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 increased 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). 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 pro-inflammatory 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

1 The abbreviations used are: ANF, atrial natriuretic factor; BSA, bovine serum albumin; DNR, daunorubicin hydrochloride; EMSA, electrophoretic motility-shift assay; ERR, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase-3; IL, interleukin; JNK, C-Jun NH2-terminal kinase; iNOS, inducible form of nitric-oxide synthase; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor κB; NRVM, neonatal rat ventricular myocytes; 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; P13P, 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→PD1K→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 Medium™ (JRH Biosciences, Lenexa, KS) and supplemented with 10% fetal bovine serum (JRH Biosciences), 0.1 mM norepinephrine, 2 mM t-glutamine (Invitrogen), and 1× antibiotic/antimycotic solution (complete media). All culture dishes and flasks were pre-coated with 0.0125% 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% CO2. At 70–80% confluency, the media were refreshed with fresh complete media containing 0.5% BSA. After overnight culture, cells were treated with IL-18 (recombinant mouse IL-18, catalog number B004-5, 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) or normal rabbit IgG (preimmune; R&D Systems) 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 MeSO) or Akt inhibitor (IL-6-hydroxy-methyl-chiro-inositol 2-R(2)-O-methyl-3-O-deacycldecarbonate; catalog number 124005, 1 μM in MeSO) 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 MeSO) 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 cardiac myocyte hypertrophy (37–41), we also examined status following IL-18 treatment, and investigated their role in IL-18-mediated cardiomyocyte hypertrophy. Cardiomyocytes were transfected with p56 siRNA (sense, 5-GCCCCAUCCCCUUAAGCA-3′; 50 nM for 48 h) or treated with p38 MAPK inhibitor (SB203580, 1 μM in MeSO for 30 min), ERK inhibitor (PD98059, 10 μM in MeSO for 1 h), or JNK inhibitor (SP600125, 10 μM in MeSO 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; SignalSilence™ Akt siRNA, targets Akt1 and Akt2; catalog number 6211, Cell Signaling Technology), PD1K (catalog number Q-0049-00-06; Dharmacon, Lafayette, CO; 150 nM), GATA4 siRNA (catalog number Q-004919-00-09; 150 nM), or negative control siRNA (catalog number D-00126-13-05; mixture of the following duplexes that will not target any genes in mammals: sense, 5′-AUGAACGUGAUAAGCUGAAAU; sense, 5′-UAAGGCGAUGAAGAGACUC; sense, 5′-AUGUUAUGGCGUAAUAGCU; sense, 5′-UAGGCAGUCCAAACGUAU; Technical Information, Dharmacon,) using Oligofectamine™ (Invitrogen). After 48 h, cells were treated with IL-18 for 6 additional 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™ 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 [3H]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 [3H]leucine was added to the culture medium, and the incubation was continued for an additional 6 h. The radioactive incorporation 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 protein extracts were prepared from treated cells using a protease inhibitor cocktail and a tissue kit (Qiagen, Valencia, CA). The [3H]leucine incorporation was normalized to DNA, and the ratio of [3H]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 changes in cell surface area, cells were grown on glass cover slips, followed by imaging with the 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 at x20 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′-TCGTTGACTAATACTTAAAAG-3′) from the ANF promoter (44). Double-stranded mutant oligonucleotides (sense, 5′-TCGTTGACTGTATACTTAAAAG-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 ((CTCGTATA)4) using Lipofectamine™ (Invitrogen). Transfected cells were grown to 80% confluency in 24-well plates with the luciferase assay after 24 h. 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 using...
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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 basal 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 [³H]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-³²P]ATP. The phosphorylated substrate was then separated from the residual [γ-³²P]ATP using Sephadex G-50 columns. 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.
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 (Ser473; catalog number 9271), PDK1 (catalog number 3062), glycogen synthase kinase (GSK)-3β (catalog number 9232), p70 S6K (catalog number 9202), phospho-p70 S6K (Thr389; catalog number 9205), ribosomal S6 protein (catalog number 2212), phospho-S6 ribosomal protein (Ser422/423; catalog number 2215), 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. 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.
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 $\gamma$-phosphate of $[^{32}P]ATP$ by S6 kinase.

Neonatal Cardiomyocyte Preparation—In order to confirm the prohypertrophic 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
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 (CCTGTGATA) were cloned upstream of the luciferase (Luc) gene and the TATA box in pLuc-MCS plasmid. IL-18 increased 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 (Luc) 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, Me2SO. B, the autoradiographic signals shown for PI3P in A were quantified, and the means ± S.E. from three
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. 3A). 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. 3B. 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 (Ser473) levels in the cytoplasmic extracts using Western blot analysis. Treatment with IL-18 rapidly induced Akt phosphorylation without modulating total Akt levels (Fig. 3C). Furthermore, IL-18 induced Akt kinase activity (Fig. 3D), 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. 4A, 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. 4A), indicating peak levels of GATA4 activity at 100 ng/ml of IL-18. In addition to the competition studies (Fig. 1A, 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. 4A, 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. 4B, 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. 4C 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. 2D, 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. 4D). Gel supershift assays revealed that the IL-18-mediated increase in GATA DNA binding activity is predominantly due to GATA4 (Fig. 4E). Because GSK3β negatively regulates GATA4 expression in the nucleus (56), we also analyzed GSK3β levels in cytoplasmic and nuclear extracts by Western blotting. Fig. 4F 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 Induces ANF Expression 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 Me2SO, significantly attenuated IL-18-mediated PI3P formation (Fig. 5A; 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. 5C; corresponding densitometric values are shown in D). Furthermore, pretreatment with PI3K and Akt inhibitors attenuated IL-18-mediated GATA4 DNA binding activity (Fig. 5E; corresponding densitometric values are shown in F). Although pretreatment with Me2SO 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. 5G; knockdown of Akt and GATA4 levels was confirmed by Western blotting, Fig. 5H), 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. 6A; corresponding densitometric values are shown in Fig. 6B). Furthermore, inhibition of PI3K, Akt, or GATA4 attenuated IL-18-mediated ANF secretion (Fig. 6C) and ANF promoter activity (Fig. 6D), indicating that IL-18 regulates its transcription and translation.

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 values 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. 28S ribosomal RNA was used as an internal control. 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.
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. 7A 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. 7B), and Akt attenuated IL-18-mediated phosphorylated p70 S6 kinase (Thr^{389}) levels. Similarly, inhibition of PI3K and Akt attenuated IL-18-mediated S6 kinase activity (Fig. 7C) and levels of phosphorylated ribosomal S6 protein (Ser^{235/236} and Ser^{240/244}; Fig. 7D). Furthermore, inhibition of PI3K, PDK1, and Akt attenuated IL-18-mediated protein synthesis (Fig. 7E) and cell size (Fig. 7F), indicating that IL-18 induces cardiomyocyte hypertrophy via activation of PI3K, PDK1, and Akt (Fig. 7G).

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. 8A shows that IL-18 indeed induced activation of NF-κB, p38 MAPK,
IL-18 induces NF-κB, p38 MAPK, ERK, and JNK activation in HL-1 cardiomyocytes. 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 phospho-p38 MAPK (Fig. 8A). 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. 8A). IL-18 also induced ERK activation. Levels of phosphorylated p42 and p44 were increased following IL-18 treatment (Fig. 8A). IL-18 also induced 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. 8B; knockdown of p65 was confirmed by Western blotting; Fig. 8C) or inhibition of p38 MAPK (Fig. 8D; p42/p44 MAPK (ERK); Fig. 8E), and JNK (Fig. 8F) failed to significantly affect IL-18-mediated protein synthesis in cardiomyocytes (Fig. 8G), 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. 8B 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. 8C), 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. 8D), 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...
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-1\(\beta\), tumor necrosis factor\(-\alpha\), and iNOS (14, 15). Both IL-1\(\beta\) and tumor necrosis factor\(-\alpha\) 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 monoand 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 IGFRI 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. As a pro-apoptotic cytokine, it induces the expression of IL-1\(\beta\), tumor necrosis factor\(-\alpha\), and iNOS (14, 15). Both IL-1\(\beta\) and tumor necrosis factor\(-\alpha\) 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 monoand 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.

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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 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 Ser235/236 and Ser240/244, 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 (Thr389) and ribosomal S6 protein (Ser235/236, Ser240/244), 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 GSKβ3 at Ser9 by PI3K and Akt renders it inactive. Recently, Haq et al. (75) have demonstrated that transfection with a GSKβ3 mutant (Ser9 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 ANP 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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