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Interleukin-18 Is a Pro-hypertrophic Cytokine That Acts through a Phosphatidylinositol 3-Kinase-Phosphoinositide-dependent Kinase-1-Akt-GATA4 Signaling Pathway in Cardiomyocytes*

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In patients with congestive heart failure, high serum levels of the proinflammatory cytokine interleukin (IL)-18 were reported. A positive correlation was described between serum IL-18 levels and the disease severity. IL-18 has also been shown to induce atrial natriuretic factor (ANF) gene expression in adult cardiomyocytes. Because re-expression of the fetal gene ANF is mostly associated with hypertrophy, a hallmark of heart failure, we hypothesized that IL-18 induces cardiomyocyte hypertrophy. Treatment of the cardiomyocyte cell line HL-1 with IL-18 induced hypertrophy as characterized by increases in protein synthesis, phosphorylated p70 S6 kinase, and ribosomal S6 protein levels as well as cell surface area. Furthermore, IL-18 induced ANF gene transcription in a time-dependent manner as evidenced by increased ANF secretion and ANF promoter-driven reporter gene activity. Investigation into possible signal transduction pathways mediating IL-18 effects revealed that IL-18 activates phosphoinositide 3-kinase (PI3K), an effect that was blocked by wortmannin and LY-294002. IL-18 induced Akt phosphorylation and stimulated its activity, effects that were abolished by Akt inhibitor or knockdown. IL-18 stimulated GATA4 DNA binding activity and increased transcription of a reporter gene driven by multimerized GATA4-binding DNA elements. Pharmacological inhibition or knockdown studies revealed that IL-18 induced cardiomyocyte hypertrophy and ANF gene transcription via PI3K, PDK1, Akt, and GATA4. Most importantly, IL-18 induced ANF gene transcription and hypertrophy of neonatal rat ventricular myocytes via PI3K-, Akt-, and GATA4-dependent signaling. Together these data provide the first evidence that IL-18 induces cardiomyocyte hypertrophy via PI3K-dependent signaling, defines a mechanism of IL-18-mediated ANF gene transcription, and further supports a role for IL-18 in inflammatory heart diseases including heart failure.

Heart failure is one of the leading causes of morbidity and mortality in the developed countries. It is characterized by increased hemodynamic overload, abnormalities in neurohormonal regulation, cell death, and pathological remodeling with compensatory hypertrophy (1). As cardiomyocytes are terminally differentiated and have limited regenerative capacity, they do not multiply further but undergo hypertrophy in response to various insults such as inflammation, infarction, hemodynamic overload following aortic banding, and exposure to vasoactive hormones such as endothelin-1, and α- and chronic β-adrenergic stimulation (2, 3). In addition to increased protein synthesis and surface area, hypertrophy is characterized by re-expression of the fetal genes such as skeletal muscle α-actin, β-myosin heavy chain, and atrial natriuretic factor (ANF).[1] These fetal genes and several other cardiac-specific genes are regulated by the coordinated interaction of various transcription factors including GATA4 (4–6).

GATA 4 is a member of the highly conserved zinc finger containing the GATA family of transcription factors that bind the consensus DNA sequence 5′-WGATAR-3′. In mammals, the GATA family consists of six members, GATA1–6. Whereas GATA1–3 are expressed predominantly in hematopoietic cells, GATA4–6 are expressed in the heart and gut (7–9). They regulate cell death, survival, differentiation, migration of cardiomyocyte precursors, and cardiomyocyte hypertrophy (10–13). Various hypertrophic stimuli activate GATA4 leading to up-regulation of its downstream gene targets.

Interleukin-18 is a pleiotropic cytokine and exerts both proinflammatory and pro-apoptotic properties (14–16). It is expressed by both immune and nonimmune cells, and plays a critical role in the pathophysiology of various diseases including myocardial ischemia, infarction, and myocarditis (17–19). Recently, Seta *et al.* (20) have described increased circulating levels of IL-18 in patients with congestive heart failure. In that study, a direct correlation was shown between serum IL-18

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[1] The abbreviations used are: ANF, atrial natriuretic factor; BSA, bovine serum albumin; DNR, daunorubicin hydrochloride; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase-3; IL, interleukin; JNK, c-Jun NH_2-terminal kinase; iNOS, inducible form of nitric-oxide synthase; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor κB; NRVM, neonatal rat ventricular myocytes; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent kinase-1; RT, reverse transcription; siRNA, small interfering RNA; ELISA, enzyme-linked immunosorbent assay; S6K, S6 kinase; IL-18R, IL-18 receptor; PI3P, phosphatidylinositol 3,4,5-phosphate.

levels and the severity of myocardial damage and dysfunction. In addition, IL-18 was shown to induce ANF gene transcription (20). Because re-expression of the fetal gene ANF is mostly associated with myocardial hypertrophy and failure (21, 22), we hypothesized that IL-18 might act as a pro-hypertrophic cytokine. Therefore, we investigated the direct effects of IL-18 on cardiomyocyte hypertrophy, and we explored the signal transduction pathways activated by IL-18 in inducing cardiomyocyte hypertrophy using the murine atrial cardiomyocyte cell line HL-1 (23). Our results reveal, for the first time, that IL-18 is indeed a pro-hypertrophic cytokine, as evidenced by increases in total protein synthesis, in the levels of the phosphorylated forms of two translational regulatory proteins p70 S6 kinase and ribosomal S6 protein, and in cell surface area. Furthermore, IL-18 induced ANF promoter activity, mRNA expression, and protein secretion. Treatment with wortmannin, LY294002, Akt inhibitor, or knockdown of PDK1, Akt, or GATA4 attenuated IL-18-mediated cardiomyocyte hypertrophy and ANF gene transcription. Most importantly, IL-18 induced ANF expression and hypertrophy of neonatal rat ventricular myocytes (NRVM). In NRVM, IL-18 induced ANF expression via PI3K, Akt, and GATA4 and increased protein synthesis via PI3K and Akt. Together, these results indicate that IL-18 signals via PI3K → PDK1 → Akt → GATA4, induces ANF gene transcription and hypertrophy of cardiomyocytes, and suggests that IL-18 may play a role in the initiation and progression of heart failure, a disease state characterized by myocardial hypertrophy.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine cardiomyocyte cell line HL-1 that maintains phenotypic characteristics of adult cardiomyocytes (23) was grown as monolayers in Claycomb Media™ (JRH Biosciences, Lenexa, KS) and supplemented with 10% fetal bovine serum (JRH Biosciences), 0.1 mM norepinephrine, 2 mM L-glutamine (Invitrogen), and 1× antibiotic/antimycotic solution (complete media). All culture dishes and flasks were pre-coated with 0.00125% fibronectin (Sigma) in 0.02% gelatin (BD Biosciences). Cells were maintained in complete media at 37 °C in a humidified atmosphere of 95% air plus 5% CO_2. At 70–80% confluency, the media were replaced with serum- and norepinephrine-free medium containing 0.5% BSA. After overnight culture, cells were treated with IL-18 (recombinant mouse IL-18, catalog number B-004-5, R & D Systems, Minneapolis, MN) for the indicated periods. Specificity of IL-18 was verified by incubating the cells with rat anti-mouse IL-18 neutralizing antibodies (catalog number D048-3, 5 μg/ml; R & D Systems) for 1 h followed by the addition of IL-18. Normal rat IgG (catalog number MAB005, R & D Systems) served as a control. In order to define the signal transduction pathway(s) involved in IL-18-mediated ANF gene expression, cardiomyocytes were pretreated with wortmannin (a PI3K inhibitor, 100 nM in Me_2SO) or Akt inhibitor (1*L*-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate; catalog number 124005; 1 μM in Me_2SO) for 1 h prior to the addition of IL-18. Akt inhibitor selectively inhibits Akt activation and at this concentration will not affect PI3K activation (24). We and others have previously used this compound to inhibit Akt activation. In human aortic smooth muscle cells, Akt inhibitor attenuated CXCL16-mediated Akt activation (25). In mouse cardiomyocytes, the Akt inhibitor inhibited β-adrenergic stimulation-mediated IL-18 induction (26). Akt inhibitor was also shown to inhibit Akt activation in fibroblasts (27), pulmonary epithelial cells (28), and cancer cells (29). In addition to wortmannin, we also treated cardiomyocytes with LY294002 (a PI3K inhibitor, 10 μM in Me_2SO) for 1 h prior to the addition of IL-18. Because IL-18 has been shown to activate diverse signaling pathways including activation of NF-κB, p38 MAPK, p42/p44 MAPK (ERK), and JNK (16, 30–36), and as some of these pathways have been implicated in cardiomyocyte hypertrophy (37–41), we also examined their activation status following IL-18 treatment, and investigated their role in IL-18-mediated cardiomyocyte hypertrophy. Cardiomyocytes were transfixed with p65 siRNA (sense, 5-GCCCUAUCCCUUUACGUCA-3; 50 nM for 48 h) or treated with p38 MAPK inhibitor (SB203580, 1 μM in Me_2SO for 30 min), ERK inhibitor (PD98059, 10 μM in Me_2SO for 1 h), or JNK inhibitor (SP600125, 10 μM in Me_2SO for 30 min) prior to the addition of IL-18. The above inhibitors were obtained from Calbiochem-Novabiochem. In addition, cells were transfected with AKT (50 nM; Signal-Silence™ Akt siRNA, targets Akt1 and Akt2; catalog number 6211, Cell Signaling Technology), PDK1 (catalog number Q-004064-00-09; Dharmacon, Lafayette, CO; 150 nM), GATA4 siRNA (catalog number Q-004919-00-09; 150 nM), or negative control siRNA (catalog number D-001206-13-05; mixture of the following duplexes that will not target any genes in mammals: sense, 5′-AUGAACGUGAAUUGCUCAAUU; sense, 5′-UAAGGCUAUGAAGAGAUACUU; sense, 5′-AUGUAUUGGCCUGUAUUAGUU; sense, 5′-UAGCGACUAAACACAUCAAUU; Technical Information, Dharmacon,) using Oligofectamine™ (Invitrogen). 48 h later, cells were treated with IL-18 for the indicated times. Knockdown of proteins following siRNA transfection was confirmed by Western blotting.

Cell Death Assay—At 70% confluency, the complete media were replaced with media containing 0.5% BSA. After 48 h, IL-18 was added, and the incubation was continued for an additional 24 h. Daunorubicin hydrochloride (DNR; Sigma) was used as a positive control. DNR (5 μM for 24 h) was shown previously to induce apoptosis in HL-1 cardiomyocytes (42). At the end of the incubation period, cells were harvested and analyzed for mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by ELISA (Cell Death Detection ELISA^{PLUS} kit; Roche Diagnostics) (25, 43).

Analysis of Protein Synthesis, DNA Levels, and Cell Surface Area—Cardiomyocyte hypertrophy was assessed by two independent methods: increased protein synthesis and cell surface area. The rate of protein synthesis was determined by the incorporation of [^{3}H]leucine. Briefly, HL-1 cardiomyocytes were plated in 24-well plates, and after overnight culture, the complete media were replaced with media containing 0.5% BSA. 24 h later, cells were treated with IL-18. Forty two hours later, 0.5 μCi of [^{3}H]leucine was added to the culture medium, and the incubation was continued for an additional 6 h. The radioactivity incorporated into the trichloroacetic acid-precipitable material was determined by using a liquid scintillation counter. In order to determine the role of PI3K signaling in IL-18-mediated protein synthesis, cells were pretreated with various pharmacological inhibitors or transfected with siRNA. Because prolonged incubation with wortmannin or LY294002 is known to exert toxic effects, we also verified their effects on cell death. Total DNA levels were analyzed in duplicate samples using the DNeasy tissue kit (Qiagen, Valencia, CA). The [^{3}H]leucine incorporation was normalized to DNA, and the ratio of [^{3}H]leucine incorporation/DNA from untreated cells was considered 1, and the results are expressed as fold increase from untreated controls. In order to investigate the role of PI3K, Akt, and GATA4, cells were pretreated with wortmannin and Akt inhibitor or transfected with siRNA prior to IL-18 treatment. To assess cell surface area, cells were grown in chamber slides (Lab-Tek™ Chamber Slide™ System, Nalge Nunc International, Rochester, NY). After overnight culture, the complete media were replaced with medium containing 0.5% BSA. 24 h later, cells were exposed to IL-18 for an additional 48 h. 100 cells from each experiment were randomly selected and digitally photographed using an Olympus CKX41 inverted microscope equipped with a Olympus digital camera (C5050 Zoom) at ×20 magnification. Surface area was measured using Adobe® Photoshop® software, and the results are expressed as % increase from cells treated with phosphate-buffered saline.

GATA DNA Binding Activity—GATA4 DNA binding activity in nuclear protein extracts was analyzed by electrophoretic mobility shift assay (EMSA) (25, 43) using double-stranded consensus GATA4-specific oligonucleotides (sense, 5′-TCGCTGGACTGATAACTTTAAAAG-3′) from the ANF promoter (44). Double-stranded mutant oligonucleotides (sense, 5′-TCGCTGGACTG*G*TAACTTTAAAAG-3′) served as controls. Gel supershift assays were performed using rabbit anti-GATA4 (sc-9053 X), GATA-5 (sc-9054 X), or GATA-6 (sc-9055 X) polyclonal antibodies (TransCruz Gel Supershift reagents; Santa Cruz Biotechnology, Inc.). Normal rabbit IgG (preimmune; R & D Systems) served as a control.

Transient Cell Transfections and Reporter Assays—In addition to EMSA, we have analyzed GATA4-driven luciferase activity in transient transfection assays using a luciferase reporter vector (pLuc-MCS; Stratagene) containing multimers of GATA4 DNA binding sequence from the ANF promoter ((CTCT**GATA**A)$_3$) using Lipofectamine™. pLuc-MCS served as a control. 24 h after transfection, cells were treated with IL-18. Each cell sample was co-transfected with 100 ng of endotoxin-free *Renilla* luciferase vector (pRL-TK vector; Promega) to normalize for any differences in transfection efficiency. At the end of the experimental period, cells were harvested for the dual luciferase assay (Promega, Madison, WI). Data were normalized by dividing firefly luciferase activity with the corresponding *Renilla* luciferase (25, 43). Transfection efficiency of HL-1 cardiomyocytes was determined by us-

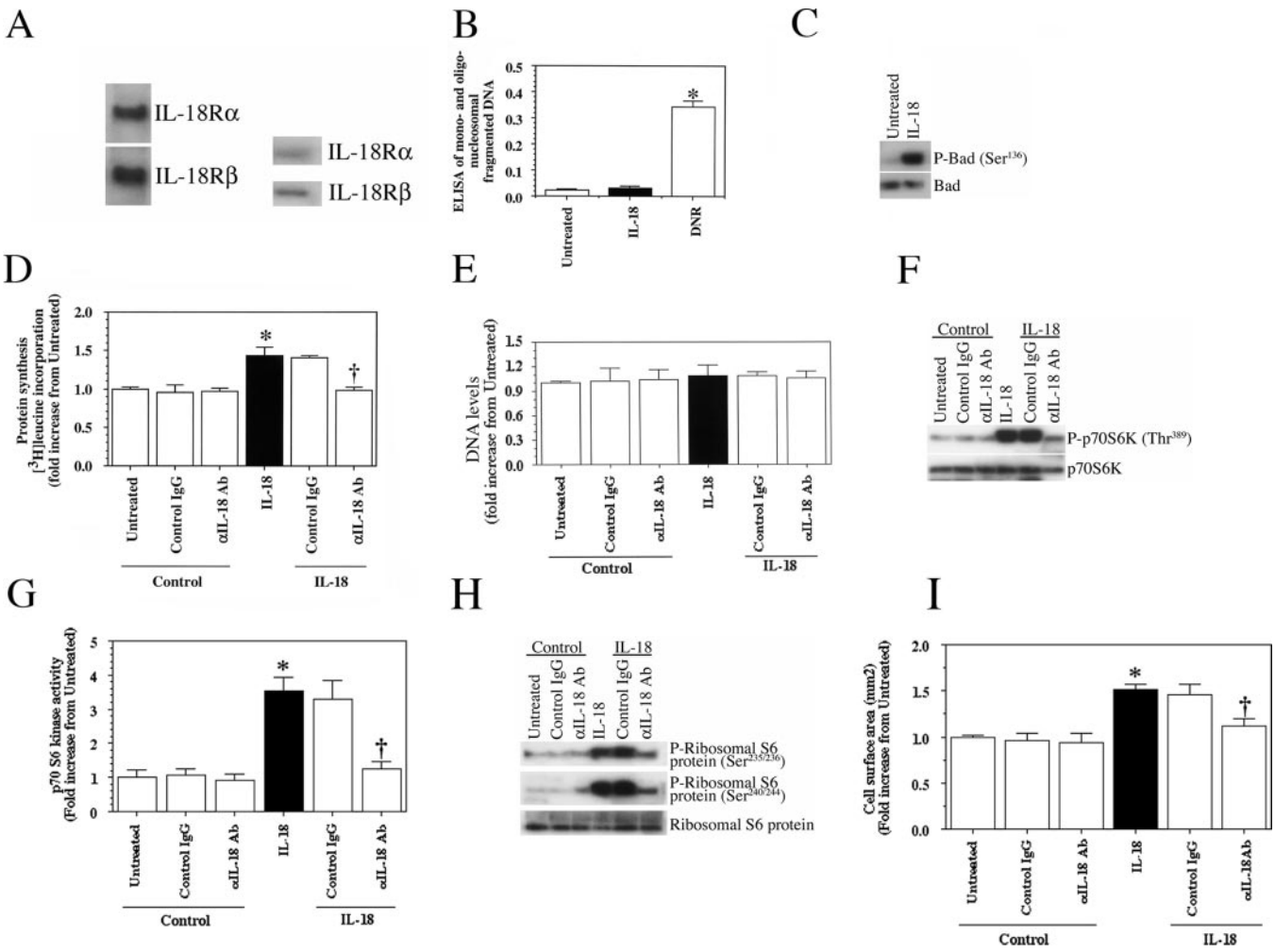

FIG. 1. **IL-18 induces hypertrophy of HL-1 cardiomyocytes. HL-1 cardiomyocytes express IL-18Rα and -β mRNA** (*A*, *upper panel*) and protein (*A*, *lower panel*) at basal conditions. HL-1 cardiomyocytes were plated in complete media supplemented with 10% serum. After overnight culture, cells were harvested for mRNA and protein extraction. Northern blot analysis was performed using 2 μg of poly(A)$^+$ RNA. IL-18 receptor protein levels were examined in the membrane fraction by Western blot analysis as described under "Experimental Procedures." *B*, IL-18 failed to induce cardiomyocyte death. Quiescent cardiomyocytes were treated with recombinant murine IL-18 (100 ng/ml) for 24 h. Levels of mono- and oligonucleosomal fragmented DNA in the cytoplasmic extracts were analyzed by an ELISA. Daunorubicin HCl was used as a positive control. Mean ± S.E. *, $p < 0.0001$ *versus* untreated and IL-18. *C*, IL-18 induces phosphorylation of Bad. Quiescent cardiomyocytes were treated with IL-18 (100 ng/ml) for 4 h. The cell lysates were immunoblotted with phospho-Bad antibody, which specifically recognizes the phosphorylated serine 136 residues. The *lower panel* shows total Bad levels. *D*, IL-18 enhances protein synthesis. Quiescent cardiomyocytes were treated with recombinant murine IL-18 (100 ng/ml). 48 h later, 0.5 μCi of [^{3}H]leucine was added. Six hours later, leucine incorporation was determined in a scintillation counter. Specificity of IL-18 was verified by incubating cells with anti-IL-18 neutralizing antibodies or control IgG for 1 h prior to the addition of IL-18. Results are mean ± S.E. of six determinations. *, $p < 0.01$ *versus* untreated; †, $p < 0.05$ *versus* IL-18. *E*, IL-18 had no effect on DNA synthesis. Quiescent cardiomyocytes were treated with IL-18. 48 h later, total DNA content was quantified. *F*, IL-18 induced p70 S6 kinase activation. Quiescent cardiomyocytes were treated with IL-18 for 30 min. Cell lysates were analyzed by Western blotting with phospho-p70 S6K antibody, which specifically recognizes the phosphorylated threonine 389 residues. The *lower panel* shows total p70 S6 kinase levels. *G*, IL-18 induced p70 S6 kinase activity. Quiescent cardiomyocytes were treated with IL-18. Cell lysates were incubated with the S6 kinase substrate peptide AKRRRLSSLRA and [γ-^{32}P]ATP The phosphorylated substrate was then separated from the residual [γ-^{32}P]ATP using P81 phosphocellulose paper and quantitated by using a scintillation counter. *, $p < 0.001$ *versus* untreated; †, $p < 0.01$ *versus* IL-18. *H*, IL-18 induced ribosomal S6 protein activation. Quiescent cardiomyocytes were treated with IL-18 for 30 min. Cell extracts were prepared and analyzed by Western blotting for total and phospho-ribosomal S6 protein. *I*, IL-18 increased cell surface area. Cardiomyocytes were treated with IL-18, and 48 h later the cell surface area was determined as described under "Experimental Procedures." Results are mean ± S.E. of quadruplicate determinations. *, $p < 0.05$ *versus* untreated; †, $p < 0.05$ *versus* IL-18.

ing pEGFP-N1 vector (Clontech) and was found to be 39 ± 3.1%.

Analysis of mRNA Expression—Expression of IL-18Rα and IL-18Rβ was analyzed by Northern blot analysis using 2 μg of poly(A)$^+$ RNA. Total RNA was extracted from HL-1 cardiomyocytes with TRIzol reagent (Invitrogen) and enriched for poly(A)$^+$ RNA with PolyATtract® mRNA Isolation System (Promega). IL-18Rα and IL-18Rβ cDNA were amplified by reverse transcription (RT)-PCR. RT-PCR was performed by using total RNA isolated from HL-1 cardiomyocytes using the following gene-specific primers (45): IL-18Rα (GenBank™ accession number BC020296.1, 427 bp), sense, 5′-CGTGACAAGCAGAGATGTTG-3′ (bases 517–536), and antisense, 5′-ATGTTGTCGTCTCCTTCCTG-3′ (bases 925–944); IL-18Rβ (GenBank™ accession number NM_010-553.1, 426 bp), sense, 5′-ATGCTCTGTTTGGGCTGGGT-3′ (bases 437–456), and antisense, 5′-CTGTCTTGATACAACAGGCCA-3′ (bases 843–863). ANF cDNA was amplified by RT-PCR using DNase (RQ1 RNase-free DNase, Promega)-treated total RNA isolated from HL-1 cardiomyocytes and the following gene-specific primers (GenBank™ accession number XM_131840.4; 458 bp): sense, 5′-ATGGGCTCCTTC-TCCATCAC-3′, (bases 114–133), and antisense, 5′-TTATCTTCGGTA-CCGGAAGCTG-3′ (bases 551–572). ANF mRNA expression was analyzed by Northern blotting using 30 μg of total RNA per lane. 28 S rRNA was used as an internal control. IL-18Rα and IL-18Rβ mRNA expression in NRVM was analyzed by Northern blot analysis using 2 μg of poly(A)$^+$ RNA. IL-18Rα and IL-18Rβ cDNA were described previously (26).

ANF Promoter Analyses—ANF promoter activity was analyzed in transient transfection assays using ANF promoter reporter construct. HL-1 cardiomyocytes were transfected with 3 μg of a 700-bp ANF-

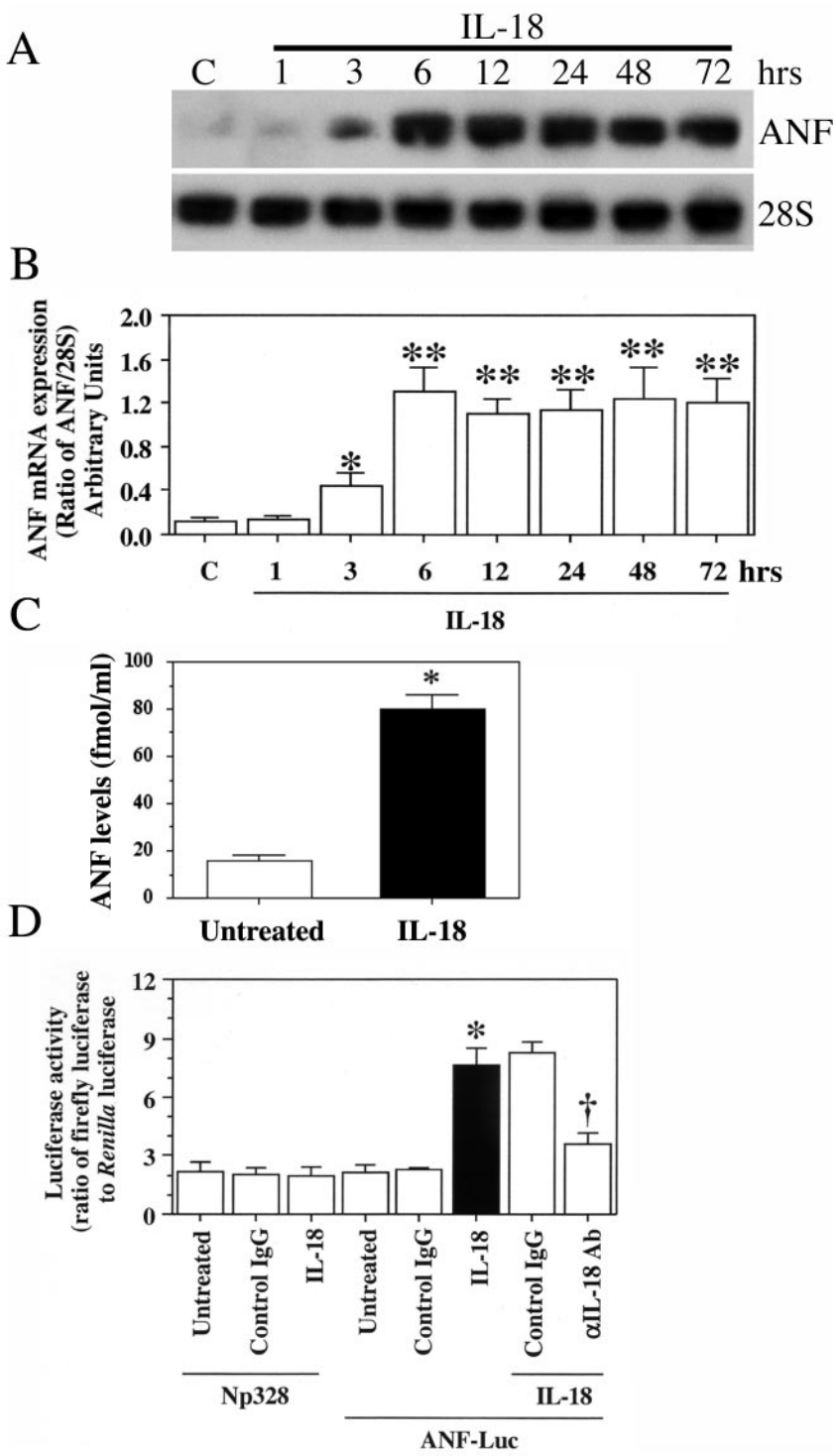

FIG. 2. **IL-18 induces ANF expression in HL-1 cardiomyocytes.** *A*, IL-18 induces ANG gene expression. Quiescent cardiomyocytes were treated with recombinant murine IL-18 (100 ng/ml) for up to 72 h. Total RNA was extracted and analyzed by Northern blotting for ANF mRNA expression. The *lower panel* shows 28 S ribosomal RNA expression. *B*, the autoradiographic bands from three independent experiments were quantified, and the results were expressed as a ratio of ANF to the corresponding 28 S rRNA. Mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* control. *C*, IL-18 induces ANF secretion. Quiescent cardiomyocytes were treated with IL-18 for 24 h. ANF levels in culture supernatants were quantified by radioimmunoassay. *, $p < 0.0001$ *versus* untreated. *D*, IL-8 induces ANF reporter gene transcription. Cardiomyocytes were transfected with ANF-Luc and plasmid. *Renilla* luciferase plasmid was also included in the transfection mixture and served as an internal control. Transiently transfected cells were treated with IL-18. Cell lysates were analyzed for firefly and *Renilla* luciferase activities as described under "Experimental Procedures." Mean ± S.E. is plotted. *, $p < 0.001$ *versus* untreated; †, $p < 0.01$ *versus* IL-18-treated ANF-Luc-transfected cells. *Ab*, antibody.

luciferase reporter. pRL-*Renilla* was used as an internal control. 24 h later, the media were changed, and the cells were treated with wortmannin or LY294002 prior to the addition of IL-18. In order to determine the role of Akt and GATA4, cells were transfected with corresponding siRNA prior to the addition of IL-18. Firefly and *Renilla* luciferase activities were analyzed at 7 h post-IL-18 treatment.

Analysis of Protein Expression—Extraction of cytoplasmic, membrane, nuclear, and whole cells homogenates, Western blotting, autoradiography, and densitometry were performed as described previously (25, 43). Protein levels were measured by BCA protein assay kit (Pierce). β-Actin was used to verify equal loading of protein per well. In addition, equal loading of protein/well was confirmed by staining the membranes with Coomassie Blue (data not shown). Polyclonal antibodies against Akt (catalog number 9272), phospho-Akt (Ser^{473}; catalog number 9271), PDK1 (catalog number 3062), glycogen synthase kinase (GSK)-3β (catalog number 9332), p70 S6K (catalog number 9202), phospho-p70 S6K (Thr^{389}; catalog number 9205), ribosomal S6 protein (catalog number 2212), phospho-S6 ribosomal protein ($Ser^{235/236}$; catalog number 2211S), phospho-S6 ribosomal protein ($Ser^{240/244}$; catalog number 2215), anti-ERKl/2 (catalog number 9102), anti-phospho ERK1/2 (catalog number 9101S), anti-p38 MAPK (catalog number 9212), anti-phospho p38 MAPK (catalog number 9211), and anti-phospho-JNK antibodies (catalog number 9251S) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-NF-κB p65 (F6; catalog number sc-8008), anti-GATA4 (C-20; catalog number sc-1237), and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. ANF protein levels in culture supernatants were measured by radioimmunoassay (catalog number RK-005–24; Phoenix Pharmaceuticals Inc., Belmont, CA) 24 h following IL-18 treatment.

Measurement of PI3K, Akt, and S6 Kinase Activities—PI3K lipid

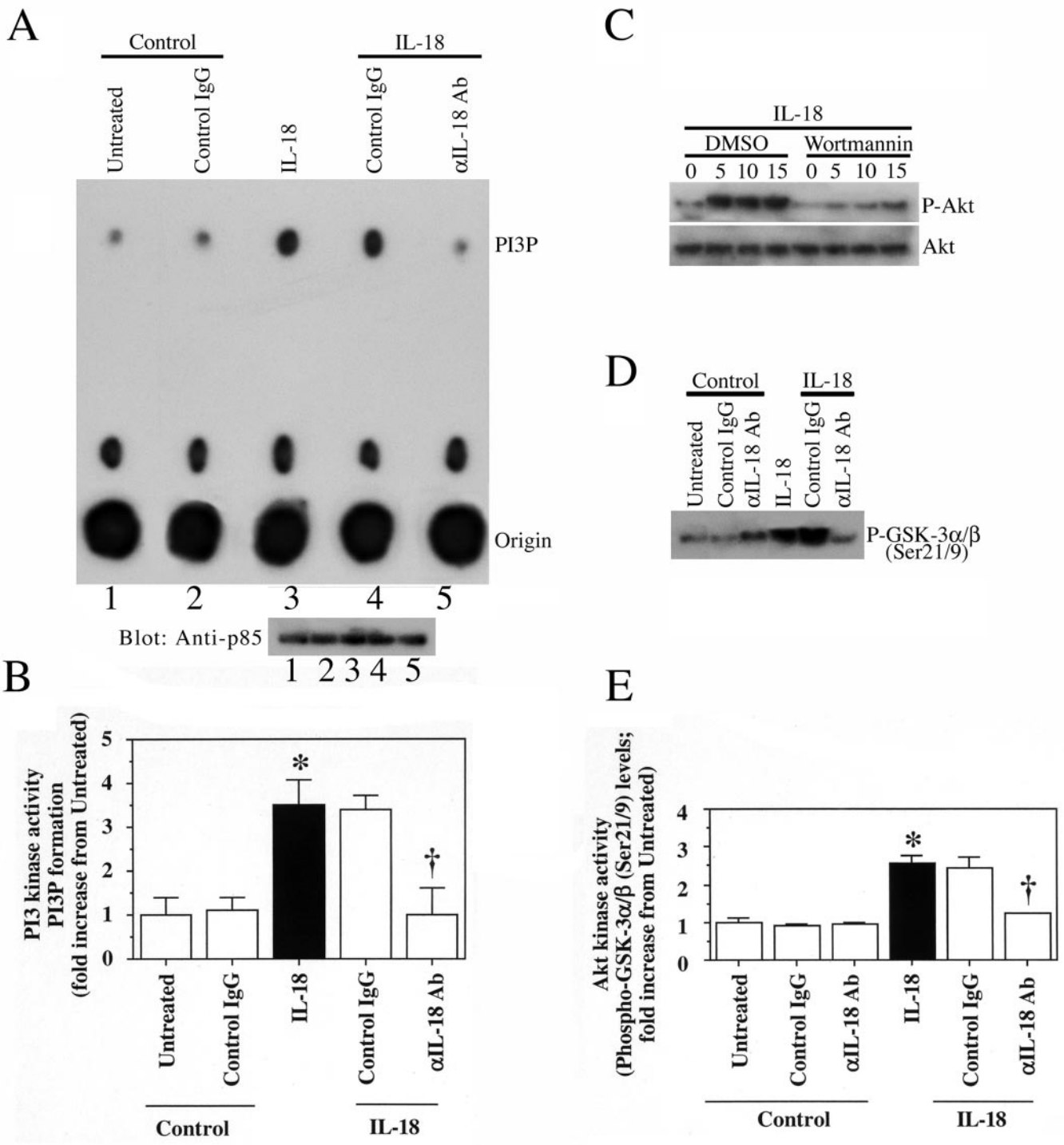

FIG. 3. **IL-18 induces PI3K and Akt kinase activation in HL-1 cardiomyocytes.** *A*, IL-18 induces PI3K activation. Quiescent cardiomyocytes were incubated with IL-18 neutralizing antibodies or control IgG for 1 h followed by the addition of IL-18 (100 ng/ml) for an additional 5 min. Equal amounts of cleared cell lysates were immunoprecipitated with anti-p85 regulatory subunit antibody (*Ab*) of PI3K, followed by immune complex kinase assay as described under "Experimental Procedures." The *bottom panel* shows immunoblot analysis of the same samples with anti-p85 antibody. *B*, PI3P levels from three independent experiments were quantified, and the results were plotted as mean ± S.E. *, $p < 0.001$ *versus* untreated; †, $p < 0.005$ *versus* IL-18. *C*, IL-18 rapidly induced Akt phosphorylation. Cardiomyocytes were treated with IL-18 for the indicated times. Cell lysates were analyzed for total Akt and phospho-Akt levels. *DMSO*, Me_2SO. *D*, IL-18 induces Akt kinase activity. Cardiomyocytes were treated with IL-18, and cell lysates were analyzed for Akt kinase activity as described under "Experimental Procedures." *E*, the autoradiographic signals in *D* were quantified, and the densitometric values from three independent experiments were plotted. *, $p < 0.01$ *versus* untreated; †, $p < 0.01$ *versus* IL-18.

kinase assays were performed using p85 immunoprecipitates (46). Akt kinase activity was performed using a commercially available kit (Cell Signaling Technology, Inc.) (25, 43); this assay is based on Akt-induced phosphorylation of GSK-3. S6 kinase activity was determined by using a commercially available kit (S6 kinase assay kit; Upstate Biotechnology, Inc., Lake Placid, NY). This assay is based on the phosphorylation of a specific substrate (AKRRRLSSLRA) using the transfer of the γ-phosphate of [γ-^{32}P]ATP by S6 kinase.

Neonatal Cardiomyocyte Preparation—In order to confirm the pro-hypertrophic effects of IL-18 in primary cells, we employed neonatal rat ventricular cardiomyocytes. NRVM were isolated as described previously (47). In brief, NRVM were prepared from 1- to 2-day-old Sprague-

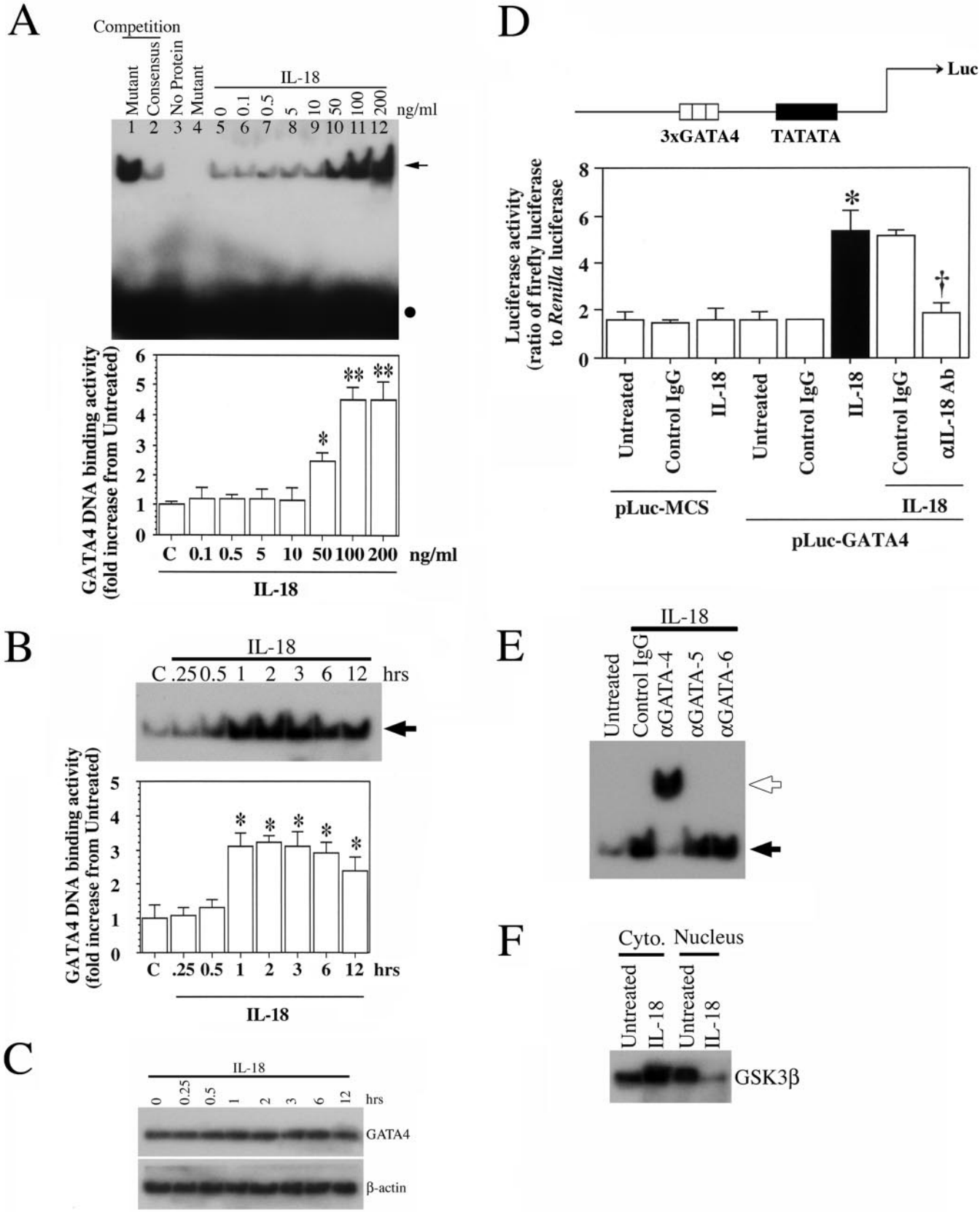

FIG. 4. **IL-18 induces GATA4 DNA binding activity and GATA-dependent luciferase activity in HL-1 cardiomyocytes.** *A*, dose-dependent effects of IL-18 on GATA4 DNA binding activity. Quiescent cardiomyocytes were incubated with IL-18. Nuclear protein extracts (10 μg) were analyzed for GATA4 DNA binding activity by EMSA as described under "Experimental Procedures." For competition, 100-fold molar excess cold consensus or mutant oligonucleotides were used with the nuclear extracts before the addition of labeled consensus oligonucleotide. The protein-DNA complexes were separated by 5% PAGE. The *arrow* denotes the specific protein-DNA complex. *Solid circle* indicates unincorporated labeled probe. In addition to competition studies, EMSA was also performed using labeled mutant oligonucleotide (*A*, *lane 4*). *B*, the autoradiographic signals from three independent experiments were semi-quantitated, and the results are plotted. Mean ± S.E. *, $p < 0.05$; **, $p < 0.001$ *versus* untreated. *C*, time course studies. Quiescent cardiomyocytes were incubated with IL-18 for up to 12 h. Nuclear protein were extracted, and

Dawley rat hearts by enzyme digestion. Hearts were collected from 4 litters of pups, ~50, trimmed of atria and excess vessels, and then rinsed in 1× ADS solution (11.6 mM NaCl, 1.8 mM HEPES, 0.1 mM NaH_2PO_4, 0.5 mM KCl, 83 μM $MgSO_4$, 55 mM glucose). The ventricles were cut into four pieces and subjected to a series of digestions in collagenase (type 2, Worthington) and pancreatin (Sigma) at a concentration of 80.6 units/ml and 62.5 μg/ml, respectively, in 1× ADS solution. The first digestion, 5 min, was discarded, and the subsequent 5–6 digestions, 20 min each, were collected into newborn calf serum. All were gently agitated in a 37 °C shaking water bath. After tissue disassociation, cells were spun for 5 min at 1750 × *g*. The resulting pellet was resuspended in 5 ml of 1× ADS and layered over Percoll gradients. The gradient was spun for 60 min at 4000 rpm (Eppendorf Centrifuge 5810R, Brinkman Instruments) with no brake. Myocyte layer was collected and washed twice in excess 1× ADS. The final pellet was resuspended in plating media (4 parts Dulbecco's modified Eagle's, 1 part M199, 10% horse serum, 5% fetal bovine serum, 1% antibiotic/antimycotic), counted with a hemocytometer, and then plated to 1% gelatin-coated dishes according to the density required. After 24 h, media were changed to maintenance media (4 parts Dulbecco's modified Eagle's, 1 part M199, 1% antibiotic/antimycotic) for the duration of the experiment. In order to verify whether IL-18 mediates hypertrophy of NRVM via activation of PI3K, Akt, and GATA4, NRVM were treated with wortmannin (100 nM in Me_2SO for 1 h) or transfected with Akt (50 nM) or GATA4 (150 nM; si*GENOME SMART* pool reagent, catalog number M-090725-00, Dharmacon) siRNA prior to the addition of IL-18. Forty two hours later, 0.5 μCi of [^{3}H]leucine was added to the culture medium, and the incubation was continued for an additional 6 h. The radioactivity incorporated into the trichloroacetic acid-precipitable material was determined by using a liquid scintillation counter.

Statistical Analyses—Data were represented as fold increase from untreated controls after normalizing the control values to 1. Data were shown as mean ± S.E. of 3–6 independent experiments. Data were subjected to analysis of variance with Student's *t* test for significance. Corrections for multiple comparisons were made using the Bonferroni factor. Probability values of 0.05 or less were considered significant.

RESULTS

IL-18 Induces Cardiomyocyte Hypertrophy—IL-18 signals via IL-18 receptor (IL-18R). IL-18R is a heterodimer comprising the ligand binding subunit IL-18Rα and the signal transducer IL-18Rβ (48–50). Therefore, we investigated whether HL-1 cardiomyocytes express IL-18Rα and -β subunits. Northern blot analysis of 2 μg of poly(A)$^+$ RNA revealed expression of both the receptors in HL-1 cardiomyocytes at basal conditions (Fig. 1*A*, *left panel*). Similarly, Western blot analysis revealed IL-18Rα and -β subunit expression in the membrane fraction (Fig. 1*A*, *right panel*). Because IL-18 is a pro-apoptotic cytokine (14–16), we investigated whether IL-18 induces cardiomyocyte death. ELISA revealed low levels of mono- and oligonucleosomal fragmented DNA in the cytoplasmic extracts from cardiomyocytes at basal conditions, and treatment with IL-18 failed to induce cell death (Fig. 1*B*). DNR, as expected, significantly increased cell death (Fig. 1*B*). In addition, IL-18 induced phosphorylation of the pro-apoptotic gene product Bad at Ser^{136} (Fig. 1*C*). Phosphorylation of Bad at Ser^{136} rendered it inactive and prevented it from inactivating Bcl-X_L or other anti-apoptotic members of the Bcl-2 family (51). Together, these results indicate that IL-18 does not induce cardiomyocyte death and that its pro-apoptotic effects may be cell type-dependent (14–16). However, treatment with IL-18 induced cardiomyocyte hypertrophy. Because hypertrophy is characterized by increased protein but not DNA synthesis, we examined protein synthesis and DNA levels following IL-18 treatment. The ratio of total protein to DNA indicated that treatment with IL-18 significantly increased protein synthesis in cardiomyocytes (Fig. 1*D*). Furthermore, neutralization of IL-18 with anti-IL-18 antibodies, but not control IgG, abrogated IL-18-induced protein synthesis, demonstrating specificity of IL-18. However, no significant increases in DNA levels were detected in cardiomyocytes following IL-18 treatment (Fig. 1*E*). Increased protein synthesis results from increased translation. Therefore, we examined the phosphorylation status of two translational regulatory proteins p70 S6 kinase and ribosomal S6 protein (52, 53). We also determined S6 kinase activity. Our results indicate that although the total levels of p70 S6 kinase remained the same, treatment with IL-18 induced phosphorylation of p70 S6 kinase (Thr^{389}) (Fig. 1*F*). Treatment with IL-18 also increased p70 S6 kinase activity (Fig. 1*G*). Similarly, IL-18 induced phosphorylation of ribosomal S6-protein (Ser$^{235/236}$ and Ser$^{240/244}$) (Fig. 1*H*), indicating activation of the translational machinery following IL-18 treatment. Increased protein synthesis without changes in DNA synthesis is expected to result in an increase in cell size. Therefore, we examined cardiomyocyte cell size after IL-18 treatment. Indeed, Fig. 1*I* shows that treatment with IL-18 significantly increases cardiomyocyte cell size, and once again neutralization of IL-18 with anti-IL-18 antibodies, but not control IgG, abrogated this effect. These results indicate that IL-18 is a pro-hypertrophic cytokine and induces cardiomyocyte hypertrophy.

IL-18 Induces ANF Expression in Cardiomyocytes—Hypertrophy is characterized by the re-expression of fetal genes including ANF. Therefore, we examined ANF mRNA expression by Northern blot analysis by using total RNA isolated from cardiomyocytes treated with IL-18. Fig. 2*A* shows low levels of ANF mRNA expression at basal conditions and a time-dependent increase following IL-18 treatment, with peak levels detected at 6 h. Its levels remained high throughout the 72-h study period (Fig. 2*A*; corresponding densitometric values are shown in *B*), indicating that IL-18 induces sustained expression of ANF in cardiomyocytes. Also, a significant increase in ANF protein levels was detected at 24 h following IL-18 treatment (Fig. 2*C*). Because IL-18 increased ANF mRNA expression and protein levels, we next examined whether IL-18 regulates ANF expression at the transcriptional level. Cardiomyocytes were transiently transfected with an ANF promoter-reporter construct. Twenty four hours later, cells were treated with IL-18. Cells transfected with empty vector (Np328) served as controls. Fig. 2*D* shows that treatment with IL-18 significantly increases ANF promoter-driven luciferase activity, and neutralization of IL-18 with anti-IL-18 antibodies, but not with control IgG, attenuated IL-18-mediated increase in ANF promoter activity. These results indicate that IL-18 is a potent inducer of ANF expression in cardiomyocytes and

EMSA was performed. *Arrow* denotes specific DNA-protein complexes. *, $p < 0.001$ *versus* control. Unincorporated labeled probe that runs to the bottom of the gel during electrophoresis is not shown. *C,* treatment with IL-18 had no effects on total GATA4 levels. Quiescent cardiomyocytes were treated with IL-18 for up to 12 h. Cell lysates were analyzed for GATA4 levels by Western blotting. *Lower panel* shows β-actin levels. *D*, *upper panel*. Structure of the 3xGATA4-Luc reporter construct. Three copies of GATA4 sequences (CTCT**GATA**A) were cloned upstream of the luciferase (*Luc*) gene and the TATA box in pLuc-MCS plasmid. IL-18 increases transcription of the reporter gene driven by GATA4. pLuc-GATA4 plasmid was transfected into cardiomyocytes. A *Renilla* luciferase plasmid was also included in the transfection mixture. Transiently transfected cells were incubated with IL-18. Cell lysates were analyzed for firefly and *Renilla* luciferase activities. Mean ± S.E. is plotted. *, $p < 0.005$ (*versus* untreated); †, $p < 0.01$ *versus* IL-18-treated pLuc-GATA4 transfected cells. *E*, gel supershift assays. Nuclear protein extracts from cardiomyocytes treated with IL-18 for 1 h were incubated with anti-GATA4, -GATA5, and -GATA6 antibodies, and EMSA was performed using labeled consensus GATA4 oligonucleotide. *Open arrow* indicates supershift, and *solid arrow* indicates GATA4-specific DNA-protein complexes. *Ab*, antibody. *F*, IL-18 decreases nuclear GSK3β with concomitant increase in cytoplasmic (*Cyto*) levels. Quiescent cardiomyocytes were treated with IL-18 for 1 h. Cytoplasmic and nuclear protein extracts were analyzed for GSK3β levels by Western blotting.

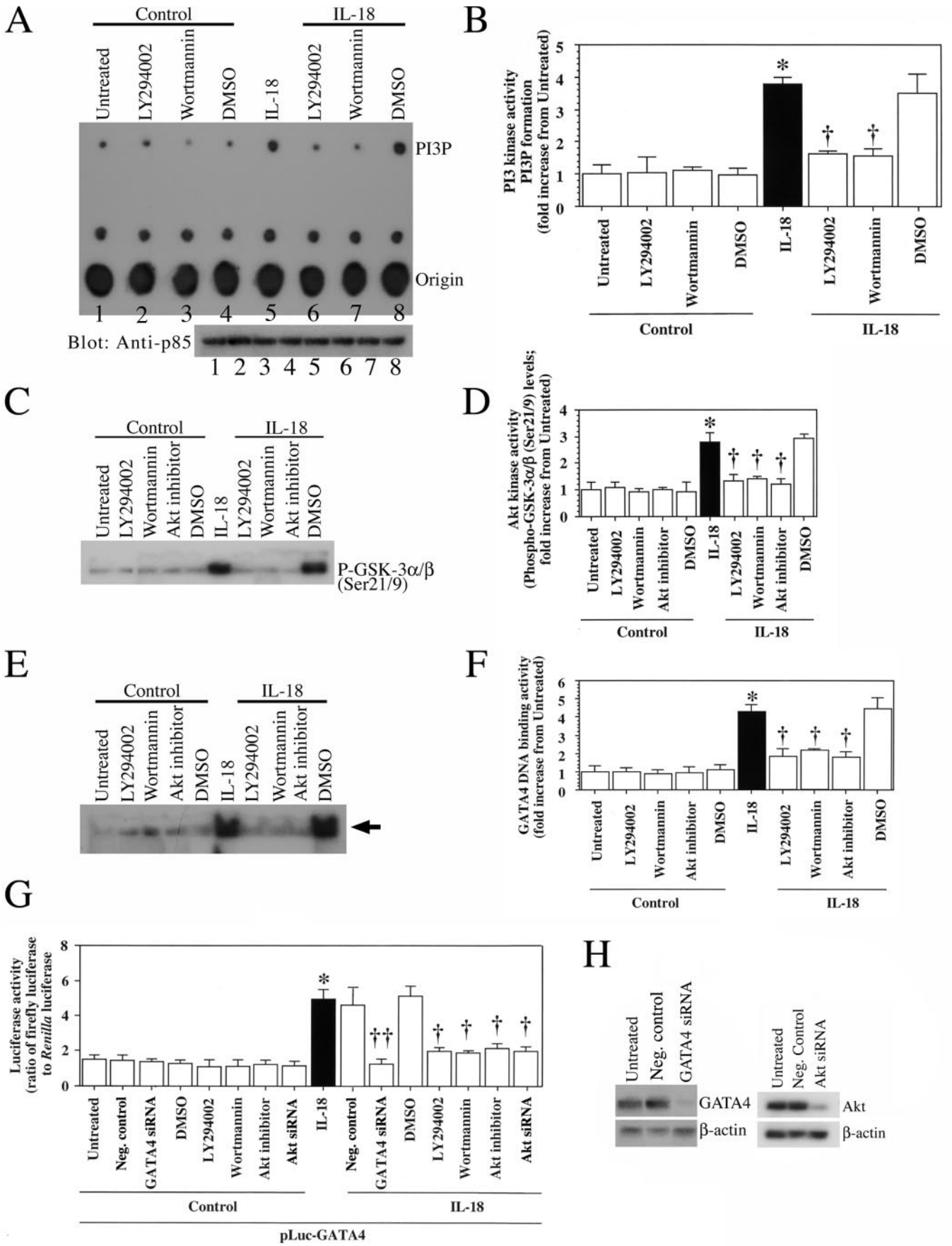

FIG. 5. **IL-18 activates GATA4 via PI3K and Akt in HL-1 cardiomyocytes.** *A*, IL-18 induces PI3K activation. Quiescent cardiomyocytes were pretreated for 1 h with wortmannin or LY294002 prior to the addition of IL-18 for 5 min. Cell lysates were analyzed for PI3K activity as described under "Experimental Procedures." The PI3P was separated by TLC. The *bottom panel* shows immunoblot analysis of the same samples with anti-p85 antibody. *DMSO*, Me_2SO. *B*, the autoradiographic signals shown for PI3P in *A* were quantified, and the means ± S.E. from three

regulates its transcription and translation.

IL-18 Induces PI3K and Akt Kinase Activation in Cardiomyocytes—Because IL-18 induced cardiomyocyte hypertrophy, we next investigated the signal transduction pathways involved in IL-18-mediated cardiomyocyte hypertrophy. IL-18 activates diverse cellular second messengers including activation of PI3K (32). Therefore, we analyzed PI3K activation following IL-18 treatment by PI3K lipid kinase assays. Our results demonstrate that treatment with IL-18 significantly ($p < 0.005$) increases PI3P formation in HL-1 cardiomyocytes (Fig. 3*A*). Whereas preincubation with control IgG failed to modulate IL-18 effects, IL-18 neutralization inhibited IL-18-mediated PI3P formation. Results from three independent experiments were quantified, and the densitometric values are shown in Fig. 3*B*. Because the serine/threonine kinase Akt/protein kinase B is one of the major downstream targets of PI3K, and transmits survival signals (54), we analyzed total Akt and phospho-Akt (Ser^{473}) levels in the cytoplasmic extracts using Western blot analysis. Treatment with IL-18 rapidly induced Akt phosphorylation without modulating total Akt levels (Fig. 3*C*). Furthermore, IL-18 induced Akt kinase activity (Fig. 3*D*, corresponding densitometric values are shown in *E*), indicating that IL-18 signals via PI3K and Akt in cardiomyocytes.

IL-18 Induces GATA4 DNA Binding Activity and Increases GATA4-dependent Luciferase Activity in Cardiomyocytes—Activation of GATA4, a member of the GATA family of zinc finger transcription factors, induces the expression of various cardiac-specific genes including ANF, a fetal gene re-expressed during hypertrophy (10, 55). Therefore, we performed a dose-response study, and we analyzed GATA4 DNA binding activity by EMSA using nuclear protein extracts isolated from cardiomyocytes treated for 1 h with various concentrations of IL-18. Our results demonstrated low levels of GATA4 DNA binding activity in cardiomyocytes at basal conditions and in cardiomyocytes treated with IL-18 for up to 10 ng/ml. However, an increase in GATA DNA binding activity was detected at 50 ng/ml. At 100 ng/ml, a robust increase in GATA4 DNA binding activity was detected (Fig. 4*A*, corresponding densitometric values are shown in the *lower panel*). Increasing IL-18 concentration to 200 ng/ml failed to further increase GATA4 DNA binding activity (Fig. 4*A*), indicating peak levels of GATA4 activity at 100 ng/ml of IL-18. In addition to the competition studies (Fig. 1*A*, *1st* and *2nd lanes*), specificity of GATA4 DNA binding activity was determined by incubating nuclear protein extracts from cardiomyocytes treated with IL-18 (100 ng/ml) for 1 h with labeled mutant oligonucleotide. In these studies, no GATA4-specific DNA binding activity was detected (Fig. 4*A*, *lane 4*). We then performed a time course study using IL-18 at 100 ng/ml. Our results indicate that treatment with IL-18 increases GATA4 DNA binding activity in a time-dependent manner with peak levels of activity detected at 1 h. Its levels remained at these high levels throughout the 12-h study period (Fig. 4*B*, corresponding densitometric values are shown in the *lower panel*). In order to confirm that these changes in GATA4 DNA binding activity are not because of variations in total GATA4 levels, we performed Western blot analysis using whole cell homogenates from controls and IL-18-treated cells. The results are shown in Fig. 4*C* and indicate no significant differences in GATA4 levels between controls and IL-18 treatment, indicating that IL-18 induces rapid and sustained activation of GATA4 in cardiomyocytes. In addition to EMSA, we also performed transient transfection assays using a GATA4 reporter construct (pLuc-GATA4; Fig. 2*D*, *schematic*). Our results indicate that IL-18 significantly increases GATA4-driven luciferase activity, and preincubation with IL-18 neutralizing antibodies, but not control IgG, attenuates IL-18-mediated GATA4-dependent luciferase activity (Fig. 4*D*). Gel supershift assays revealed that the IL-18-mediated increase in GATA DNA binding activity is predominantly due to GATA4 (Fig. 4*E*). Because GSK3β negatively regulates GATA4 expression in the nucleus (56), we also analyzed GSK3β levels in cytoplasmic and nuclear extracts by Western blotting. Fig. 4*F* demonstrates that while reducing nuclear levels of GSK3β, treatment with IL-18 increases its cytoplasmic levels, indicating that treatment with IL-18 decreases nuclear levels of GSK3β, a negative regulator of GATA4.

IL-18 Activates GATA4 via PI3K and Akt—We demonstrated that IL-18 activates PI3K, Akt, and GATA4 (Figs. 3 and 4). We next investigated whether IL-18 induces GATA4 activation via PI3K and Akt. Our results indicate that pretreatment with wortmannin or LY294002, but not with their solvent control Me_2SO, significantly attenuated IL-18-mediated PI3P formation (Fig. 5*A*; densitometric values from three independent experiments are shown in *B*). To confirm that variations in PI3P formation are not because of variations in the amounts of immunoprecipitates used, we performed Western blot analysis using anti-p85 antibodies. Our results indicate similar levels of p85 in all the lanes (Fig. 5, shown below *A*). Treatment with PI3K and Akt inhibitors attenuated IL-18-mediated Akt kinase activity, as seen by reduced levels of phosphorylated GSK levels (Fig. 5*C*; corresponding densitometric values are shown in *D*). Furthermore, pretreatment with PI3K and Akt inhibitors attenuated IL-18-mediated GATA4 DNA binding activity (Fig. 5*E*; corresponding densitometric values are shown in *F*). Although pretreatment with Me_2SO or control siRNA failed to modulate, treatment with LY294002, wortmannin, Akt inhibitor, or knockdown of Akt or GATA4 by respective siRNA significantly attenuated IL-18-mediated GATA4-driven luciferase activity (Fig. 5*G*; knockdown of Akt and GATA4 levels was confirmed by Western blotting, Fig. 5*H*), indicating that IL-18 signals via PI3K and Akt and stimulates GATA4 DNA binding activity and GATA4-driven luciferase activity.

IL-18 Induces ANF Expression via PI3K, Akt, and GATA4—We have demonstrated that IL-18 induces ANF gene transcription (Fig. 2). We next explored the role of PI3K, Akt, and GATA4 in IL-18-mediated ANF expression. Our results indicate that treatment with LY294002, wortmannin, or Akt inhibitor or knockdown of Akt or GATA4 by the respective siRNA significantly attenuated IL-18-mediated ANF mRNA expression. 28 S rRNA, used as an internal control, was not affected by various treatments (Fig. 6*A*; corresponding densitometric values are shown in Fig. 6*B*). Furthermore, inhibition of PI3K, Akt, or GATA4 attenuated IL-18-mediated ANF secretion (Fig. 6*C*) and ANF promoter activity (Fig. 6*D*), indicat-

independent experiments were plotted. *C*, IL-18 induced Akt kinase activity. Quiescent cardiomyocytes were re-treated with IL-18 with and without pretreatment for 1 h with wortmannin, LY294002, or Akt inhibitor. Akt activity was determined by an *in vitro* kinase assay (*C*, corresponding densitometric values are shown in *D*). *E*, IL-18 increased GATA4 DNA binding activity in PI3K- and Akt-dependent manner. Quiescent cardiomyocytes were treated with wortmannin, LY294002, or Akt inhibitor for 1 h followed by IL-18 for an additional 1 h. Nuclear protein extracts were subjected to EMSA. *Arrow* indicates specific DNA-protein complexes. Corresponding densitometric vales are shown in *F*. *G*, IL-18 increases GATA4-dependent promoter reporter activity in a PI3K- and Akt-dependent manner. Quiescent cardiomyocytes were either treated with pharmacological inhibitors or transfected with siRNA prior to IL-18 addition. Cell lysates were analyzed for firefly and *Renilla* luciferase activities. Results are mean ± S.E. of six determinations. *, at least $p < 0.01$ (*versus* untreated); †, $p < 0.05$; ††, $p < 0.01$ *versus* IL-18. *H*, knockdown of GATA4 and Akt were confirmed by Western blotting. β-Actin was used as an internal control.

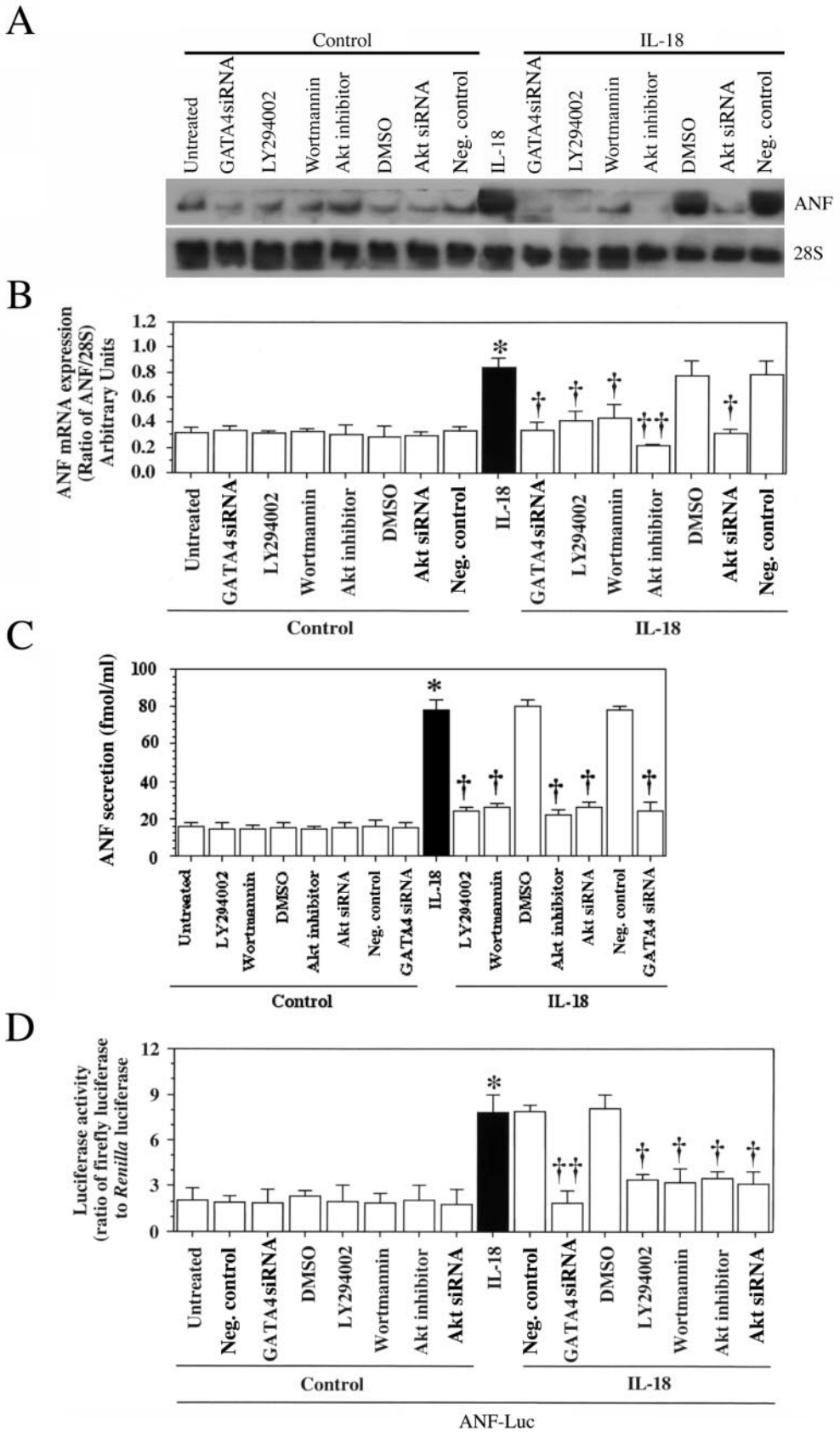

FIG. 6. **IL-18 induces ANF expression in HL-1 cardiomyocytes via PI3K, Akt, and GATA4.** *A*, IL-18 induces ANF mRNA expression via PI3K, Akt, and GATA4. Quiescent cardiomyocytes were treated with wortmannin (1 h), LY294002 (1 h), Akt inhibitor (1 h), or transiently transfected with GATA4 or Akt siRNA (48 h) followed by the addition of IL-18 for 6 h. ANF mRNA expression was analyzed by Northern blotting. 28 S ribosomal RNA was used as an internal control. *DMSO*, Me_2SO. *B*, the autoradiographic signal shown in *A* were quantified, and the means ± S.E. of three independent experiments are plotted. *C*, IL-18 increased ANF secretion in PI3K-, Akt-, and GATA4-dependent manner. Experimental conditions were as described under *A*. ANF levels in culture supernatants were analyzed by radioimmunoassay at 24 h following IL-18 treatment. *D*, IL-18 increases ANF promoter-driven luciferase activity in PI3K-, Akt-, and GATA4-dependent manner. *, at least $p < 0.05$ *versus* untreated; †, $p < 0.025$; ††, $p < 0.01$ *versus* IL-18 in *A*, *C*, and *D*.

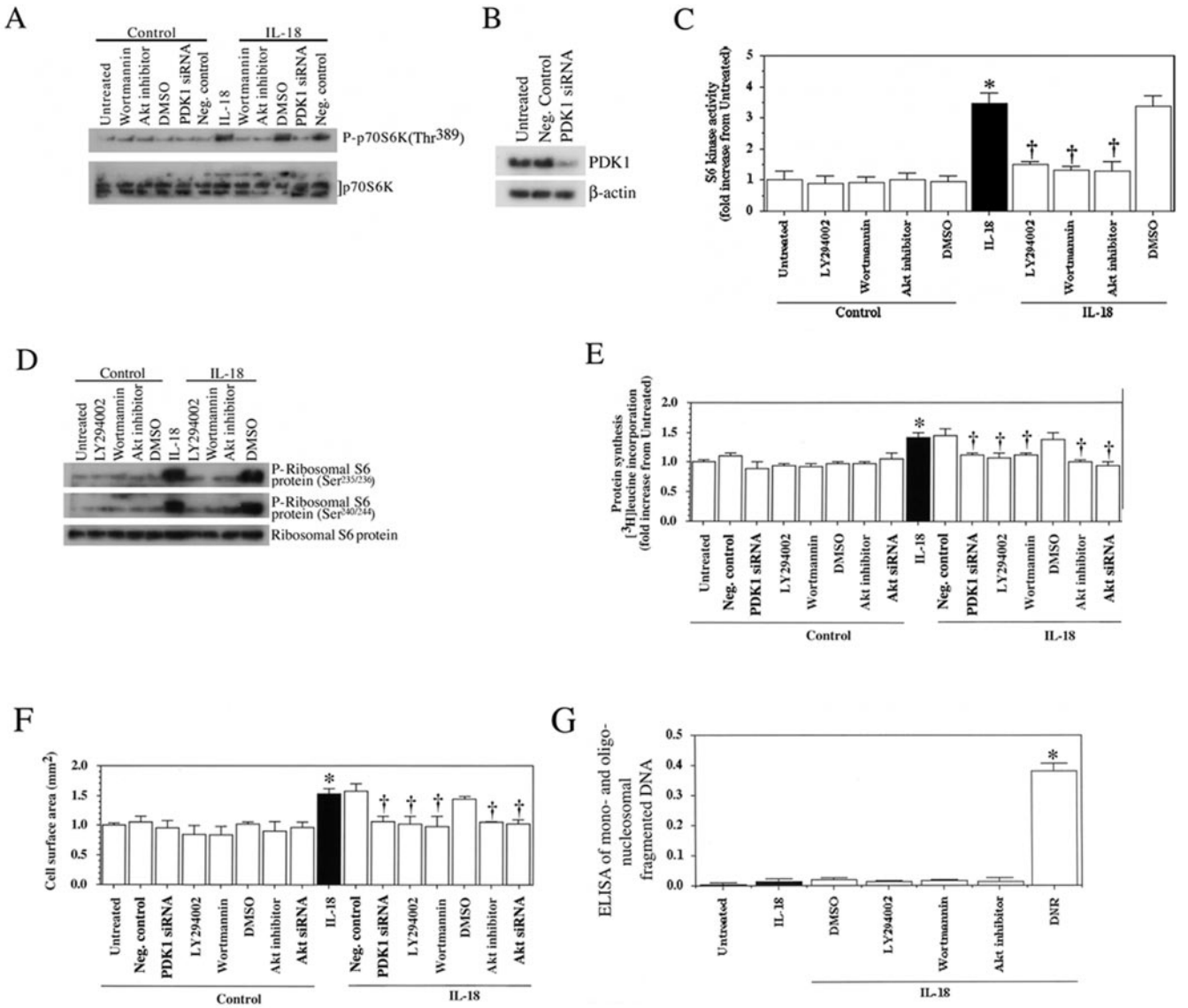

FIG. 7. **IL-18 induces hypertrophy of HL-1 cardiomyocytes via PI3K-, PDK1-, and Akt-dependent signaling.** *A–C*, IL-18 induces phosphorylation (*A*) and activation (*C*) of p70 S6 kinase via PI3K, PDK1, and Akt. Quiescent cardiomyocytes were treated with PI3K inhibitors (wortmannin and LY294002), Akt inhibitor, or transfected with PDK1 siRNA followed by the addition of IL-18 (100 ng/ml). Cell lysates were analyzed by Western blotting for total p70 S6 kinase and phospho-p70 S6 kinase or Akt kinase activity as described in the legend to Figs. 1 and 3. *DMSO*, Me_2SO. *B*, knockdown to PDK1 was confirmed by Western blotting. β-Actin shown in the *lower panel* served as an internal control. *, $p < 0.001$ *versus* untreated; †, $p < 0.01$ *versus* IL-18. *D*, IL-18 induced ribosomal S6 protein activation. Quiescent cardiomyocytes were treated as described in *A*. Cell lysates were analyzed by Western blotting for total and phosphorylated ($Ser^{235/236}$ and $Ser^{240/244}$) forms of ribosomal S6 protein levels. *E* and *F*, IL-18 increased protein synthesis (*E*) and cell surface area (*F*) via PI3K, PDK1, and Akt. Experimental conditions for protein synthesis and cell surface area are described in the legend to Fig. 1. *, $p < 0.01$ *versus* untreated; †, $p < 0.05$ *versus* IL-18. *G*, inhibition of PI3K or Akt failed to induce cell death in IL-18-treated cardiomyocytes. Quiescent cardiomyocytes were pretreated with PI3K inhibitors wortmannin or LY294002 or Akt inhibitor prior to the addition of IL-18 for an additional 24 h. DNR was used as a positive control. Cell death was analyzed by an ELISA as described under "Experimental Procedures." *, $p < 0.001$ *versus* untreated.

ing that IL-18 induces ANF gene transcription via activation of PI3K, Akt, and GATA4.

IL-18 Induces Cardiomyocyte Hypertrophy via PI3K, PDK1, and Akt—In the next series of experiments, we investigated whether PI3K, PDK1, and Akt play a role in IL-18-mediated cardiomyocyte hypertrophy. Fig. 7*A* shows total and phosphorylated p70 S6 kinase levels as analyzed by Western blotting. Although treatment with IL-18 had no effect on total p70 S6 kinase levels, inhibition of PI3K, PDK1 (knockdown of PDK1 was confirmed by Western blotting, Fig. 7*B*), and Akt attenuated IL-18-mediated phospho-p70 S6 kinase (Thr^{389}) levels. Similarly, inhibition of PI3K and Akt attenuated IL-18-mediated S6 kinase activity (Fig. 7*C*) and levels of phosphorylated ribosomal S6 protein ($Ser^{235/236}$ and $Ser^{240/244}$; Fig. 7*D*). Furthermore, inhibition of PI3K, PDK1, and Akt attenuated IL-18-mediated protein synthesis (Fig. 7*E*) and cell size (Fig. 7*F*), indicating that IL-18 induces cardiomyocyte hypertrophy via activation of PI3K, PDK1 and Akt (Fig. 7*G*).

IL-18 Activates NF-κB, p38 MAPK, ERK, and JNK—The above series of experiments demonstrated that IL-18 induced cardiomyocyte hypertrophy via activation of PI3K, PDK1, Akt, and GATA4. However, IL-18 is known to activate diverse signaling pathways, including activation of NF-κB, p38 MAPK, ERK, and JNK (16, 30–36). As these signaling pathways are also involved in cardiomyocyte hypertrophy (37–41), we next examined whether IL-18-mediated cardiomyocyte hypertrophy involves NF-κB, p38 MAPK, ERK, and JNK. Fig. 8*A* shows that IL-18 indeed induced activation of NF-κBp65, p38 MAPK,

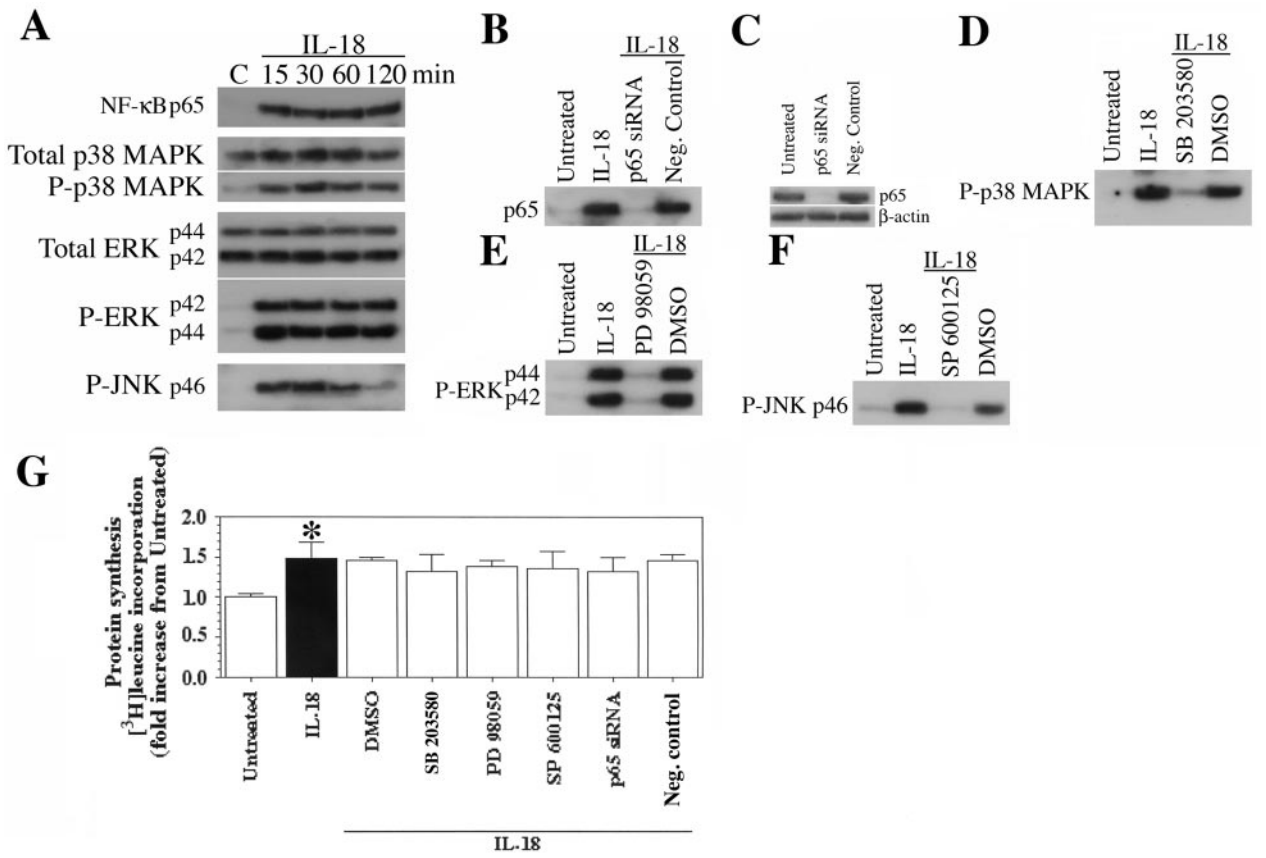

FIG. 8. **IL-18 induces NF-κB, p38 MAPK, ERK, and JNK activation in HL-1 cardiomyocytes.** *A*, IL-18 induced NF-κB, p38 MAPK, ERK, and JNK activation. Quiescent cardiomyocytes were treated with IL-18 for the indicated times. Nuclear protein extract (20 μg; p65) or whole cell homogenates (50 μg; p38 MAPK, ERK, and JNK) were prepared and separated on 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose sheet and probed with rabbit polyclonal antibodies against p65, p38 MAPK, phospho-p38 MAPK, p42/44 MAPK (ERK), phospho-p42/44 MAPK, and phospho-JNK as described under "Experimental Procedures." *B–F*, inhibition of p65 (p65 siRNA; *B*, knockdown of p65 was confirmed by Western blotting; *C*, the p38 MAPK (SB203580); *D*, p42/44 MAPK; *E*, PD98059; or JNK (SP 600125; *F*) pathways do not impair IL-18 induced protein synthesis. *G*, protein synthesis was measured by [^{3}H]leucine incorporation as described in the legend to Fig. 1*D*. *, $p < 0.01$ *versus* untreated. *DMSO*, Me_2SO.

ERK, and JNK. Western blot analysis revealed increased levels of NF-κBp65 in nuclear protein extracts, indicating that IL-18 induced NF-κB activation. Similarly, IL-18 induced p38 MAPK activation as evidenced by an increase in the levels of phosphorylated p38 MAPK (Fig. 8*A*). However, total p38 MAPK levels were not altered following IL-18 treatment. Treatment with IL-18 induced ERK activation. Levels of phosphorylated p42 and p44 were increased following IL-18 treatment (Fig. 8*A*). IL-18 also increased levels of phosphorylated JNK p46 in cardiomyocytes. These results indicate that IL-18, in addition to activation of PI3K-PDK1-Akt-GATA4 signaling, also induces NF-κB, p38 MAPK, ERK, and JNK activation in cardiomyocytes. However, p65 knockdown (Fig. 8*B*; knockdown of p65 was confirmed by Western blotting, Fig. 8*C*) or inhibition of p38 MAPK (Fig. 8*D*), p42/p44 MAPK (ERK; Fig. 8*E*), and JNK (Fig. 8*F*) failed to significantly affect IL-18-mediated protein synthesis in cardiomyocytes (Fig. 8*G*), suggesting that NF-κB, p38 MAPK, ERK, and JNK may not play a significant role in IL-18-mediated cardiomyocyte hypertrophy.

IL-18 Induces ANF Expression and Neonatal Rat Ventricular Myocytes Hypertrophy via Activation of PI3K, Akt, and GATA4 Signaling—In the next series of experiments, we investigated whether IL-18 induces hypertrophy of primary cardiomyocytes. We examined IL-18-mediated hypertrophy of NRVM. We also examined for ANF secretion. Because IL-18 signals via IL-18Rα and -β, we examined their mRNA expression. Northern blot analysis of poly(A)$^+$ RNA isolated from NRVM demonstrated that expression of both subunits of IL-18 receptor at basal conditions indicating that IL-18 signaling is normal in NRVM. We then investigated whether IL-18 induces ANF secretion. Fig. 9*B* shows a significant increase ($p < 0.001$) in ANF levels in culture supernatants following IL-18 treatment. Furthermore, IL-18-mediated ANF secretion was inhibited by wortmannin, Akt inhibitor, Akt siRNA, and GATA4 siRNA (knockdown of Akt and GATA4 was confirmed by Western blotting, Fig. 9*C*), indicating that IL-18 induces ANF expression via PI3K, Akt, and GATA4. Furthermore, IL-18 induced NRVM hypertrophy as evidenced by a significant increase ($p < 0.001$) in protein synthesis (Fig. 9*D*), and pretreatment with wortmannin or transfection with Akt or GATA4 siRNA inhibited IL-18-mediated increases in protein synthesis. Together, our studies demonstrated for the first time the pro-hypertrophic effects of IL-18 in both HL-1 cardiomyocytes and NRVM. Our studies also demonstrated that IL-18 induces cardiomyocyte hypertrophy via PI3K → PDK1 → Akt → GATA4 signaling.

DISCUSSION

Our results indicate for the first time that IL-18 is a pro-hypertrophic cytokine. IL-18 induces cardiomyocyte hypertrophy via activation of PI3K → PDK1 → Akt → GATA4 signaling. IL-18 increases total protein synthesis, phosphorylation of the translational regulatory proteins p70 S6 kinase and ribosomal S6 protein, and increases cell surface area. Also, IL-18 induces promoter activity, mRNA expression, and protein secretion of ANF, a fetal gene re-expressed

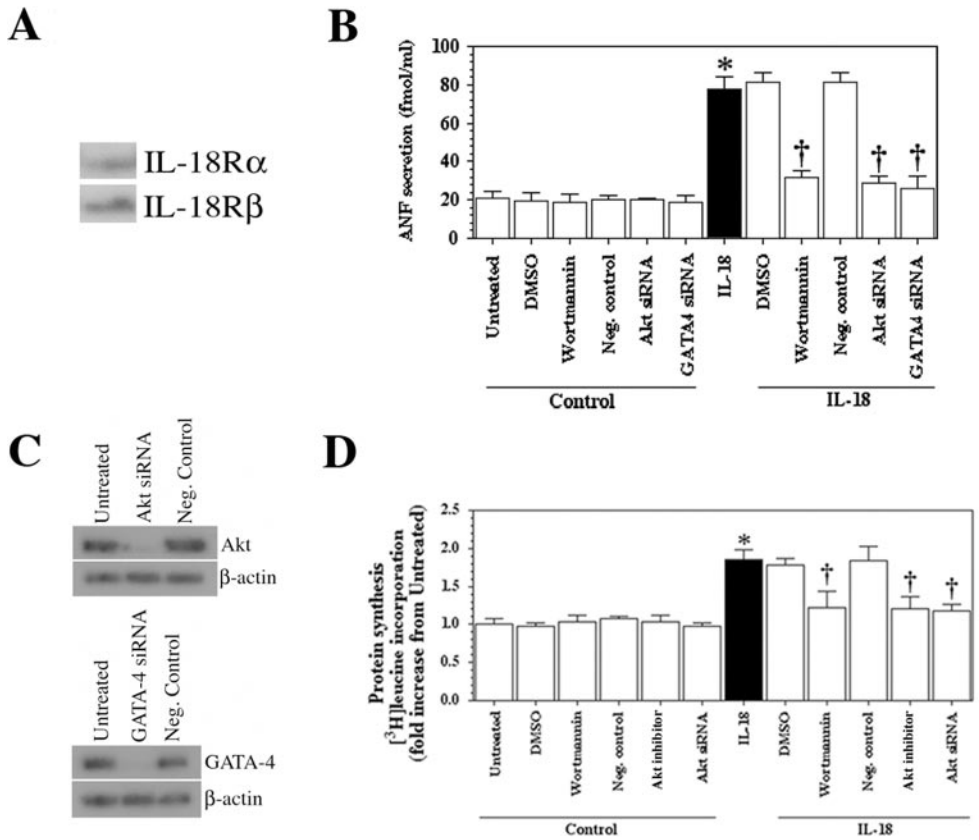

FIG. 9. **IL-18 induces neonatal rat ventricular myocytes hypertrophy via PI3K, Akt, and GATA4 signaling.** *A*, NRVM express IL-18 receptor α and β. IL-18Rα and -β expression was analyzed by Northern blotting using 2 μg of poly(A)$^+$ RNA from quiescent NRVM. *B*, IL-18 increases ANF secretion. Quiescent NRVM were treated with wortmannin (1 h), Akt inhibitor (1 h), or transfected with Akt or GATA4 siRNA (48 h) followed by IL-18 treatment for 24 h. ANF levels in culture supernatants were analyzed by radioimmunoassay. *, $p < 0.001$ *versus* untreated; †, $p < 0.01$ *versus* corresponding controls. *DMSO*, Me_2SO. *C*, knockdown of Akt and GATA4 was confirmed by Western blotting. β-Actin served as a control. *D*, IL-18 increased protein synthesis in NRVM via activation of PI3K and Akt. Quiescent NRVM were treated with IL-18 with and without pretreatment with pharmacological inhibitors or siRNA as described in legend to Fig. 7*E*. *, $p < 0.01$ *versus* untreated; †, $p < 0.05$ *versus* IL-18.

during hypertrophy, via activation of PI3K, Akt, and GATA4 signaling. Furthermore, IL-18 induces hypertrophy of neonatal rat ventricular myocytes via PI3K-dependent signaling. Collectively, these data indicate that IL-18 may play a role in myocardial remodeling and failure, disease states characterized by cardiomyocyte hypertrophy.

IL-18 is a pleiotropic cytokine with proinflammatory and pro-apoptotic properties (14–16). As a proinflammatory cytokine, it induces the expression of IL-1β, tumor necrosis factor-α, and iNOS (14, 15). Both IL-1β and tumor necrosis factor-α act as negative myocardial inotropes, and induction of iNOS and iNOS-mediated nitric oxide generation play a critical role in myocardial dysfunction (57–62). Recently, administration of IL-18 has been shown to induce myocardial contractile function *in vivo* and cardiomyocyte contractility *in vitro* (18), suggesting that IL-18 may play a role in post-ischemic myocardial dysfunction. As a pro-apoptotic cytokine, IL-18 induces cell death in both immune and nonimmune cells via the Fas-Fas-L pathway (63, 64). We have demonstrated recently (16) that IL-18 induces cardiac derived endothelial cell death via activation of both intrinsic and extrinsic pro-apoptotic signaling pathways. However, it is not known whether IL-18 induces cardiomyocyte death. Our present studies demonstrate that treatment with IL-18, at the indicated concentrations, failed to induce cardiomyocyte death as evidenced by low levels of mono- and oligonucleosomal fragmented DNA in the cytoplasmic extracts. In addition, IL-18 induced phosphorylation of Bad, a pro-apoptotic gene product, at Ser^{136}. Phosphorylation of Bad renders it inactive and makes it unavailable to the pro-apoptotic machinery (65, 66). Furthermore, IL-18 induced activation of the pro-survival factor PI3K in cardiomyocytes.

Activation of PI3K plays a critical role in a diverse array of biological responses including cell survival (67). In addition, PI3K has been shown to determine the size of an organ or cell. In transgenic mouse models, cardiac specific overexpression of the constitutively active PI3K has been shown to increase heart size as a result of an increase in cardiomyocyte size (68, 69). In contrast, overexpression of the dominant negative mutant of PI3K that lacks kinase activity reduces heart and cardiomyocyte size, indicating that activation of PI3K plays an important role in determining organ or cell size. Similarly, cardiac specific overexpression of IGFR1 induced myocardial hypertrophy via activation of PI3K, Akt, and p70 S6K (70). In the present study, we demonstrated that IL-18 induces cardiomyocyte hypertrophy via activation of PI3K. Together, these results indicate that activation of PI3K and its downstream signaling molecules play a role in both physiological and pathological hypertrophy.

PI3K is a heterodimer comprising of a catalytic 110-kDa subunit and a regulatory subunit of 85 or 55 kDa. Following activation, PI3K phosphorylates the inositol ring in various phosphatidylinositol phosphates including phosphatidylinositol 4,5-phosphate forming PI3P (71, 72). PI3P in turn binds Akt, resulting in the translocation of Akt from the cytoplasm to the plasma membrane. In addition, binding of PI3P brings about conformational changes in Akt and is activated in a PDK1-dependent manner (73). Results from the present study

indicate that treatment with IL-18 induces Akt phosphorylation and Akt kinase activity, and knockdown of PDK1 inhibits IL-18-mediated Akt activation. Furthermore, inhibition of PDK1 and Akt attenuates IL-18-induced Akt-dependent cardiomyocyte hypertrophy.

Activation of Akt plays an important role in various cellular processes including cell death, survival, proliferation, differentiation, and cell size through activation of diverse downstream signaling pathways (54). Activation of Akt has been shown to promote cell survival in a cell- and stimulus-specific manner. In addition to survival, and similar to PI3K, activation of Akt has been shown to regulate cell size. Transgenic overexpression of constitutively active Akt in a cardiac specific manner increased heart size and cardiomyocyte cell size (74). In these mice, p70 S6 kinase activity was enhanced in heart homogenates indicating activation of the translational machinery. Activation of p70 S6 kinase induces phosphorylation and activation of ribosomal S6 protein that are involved in translation (51, 53). Results from the present study indicate that treatment with IL-18 not only increased p70 S6 kinase activity and phosphorylation, it induced phosphorylation of ribosomal S6 protein at $Ser^{235/236}$ and $Ser^{240/244}$, indicating hyperphosphorylation. These effects were completely blocked by PI3K and Akt inhibition, indicating that IL-18 induces ribosomal S6 protein activation via PI3K and Akt. Increased protein but not DNA synthesis results in increased cell size, and our results clearly indicate that treatment with IL-18 significantly increases protein synthesis and cell surface area. Together, our results indicate that IL-18 induces cardiomyocyte hypertrophy as seen by increases in protein synthesis, the levels of phosphorylated p70 S6 kinase (Thr^{389}) and ribosomal S6 protein ($Ser^{235/236}$, $Ser^{240/244}$), and cell surface area.

Hypertrophy is characterized by the re-expression of various fetal genes including ANF. In fact treatment with IL-18 increased ANF promoter activity, mRNA expression, and protein secretion, effects that were blocked by the inhibition of PI3K, Akt, and GATA4. GATA4 is a zinc finger transcription factor involved in the induction and regulation of various cardiac specific genes including ANF. In the present study, we demonstrate that treatment with IL-18 increases GATA4 DNA binding activity and GATA4-dependent luciferase activity. In addition, treatment with IL-18 increases its cytoplasmic levels, while reducing nuclear levels of GSK3β. GSK3β, a protein kinase involved in various cellular processes including proliferation, has been shown to act as a negative regulator of hypertrophy (56). Phosphorylation of GSK3β at Ser^{9} by PI3K renders it inactive. Recently, Haq *et al.* (75) have demonstrated that transfection with a GSK3β mutant (Ser^{9} to alanine) that fails to phosphorylate in response to hypertrophic stimuli prevented endothelin-1- or phenylephrine-mediated cardiomyocyte hypertrophy by inhibiting nuclear export of the transcription factor nuclear factor of activated T cells. In addition, GSK3β has been shown as a negative regulator of GATA4 in cardiomyocytes (56). It prevented nuclear localization of GATA4 in cardiomyocytes following β-adrenergic stimulation (56). In the present study, we demonstrate that IL-18 blocks nuclear localization of GSK3β in cardiomyocytes while increasing its cytoplasmic levels.

Activation of PI3K has also been shown to activate various transcription factors, including GATA4 and the cardiac homeobox transcription factor Csx/Nkx-2.5, that are involved in cardiomyocyte hypertrophy and differentiation. Recently, Naito *et al.* (76) have demonstrated that specific inhibition of PI3K by LY294002 inhibited early stages of cardiomyocyte differentiation by suppressing Csx/Nkx-2.5 and GATA4 expression. Furthermore, in a transgenic mouse model that overexpresses constitutively active Akt in a cardiac specific manner, Condorelli *et al.* (74) have demonstrated improved myocardial contractile function and increased cardiomyocyte cell size. Myocardial extracts from these mice showed phosphorylation of GSK3β. In addition, GATA4 levels in the nuclei of these mice were increased, suggesting that Akt lies upstream of GATA4, and activation of Akt promotes GATA4 nuclear localization by phosphorylating and inhibiting GSK3β. Similarly, Morisco *et al.* (56) have demonstrated that activation of PI3K and PI3K-dependent Akt kinase activation positively regulate GATA4 transactivation in cardiomyocytes via phosphorylation and inactivation of GSK-3β. In the present study, we demonstrated that treatment with IL-18 stimulated GATA4 DNA binding activity and inhibited nuclear GSK3β levels, and inhibition of PI3K and Akt attenuated IL-18-mediated cardiomyocyte hypertrophy and ANF expression. We also demonstrated that IL-18 activates NF-κB, p38 MAPK, ERK, and JNK in cardiomyocytes, and inhibition of these signaling pathways had minimal effects on IL-18-mediated cardiomyocyte hypertrophy, indicating that PI3K, Akt, and GATA4 signaling may be the predominant signal transduction pathway involved in IL-18-mediated cardiomyocyte hypertrophy. Our studies also demonstrated the pro-hypertrophic effects of IL-18 in neonatal rat ventricular cardiomyocytes. IL-18 induced hypertrophy of NRVM via PI3K, Akt, and GATA4 signaling. Collectively, these data provide the first evidence that IL-18 is a pro-hypertrophic cytokine. IL-18 induces cardiomyocyte hypertrophy via activation of PI3K → PDK1 → Akt → GATA4 signaling and suggests that IL-18 may play a role in inflammatory cardiac diseases and heart failure.

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