody (New England Biolab), or anti-

phosphotyrosine antibody (4G10) was used as a primary antibody. A peroxidase-conjugated goat anti-rabbit IgG or a peroxidase-conjugated rabbit anti-mouse IgG was used as a secondary antibody. Signals were visualized with enhanced chemiluminescence (Amersham).

**Kinase activity assays for Raf-1 and ribosomal S6 kinases.** Immunoprecipitates were washed three times with lysis buffer and three times with kinase buffer containing 25 mM Tris·HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 0.5 mM EGTA and 2  $\mu$ M protein kinase inhibitor peptide and then incubated with 2.5 nmol of syntide-2, a synthetic Raf-1-specific substrate (Santa Cruz), or 2.5 nmol of S6 peptide (UBI) in the presence of 40  $\mu$ M ATP and 2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After a 20-min incubation at 25°C, aliquots of supernatant were spotted on P81 paper (Whatman), washed five times in 0.75% phosphoric acid, dried, and counted by the Cerenkov technique.

**Kinase assay in myelin basic protein-containing gel.** Activities of ERKs were assayed by the “in-gel” method with use of myelin basic protein (MBP)-containing gels, as described previously (41). Cell lysates were separated on an SDS-polyacrylamide gel containing 0.5 g/l MBP (Sigma Chemical). ERKs in the gels were denatured in 6 M guanidine HCl and renatured in 50 mM Tris·HCl (pH 8.0) containing 0.04% Tween 20 and 5 mM 2-mercaptoethanol. The phosphorylative activity of ERKs was assayed by incubating the gel with [ $\gamma$ -<sup>32</sup>P]ATP at 30°C for 1 h. After incubation, the gel was extensively washed, dried, and subjected to autoradiography.

**Gel mobility shift assay.** Cardiomyocytes were rinsed with PBS at 0°C and scraped into the same buffer. Nuclear extracts were prepared according to standard methods described previously (13, 14). Five micrograms of nuclear extract were incubated with 1  $\mu$ g of poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech) with or without competitor oligonucleotide in 20  $\mu$ l of 10 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, and 10% glycerol for 20 min at 25°C. The samples were incubated with 1 or 2 fmol of radiolabeled probes (~5,000 cpm) for 10 min at 25°C. The probes were purchased from Santa Cruz Biotechnology, and their sequences have been described (SIE-DNA, 5'-CAGTTCCTCAATC-3'). Binding reactions were resolved by a 4% native PAGE.

**Immunofluorescence photography and cell-sizing protocol.** Cells grown on glass coverslips were permeabilized in 1% formaldehyde-PBS for 10 min. After fixation, cells were stained with antisarcomeric myosin antibodies (MF20), as described previously (13). The sizes (surface area and perimeter) of the cardiomyocytes were measured using enlarged, calibrated fluorescent photomicrographs and quantitated and validated with a Power Macintosh computer (model G4) and an Epson scanner (model GT-9000) with Adobe Photoshop version 5.0J and NIH image version 1.56 software.

**Incorporation of [<sup>3</sup>H]phenylalanine.** The effects of LIF on [<sup>3</sup>H]phenylalanine uptake were determined in gelatin-coated 24-well plates. Serum-starved cardiomyocytes were stimulated with LIF (1,000 U/ml) in the presence or absence of PD-98059, wortmannin, and AG-490. After 48 h of LIF stimulation, [<sup>3</sup>H]phenylalanine uptake was measured as described previously (13). The results were expressed as disintegrations per minute per well. Each data point was the mean of six separate experiments.

**RNA extraction and Northern blot analysis.** Total RNA was isolated using TRIzol reagent. Rat ANP cDNA was obtained by RT-PCR from the heart RNA and cloned into the pCR II plasmid. PCR fragments of the rat skeletal  $\alpha$ -actin cDNA were kindly provided by Hiroshi Ito. Rat glyceraldehyde 3-phosphate dehydrogenase cDNA was used as an internal control. Inserts were labeled with [ $\gamma$ -<sup>32</sup>P]dCTP by the

random priming technique. A 20- $\mu$ g sample of total RNA was run on a 1% MOPS-formaldehyde-agarose gel, and Northern blots were performed as described previously (13).

**Statistical analysis.** Values are means  $\pm$  SD. Statistical significance among mean values was evaluated with an ANOVA. Student's *t*-test was used when two values were compared. Differences were considered significant when *P* < 0.05.

## RESULTS

*LIF activates the Raf-1/MEK/ERK/p90<sup>RSK</sup> cascade in cardiomyocytes.* To demonstrate the signal transduction pathways involved in LIF-induced cardiac hypertrophy, we elucidated the protein kinase pathway of phosphorylation by examining the time course of activation of Raf-1, MEK1/2, and ERK1/2. We initially observed a rapid increase in the MAPKKK activity of Raf-1 from 2 min after LIF stimulation and a gradual decrease thereafter (Fig. 1). We confirmed that equal amounts of Raf-1 proteins were immunoprecipitated in each reaction.

We next observed LIF-induced activation of MEK1/2. Because activation of MEK1/2 occurs through phosphorylation of serine residues 217 and 221, Western blot analysis was performed using anti-phospho-MEK antibody. Serine phosphorylation of MEK1/2 increased at 2 min, peaked at 5 min, and returned to the control level at 30 min (Fig. 2A). The MAPKK activity of MEK was also increased. MAPKK activity of MEK led to maximal levels at 5 min (data not shown).

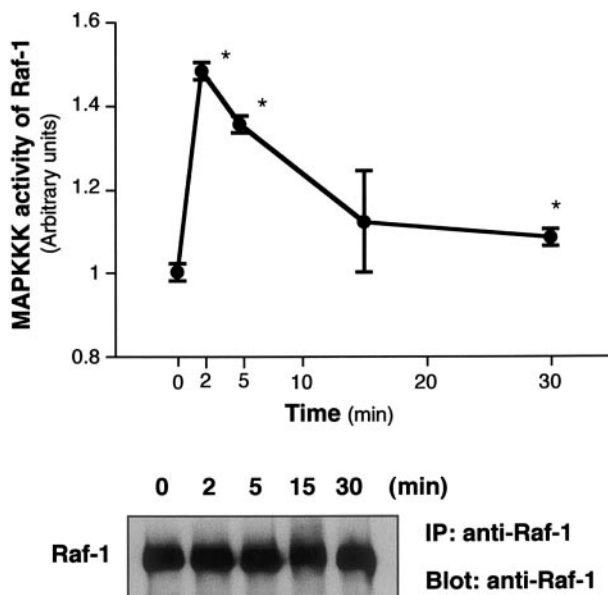


Fig. 1. Leukemia inhibitory factor (LIF) stimulated mitogen-activated protein kinase kinase (MAPKKK) activity of Raf-1 in cardiomyocytes. Confluent, serum-starved cardiomyocytes were stimulated with LIF (1,000 U/ml) for 2–30 min. Cell lysates were immunoprecipitated with an anti-Raf-1 antibody and incubated with Raf-1-specific substrate and [ $\gamma$ -<sup>32</sup>P]ATP. Results are means of 4 separate experiments, in which each experiment showed similar results. \**P* < 0.01. To confirm that equal amounts of protein were immunoprecipitated in each reaction, aliquots of the samples were also immunoprecipitated and subjected to Western blot analysis with anti-Raf-1 antibody (*bottom*). IP, immunoprecipitation.

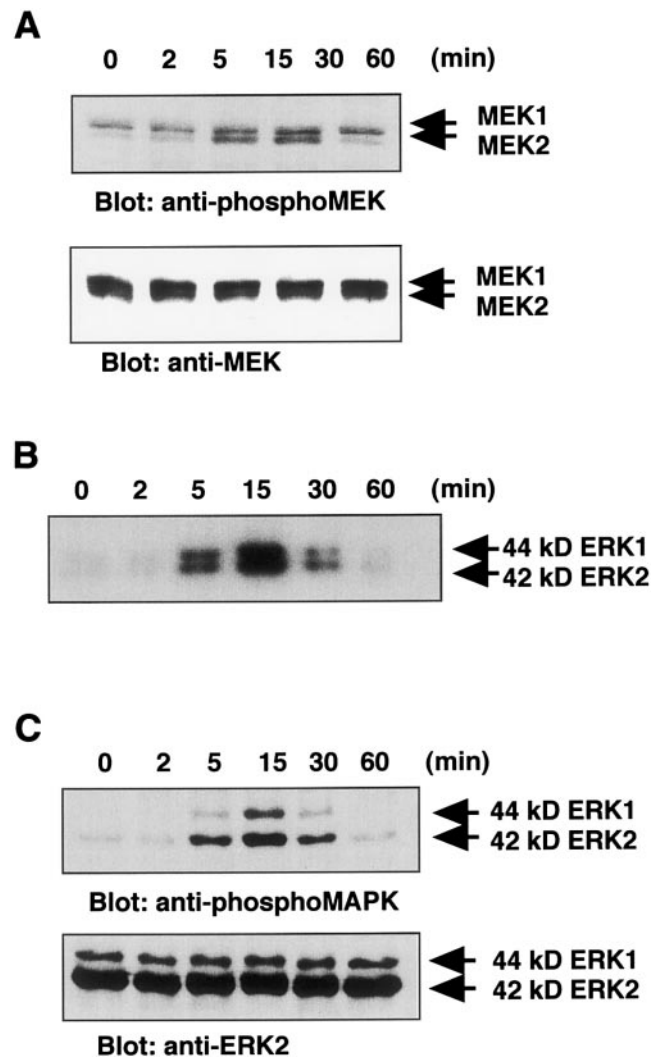


Fig. 2. LIF sequentially activates mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (MEK1/2) and extracellular signal-regulated protein kinase (ERK1/2) in cardiomyocytes. **A:** LIF phosphorylated and activated MEK1/2 in cardiomyocytes. Confluent, serum-starved cardiomyocytes were stimulated with LIF (1,000 U/ml) for 2–60 min. Cell lysates were separated and blotted with anti-phospho-MEK antibody (*top*) and anti-MEK antibody (*bottom*). Positions of MEKs are indicated by arrows at *right*. Serine phosphorylation of MEK1/2 increased after 2 min and peaked at 5 min. Each lane contained equal amounts of MEK1 and MEK2 (*bottom*). **B:** LIF stimulated myelin basic protein (MBP) kinase activity of ERK1/2 in cardiomyocytes. Cell lysates were resolved on a 10% SDS-polyacrylamide gel containing MBP (0.5 g/l). After denaturation and renaturation, phosphorylative activities of ERKs were assayed by incubating the gel with [ $\gamma$ -<sup>32</sup>P]ATP. MBP kinase activity of ERK increased at 5 min after LIF stimulation and peaked at 15 min. A representative autoradiogram from 3 independent experiments is shown. **C:** LIF induced tyrosine phosphorylation of ERK1/2 in cardiomyocytes. Cell lysates were separated and blotted with anti-phospho-ERK antibody (*top*) and anti-ERK antibody (*bottom*). Positions of ERKs are indicated by arrows at *right*. Tyrosine-phosphorylated ERK1 and ERK2 increased after 5 min and peaked at 15 min. Each lane contained equal amounts of ERK1 and ERK2 (*bottom*).

We measured the activities of ERKs using the in-gel method. MBP kinase activity migrating at 42 and 44 kDa increased after LIF stimulation, reached a maximum level at 15 min, and gradually decreased there-

after (Fig. 2B). Western blot analysis with anti-phospho-ERK antibody showed that ERK1/2 were unphosphorylated in unstimulated cells (Fig. 2C). Tyrosine phosphorylation of ERK1/2 increased at 5 min, peaked at 15 min, and returned to the control level at 30 min. This observation is consistent with the notion that phosphorylation of ERKs reflects their activation (21).

Ribosomal S6 kinases (p70 S6K and p90<sup>RSK</sup>) have been thought to be key enzymes in protein synthesis and are related to the ERK or PI3-K signal transduction pathways (12, 19). There is accumulating evidence to suggest that p70 S6K (3, 28) and p90<sup>RSK</sup> (29) were critically involved in cardiac hypertrophy. However, the localization and the precise role of ribosomal S6 kinases in cardiac hypertrophy are not well understood. To verify that p90<sup>RSK</sup> is activated by LIF and is downstream of ERK, but not of JAK/STAT or PI3-K, in cardiomyocytes, S6 kinase activity was measured in the presence and absence of PD-98059, wortmannin, or AG-490. The S6 kinase activity of p90<sup>RSK</sup> increased as early as 5 min and peaked at 15 min. LIF-induced activation of p90<sup>RSK</sup> was significantly inhibited by PD-98059 ( $n = 5$ ,  $P < 0.01$ ), but not by wortmannin or AG-490 (Fig. 3). Taken together, these results indicate that LIF activated the Raf-1/MEK/ERK/p90<sup>RSK</sup> cascade and that p90<sup>RSK</sup> is independent of the JAK/STAT and PI3-K pathways.

*LIF-induced activation of PI3-K/p70 S6K pathway was independent of the JAK/STAT or Raf-1/MEK/ERK/p90<sup>RSK</sup> pathway.* A previous report showed that LIF activated the PI3-K/p70 S6K pathway in rat cardiomyocytes (22). To confirm that this pathway was

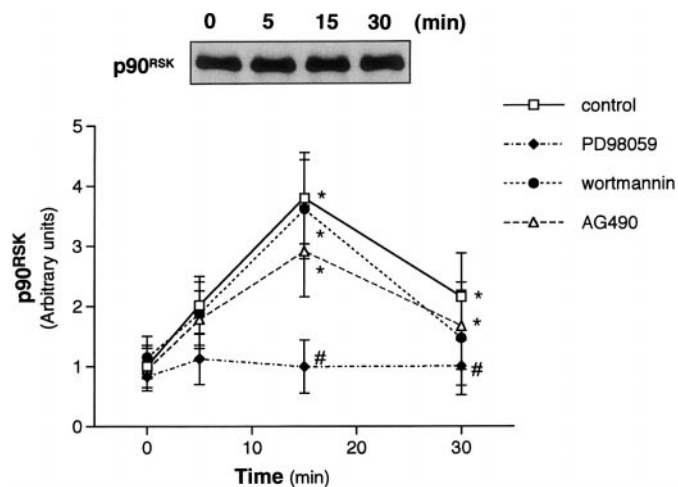


Fig. 3. LIF-induced activation of p90<sup>RSK</sup> was mediated by MEK/ERK pathway, but not by phosphatidylinositol 3-kinase (PI3-K) or JAK/STAT pathway. S6 kinase activity was measured as described in METHODS. S6 kinase activity of p90<sup>RSK</sup> was increased by LIF as early as 5 min and peaked at 15 min (control). Equal amounts of p90<sup>RSK</sup> were immunoprecipitated in each reaction (control; top). To determine the upstream signal of p90<sup>RSK</sup>, S6 kinase activity was measured in the presence and absence of PD-98059 or wortmannin. LIF-induced activation of p90<sup>RSK</sup> was significantly inhibited by PD-98059, but not by AG-490 or wortmannin. \* $P < 0.01$  vs. time 0; # $P < 0.01$  vs. LIF stimulation ( $n = 5$ ).

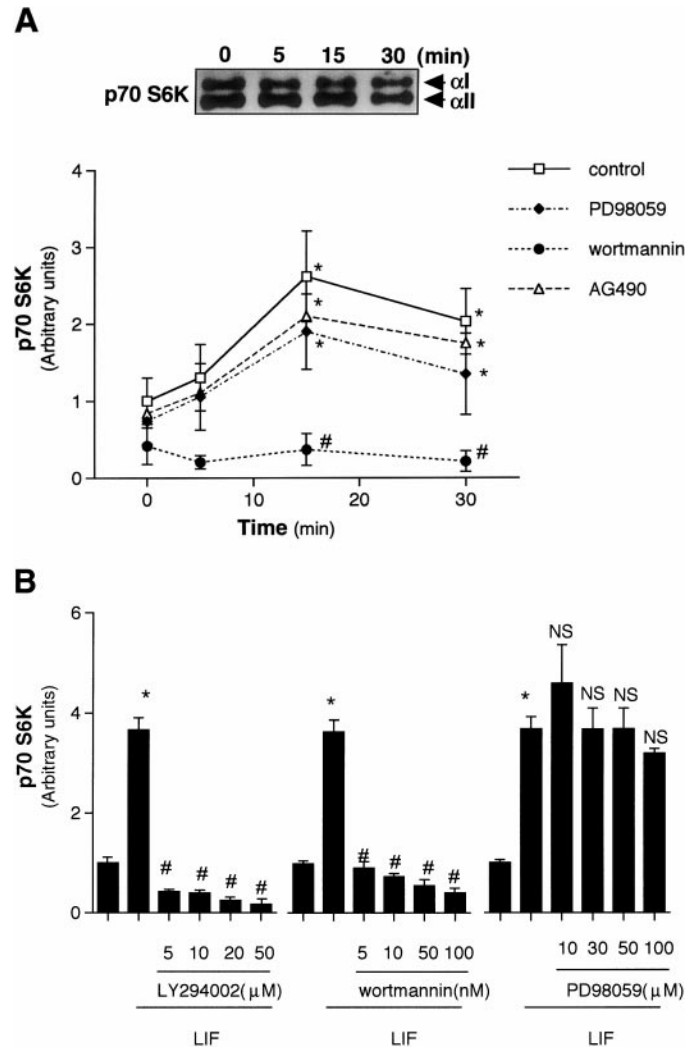


Fig. 4. LIF-induced activation of p70 S6K was mediated by PI3-K, but not by the MEK/ERK pathway or JAK/STAT pathways. A: time course of the kinase activity of p70 S6K and the effect of wortmannin, AG-490, and PD-98059 on LIF-stimulated cardiomyocytes. p70 S6K kinase activity was increased by LIF as early as 5 min and peaked at 15 min (control). Equal amounts of p70 S6K were immunoprecipitated in each reaction (control; top). The positions of the 85-kDa form ( $\alpha$ I) and the 70-kDa form ( $\alpha$ II) of p70 S6K are indicated. To determine the upstream signal of p70 S6K, S6 kinase activity was measured in the presence and absence of PD-98059 or wortmannin. LIF-induced activation of p70 S6K was significantly inhibited by wortmannin, but not by AG-490 or PD-98059. Results are means of 5 separate experiments. \* $P < 0.01$  vs. time 0; # $P < 0.01$  vs. LIF stimulation. B: dose-inhibitory relationship between wortmannin, LY-294002, and PD-98059 and p70 S6K activity in LIF-stimulated cardiomyocytes. Cells were preincubated with various concentrations of these inhibitors and stimulated with LIF, and the kinase activity of p70 S6K was measured 15 min after the stimulation. NS, not significant.

independent of the JAK/STAT or the Raf-1/MEK/ERK/p90<sup>RSK</sup> pathway, we performed a kinase activity assay for p70 S6K under various concentrations of wortmannin, LY-294002, AG-490, and PD-98059. LIF activated p70 S6K as early as 5 min, and activation peaked at 15 min (Fig. 4A). LIF-induced activation of p70 S6K was significantly inhibited by wortmannin, but not by PD-98059 or AG-490. Figure 4B shows the effect of various

concentrations of LY-294002, wortmannin, and PD-98059 on p70 S6K in LIF-stimulated cells. LY-294002 and wortmannin completely blocked p70 S6K even at a lower concentration. Neither PD-98059 nor AG-490 (data not shown) attenuated p70 S6K activity even at a higher concentration. These findings indicated that the PI3-K/p70 S6K pathway was independent of the other two pathways.

*Independence of the Raf-1/MEK/ERK/p90<sup>RSK</sup>, JAK/STAT, and PI3-K/p70 S6K pathways.* Because this study is based on the effect of inhibitors of each pathway, it was important to clarify the specificity of the inhibitors and independence of these three pathways. To demonstrate that PD-98059, wortmannin, and AG-490 (20) work as specific inhibitors of the Raf-1/MEK/ERK/p90<sup>RSK</sup>, PI3-K/p70 S6K, and JAK/STAT pathways, respectively, we preincubated the cells with these inhibitors before LIF stimulation and detected in-gel phosphorylation of MBP by ERK, tyrosine phosphorylation of STAT3, and p70 S6K activity and performed a gel mobility shift assay.

AG-490 inhibited the tyrosine phosphorylation of STAT3, whereas PD-98059 or wortmannin did not (Fig. 5A). Densitometric analysis revealed that AG-490 inhibited LIF-induced phosphorylation of STAT3 in cardiomyocytes by  $88.4 \pm 7.8\%$ . Gel mobility shift assay revealed that STAT3 activated by LIF bound to its consensus SIE site and that AG-490 almost completely inhibited this process, whereas wortmannin and PD-98059 did not (Fig. 5B). Figure 5C shows the in-gel phosphorylation of MBP by ERK after preincubation with these inhibitors in LIF-stimulated cells. Figure 5D shows the dose dependency of the inhibitors. PD-98059 at 10  $\mu\text{M}$  clearly inhibited the LIF-induced MBP phosphorylation by ERK, whereas AG-490 did not. Previous reports showed that wortmannin at lower concentrations (5–10 nM) specifically inhibited PI3-K (18), but wortmannin at higher concentrations (50–100 nM) also inhibited ERK in various cell types (33). Figure 5D revealed that 10 nM wortmannin had no effect on MBP phosphorylation, whereas 100 nM wortmannin attenuated MBP phosphorylation in LIF-stimulated cardiomyocytes. These findings indicated that PD-98059, wortmannin (5–10 nM), and AG-490 could be used as specific inhibitors of the Raf-1/MEK/ERK/p90<sup>RSK</sup>, PI3-K/p70 S6K, and JAK/STAT pathways, respectively.

*LIF-induced increase in [<sup>3</sup>H]phenylalanine uptake is predominantly mediated by the Raf-1/MEK/ERK/p90<sup>RSK</sup> cascade.* We tried to determine which of these pathways plays an important role in LIF-induced cardiac hypertrophy. The effect of PD-98059, wortmannin, or AG-490 on LIF-induced protein synthesis was investigated by measuring [<sup>3</sup>H]phenylalanine uptake. LIF caused a 67% increase in [<sup>3</sup>H]phenylalanine uptake compared with the control (Fig. 6). PD-98059, wortmannin, and AG-490 inhibited the LIF-induced [<sup>3</sup>H]phenylalanine uptake by 54.7, 21.5, and 25.6%, respectively, whereas these inhibitors at this concentration had minimal effects on the basal [<sup>3</sup>H]phenylalanine uptake. The results were fully reproducible

and indicated that the LIF-induced increase in protein synthesis in cardiomyocytes was mediated by all these pathways and that the Raf-1/MEK/ERK/p90<sup>RSK</sup> pathway plays the most important role in protein synthesis among these three pathways.

*Effect of PD-98059, wortmannin, and AG-490 on LIF-induced increase in cell size.* Morphometric analysis was used to evaluate any effect of the signal transduction inhibitors on the LIF-induced increase in cardiomyocytes. LIF caused a 35 and 43% increase in cell area and perimeter compared with the control cells, respectively. PD-98059, wortmannin, and AG-490 significantly decreased the increase in cell area by 61.2, 42.8, and 39.2%, respectively (Fig. 7A). PD-98059, wortmannin, and AG-490 significantly decreased the LIF-induced increase in perimeter of the cells by 38.8, 31.0, and 34.6%, respectively (Fig. 7B). These inhibitors at this concentration did not have any substantial effects on the basal size of the unstimulated cells. These results indicated that all these pathways were involved in the induction of cardiac hypertrophy and that, again, the Raf-1/MEK/ERK/p90<sup>RSK</sup> cascade plays the most important role in cell size increase.

Figure 8 shows representative immunofluorescence photographs of cardiomyocytes stimulated with LIF for 48 h in the presence of the various signal transduction inhibitors. LIF clearly caused reorganization of the myofilaments. AG-490 strongly inhibited the reorganization of myofilaments, whereas PD-98059 and wortmannin also inhibited reorganization, but to a lesser extent.

*Differential regulation of hypertrophic marker gene expression in gp130-mediated signaling.* To determine which pathway may mediate activation of hypertrophic marker genes such as *c-fos*, brain natriuretic peptide (BNP), skeletal  $\alpha$ -actin, and ANP, we performed Northern blot analysis on LIF-stimulated cells in the presence and absence of PD-98059, wortmannin, and AG-490. LIF activated *c-fos* (30 min), BNP (1 h), skeletal  $\alpha$ -actin (24 h), and ANP (24 h; Fig. 9). Expression of *c-fos* was strongly inhibited by PD-98059 and moderately inhibited by wortmannin and AG-490. BNP expression was markedly inhibited by PD-98059 but was only slightly inhibited by wortmannin and AG-490. Skeletal  $\alpha$ -actin expression was strongly inhibited by PD-98059 and wortmannin and was not affected by AG-490. In contrast, ANP expression was significantly inhibited by AG-490 but was not affected by PD-98059 or wortmannin. These findings indicated that various hypertrophic marker genes were differentially regulated by these three pathways in gp130-mediated cardiac hypertrophy.

## DISCUSSION

The relay system that transmits signals from gp130 to the nucleus involves at least three distinct pathways of protein phosphorylation: the JAK/STAT (13, 16), PI3-K/p70 S6K (22, 30) and ERK pathways (16, 30). We investigated the role of the ERK pathway in gp130-mediated cardiac hypertrophy and found that 1) LIF

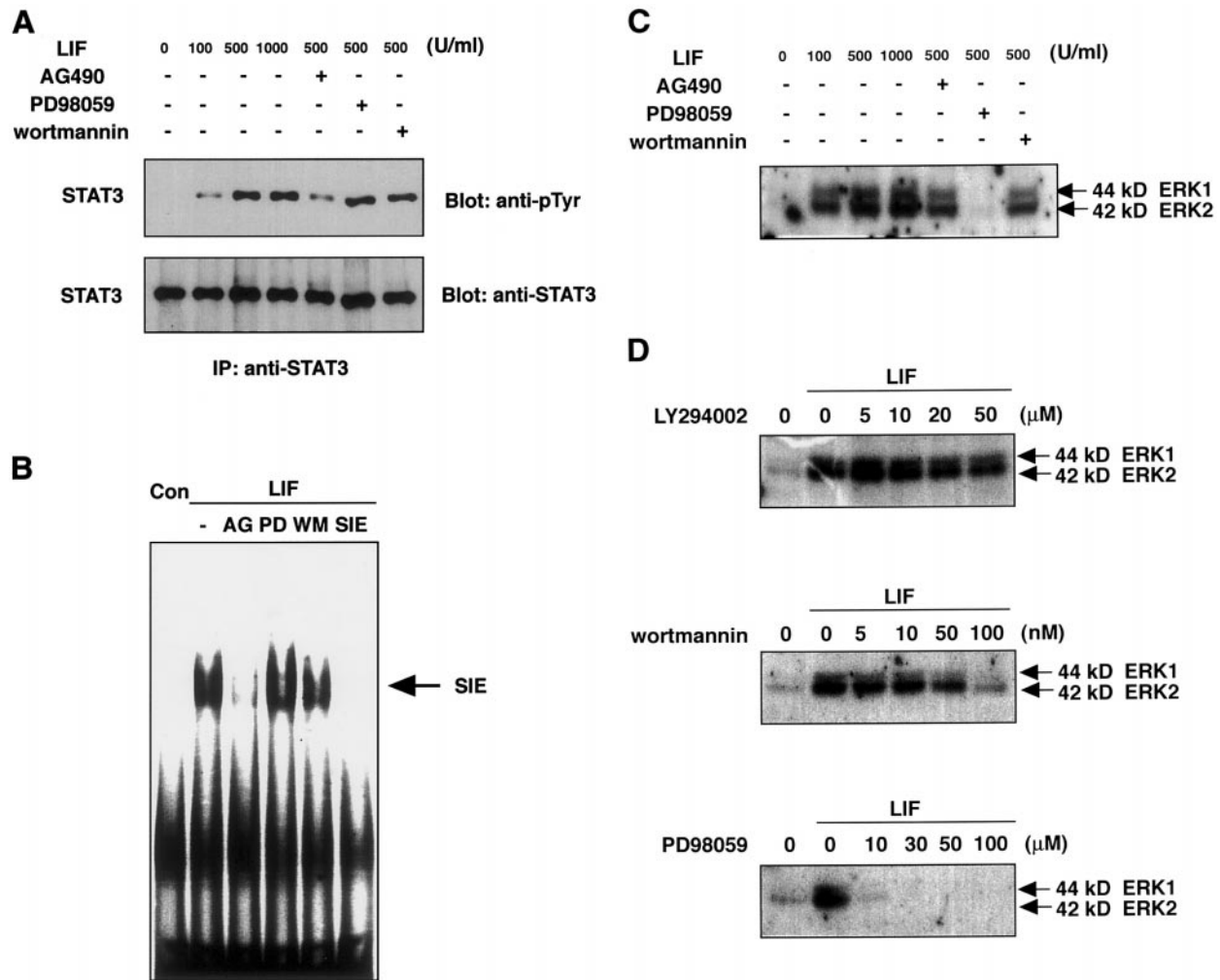


Fig. 5. Inhibitory effects of AG-490 (AG), PD-98059 (PD), and wortmannin (WM) on activation of signal transduction and activation of transcription (STAT3) and ERK1/2. **A**: tyrosine phosphorylation of STAT3 was inhibited by AG-490, but not by PD-98059 or wortmannin. Confluent, serum-starved cardiomyocytes were stimulated with LIF (100–1,000 U/ml) for 5 min in the presence and absence of AG-490, PD-98059, or wortmannin. Tyrosine phosphorylation of STAT3 was investigated by IP-Western blot analysis. AG-490 decreased LIF-induced phosphorylation of STAT3 by  $88.4 \pm 7.8\%$ , whereas PD-98059 or wortmannin did not affect the phosphorylation of STAT3. To confirm that equal amounts of STAT3 were immunoprecipitated in each reaction, the same membranes were reprobbed with anti-STAT3 antibody. Similar results were obtained from 3 independent experiments. **B**: LIF-induced gel mobility shift of SIE was inhibited by AG-490, but not by PD-98059 or wortmannin. Gel mobility shift assay was performed on cells that had been preincubated with or without AG-490 (20  $\mu$ M), wortmannin (10 nM), or PD-98059 (30  $\mu$ M) and stimulated with LIF. *Right lane*, competition by unlabeled SIE probe to demonstrate that this band was specific. **C**: ERK1/2 activity was inhibited by PD-98059, but not by wortmannin or AG-490. Confluent, serum-starved cardiomyocytes were stimulated with LIF (100–1,000 U/ml) for 15 min in the presence and absence of AG-490, PD-98059, or wortmannin. Activities of ERK1/2 were assayed by the “in-gel” method. PD-98059 clearly inhibited the LIF-induced activation of ERK1/2, whereas AG-490 and wortmannin did not significantly affect the activation of ERK1/2. A representative autoradiogram from 3 independent experiments is shown. **D**: dose-inhibitory relationship between wortmannin and LY-294002 and ERK. In-gel MBP phosphorylation activity was measured under various concentrations of wortmannin, LY-294002 and ERK. A low dose of wortmannin (5–10 nM) had no effect on LIF-induced ERK activation; a high dose (100 nM) inhibited ERK activation. LY-294002 did not attenuate this activation. PD-98059 at 10  $\mu$ M was sufficient to inhibit ERK in LIF-stimulated cardiomyocytes.

sequentially activates the Raf-1/MEK/ERK-p90<sup>RSK</sup> cascade in rat cardiomyocytes, 2) activation of these three pathways was basically independent, 3) PD-98059, wortmannin, and AG-490 inhibited LIF-induced increase in [<sup>3</sup>H]phenylalanine uptake and cell area, 4) LIF-induced reorganization of the myofilament was apparently suppressed by AG-490, but PD-98059

or wortmannin only had a minimal effect, and 5) LIF-induced expression of *c-fos*, BNP, and skeletal  $\alpha$ -actin was markedly suppressed by PD-98059 and was moderately suppressed by wortmannin and AG-490, but, in contrast, LIF-induced expression of ANP was significantly suppressed by AG-490 but was not suppressed by PD-98059 or wortmannin. These findings indicated

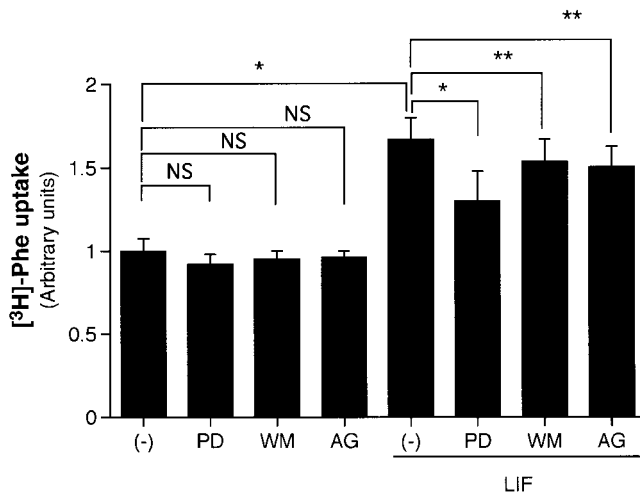


Fig. 6. Effects of PD-98059 (PD), wortmannin (WM), and AG-490 (AG) on LIF-induced increase in [<sup>3</sup>H]phenylalanine uptake. Serum-starved cardiomyocytes were stimulated with LIF (1,000 U/ml) in the presence and absence of PD-98059, wortmannin, and AG-490, and [<sup>3</sup>H]phenylalanine uptake was measured at 48 h after stimulation. The LIF-induced increase in [<sup>3</sup>H]phenylalanine uptake was inhibited by PD-98059, wortmannin, and AG-490 by 54.7, 21.5, and 25.6%, respectively. Each bar represents mean of 6 separate experiments. \**P* < 0.01; \*\**P* < 0.05.

that the Raf-1/MEK/ERK-p90<sup>RSK</sup> pathway was critically involved in the progression of gp130-mediated cardiac hypertrophy in addition to the other two pathways and that each pathway induces different hypertrophic marker genes.

There is conflicting evidence on the role of the ERK cascade in the development of cardiac hypertrophy. The Raf-1/MEK/ERK cascade, also known as the ERK module, can be activated by various hypertrophic stimuli, including phenylephrine, endothelin-1, ANG II, and mechanical stress (2, 5, 29, 40, 41), and has been shown to play an important role in cardiac hypertrophy. Transfection of constructs encoding active Ras, Raf-1, or MEK can induce ANP,  $\beta$ -myosin heavy chain, skeletal  $\alpha$ -actin, and myosin light chain-2v promoter activities (34, 36, 37). Dominant-negative Ras or Raf-1 can inhibit phenylephrine-induced ERK and cardiac hypertrophic gene promoter activities (34, 36). These findings suggested that the Raf-1/MEK/ERK pathway was critical to the development of cardiac hypertrophy.

In contrast, Thorburn et al. (35) reported that over-expression of the active forms of Raf-1 or ERK does not cause the sarcomeric organization typical of hypertrophic growth, and Post et al. (25) reported that inhibition of MEK by PD-98059 did not suppress phenylephrine-induced sarcomeric organization or ANP gene expression. Moreover, they showed that ATP and carbachol activated the Raf-1/MEK/ERK cascade, although neither of these reagents could cause cardiac hypertrophy. These findings suggested that the activation of ERK alone was not sufficient for the induction of cardiac hypertrophy and hypertrophic gene expression and suggested that the role of this pathway was distinct from ligand to ligand.

The present study compared the roles of these three pathways in LIF-induced cardiac hypertrophy with use of specific inhibitors. We previously observed that AG-490, wortmannin (10 nM), and PD-98059 specifically blocked the JAK/STAT, PI3-K/p70 S6K, and Raf-1/MEK/ERK/p90<sup>RSK</sup> pathways, respectively, but did not attenuate other pathways. These findings suggested that these three pathways were mutually independent. However, the findings that all these pathways attenuated hypertrophic marker gene expression indicated that these pathways cooperatively regulated hypertrophic gene expression.

The present data indicated that inhibition of Raf-1/MEK/ERK/p90<sup>RSK</sup> cascade in gp130-mediated cardiac hypertrophy suppressed protein synthesis and induction of *c-fos*, BNP, and skeletal- $\alpha$ -actin, whereas it had a minimal effect on ANP gene expression and myofil-

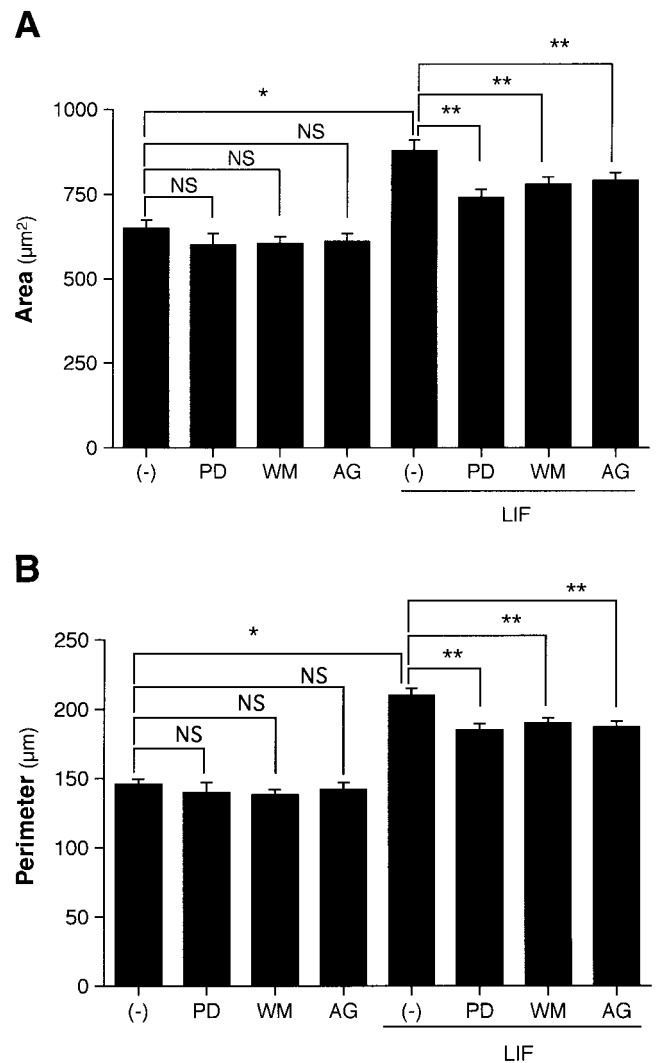


Fig. 7. Effect of PD-98059 (PD), wortmannin (WM), and AG-490 (AG) on LIF-induced increase in cell area (A) and perimeter (B). Cardiomyocytes were stimulated with LIF for 48 h in the presence and absence of PD-98059, wortmannin, and AG-490. Surface area and perimeter of the cardiomyocytes were measured using calibrated fluorescent photomicrographs of the sarcomeric myosin, which were quantitated and validated. \**P* < 0.01; \*\**P* < 0.05.

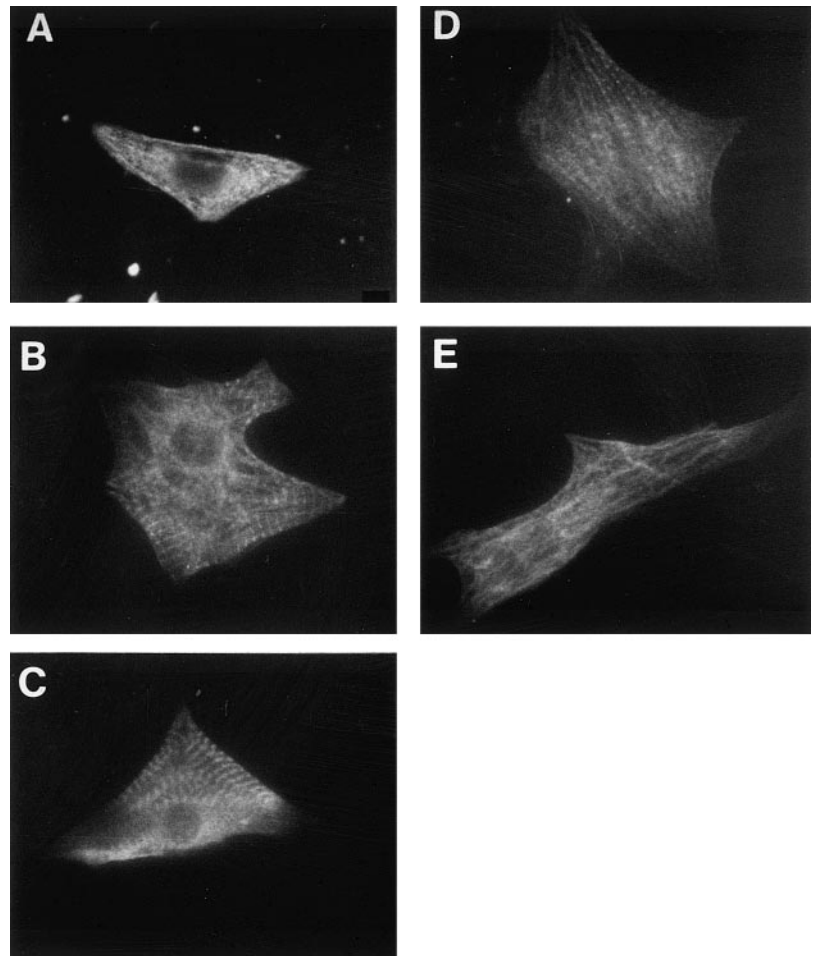
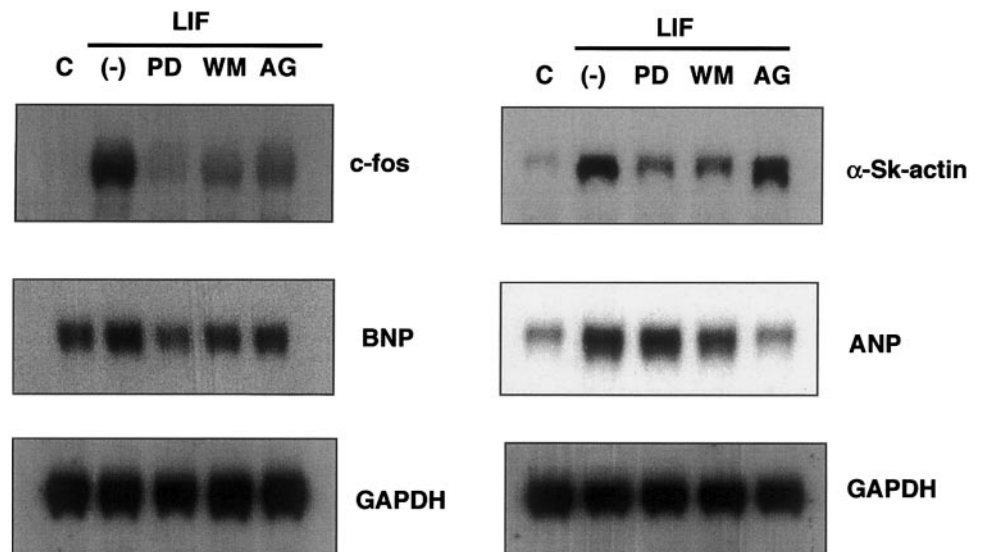


Fig. 8. Effect of PD-98059, wortmannin, and AG-490 on LIF-induced increase in myofilament reorganization. Representative immunofluorescent photographs of cardiomyocytes stained with antisarcomeric myosin are shown. Serum-starved cardiomyocytes were stimulated with LIF (1,000 U/ml) for 48 h in the presence and absence of PD-98059, wortmannin, and AG-490. LIF caused an increase in cell size and reorganization of myofilaments. A: control; B: LIF; C: LIF + PD-98059; D: LIF + wortmannin; E: LIF + AG-490.

ament reorganization. The finding that the inhibition of this cascade did not affect myofilament reorganization or ANP gene induction in gp130-mediated cardiac hypertrophy is in accordance with results obtained in phenylephrine-induced cardiac hypertrophy (25, 35). However, the finding that inhibition of this cascade

suppressed protein synthesis and induction of *c-fos*, BNP, and skeletal- $\alpha$ -actin strongly indicates that this pathway was critically involved in gp130-mediated cardiac hypertrophy. Together, these results suggest that this cascade plays a crucial role in gp130-mediated cardiac hypertrophy, although this pathway alone may

Fig. 9. Effect of PD-98059 (PD), wortmannin (WM), and AG-490 (AG) on LIF-induced gene expression. Cardiomyocytes were stimulated with LIF in the presence and absence of PD-98059, wortmannin, and AG-490, and total RNA was extracted at 30 min (*c-fos*), 1 h [brain natriuretic peptide (BNP)], or 24 h [skeletal  $\alpha$ -actin and atrial natriuretic peptide (ANP)] after stimulation. LIF-induced *c-fos* and skeletal  $\alpha$ -actin expression was strongly inhibited by PD-98059 and moderately by wortmannin and AG-490. BNP expression was strongly inhibited by PD-98059, but only slightly by wortmannin and AG-490. In contrast, LIF-induced expression of ANP was strongly inhibited by AG-490. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



not be sufficient for the development of cardiac hypertrophy. Recent studies demonstrated that gp130-mediated cardiac hypertrophy has a phenotype distinct from that for phenylephrine-induced cardiac hypertrophy (39). It was interesting that the inhibition of this cascade caused a similar response, although these two ligands caused quite distinctive hypertrophic phenotypes.

In the present study we observed a role for the PI3-K and JAK/STAT pathways in hypertrophic marker gene induction. Oh et al. (22) revealed that inhibition of PI3-K resulted in suppression of protein synthesis and partial inhibition of *c-fos* induction in LIF-mediated cardiac hypertrophy, but they did not find a role for this pathway in induction of other hypertrophic marker genes. The present study revealed that inhibition of the PI3-K/p70 S6K pathway caused partial inhibition of BNP, skeletal  $\alpha$ -actin, and ANP. Kunisada et al. (17) showed that adenovirus-mediated transfer of dominant-negative mutants of STAT3 partially attenuated *c-fos* and ANP expression in cardiomyocytes. We have observed the effect of a JAK2 inhibitor on hypertrophic marker gene induction. It was quite interesting that expression of ANP was more strongly inhibited by a JAK2 inhibitor than MEK or PI3-K inhibitors, whereas expression of other hypertrophic marker genes such as *c-fos*, BNP, and skeletal  $\alpha$ -actin was strongly inhibited by a MEK inhibitor. Because the rat ANP promoter region does not contain an SIE consensus sequence (32), AG-490 did not directly inhibit the transcription of ANP. Furthermore, myofibrillar reorganization induced by LIF was more strongly disrupted by AG-490 than by PD-98059. These findings indicated that the JAK/STAT pathway and the Raf-1/MEK/ERK pathway play different roles in gp130-mediated cardiac hypertrophy, but further studies are needed to clarify the precise role of each pathway.

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