Cardiotrophin-1 Increases Angiotensinogen mRNA in Rat Cardiac Myocytes Through STAT3
An Autocrine Loop for Hypertrophy


Abstract—Cardiotrophin-1, an interleukin-6–related cytokine, stimulates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway and induces cardiac myocyte hypertrophy. In this study, we demonstrate that cardiotrophin-1 induces cardiac myocyte hypertrophy in part by upregulation of a local renin-angiotensin system through the JAK/STAT pathway. We found that cardiotrophin-1 increased angiotensinogen mRNA expression in cardiac myocytes via STAT3 activation. Tyrosine phosphorylation of STAT3 by cardiotrophin-1 treatment resulted in STAT3 homodimer binding to the St-domain in the angiotensinogen gene promoter, which lead to promoter activation in a transient transfection assay. Cardiotrophin-1–induced STAT3 tyrosine phosphorylation and binding to the St-domain were suppressed by AG490, a specific JAK2 inhibitor, which also attenuated cardiotrophin-1–stimulated angiotensinogen promoter activity. Cardiotrophin-1 did not activate the angiotensinogen gene promoter that contained a substitution mutation within the St-domain. Finally, losartan, an angiotensin II type 1 receptor antagonist, significantly attenuated cardiotrophin-1–induced hypertrophy of neonatal rat cardiac myocytes. Angiotensin II is known to induce cardiac myocyte hypertrophy by activating the G-protein–coupled angiotensin II type 1 receptor. Our results suggest that upregulation of angiotensinogen and angiotensin II production contribute to cardiotrophin-1–induced cardiac myocyte hypertrophy and emphasize an important interaction between G-protein–coupled and cytokine receptors. (Hypertension. 2000;35:1191-1196.)

Key Words: angiotensinogen ■ gene expression ■ promoter regions ■ motifs ■ Janus kinases ■ STAT pathway ■ cardiac myocyte

Cardiotrophin-1 (CT-1), a newly isolated member of the interleukin (IL)-6–related cytokine family, which includes IL-6 and leukemia inhibitory factor (LIF), is a potent inducer of cardiac myocyte hypertrophy and gene expression.1,2 CT-1, via coupling through the LIF receptor and gp130, has been shown to activate a number of signaling pathways in cardiac myocytes, including mitogen-activated protein kinases and the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway.3,4 On tyrosine phosphorylation by JAK, STAT proteins undergo dimerization, translocate to the nucleus, and activate expression of target genes.5 Recent reports implicate the JAK/STAT pathway in cardiac hypertrophy: 2 potent hypertrophic stimuli, acute pressure overload and mechanical stretch, activate the JAK/STAT pathway in cardiac myocytes: 2 potent hypertrophic stimuli, acute pressure overload and mechanical stretch, activate the JAK/STAT pathway in cardiac myocytes,6–8 and nuclear extracts from hypertrophied hearts of genetically hypertensive SHR rats exhibit enhanced STAT binding activity compared with extracts of aged-matched normotensive Wistar-Kyoto rats.9 Moreover, LIF was shown to induce cardiac myocyte hypertrophy through the JAK/STAT pathway.10,11 LIF-induced cardiac myocyte hypertrophy and expression of c-fos and atrial natriuretic factor mRNA were amplified by STAT3 overexpression and attenuated by a STAT3-dominant negative mutant.12 However, the process by which the JAK/STAT signaling pathway couples to hypertrophic growth in cardiac myocytes has not been defined.

In the present study, we hypothesized that CT-1 activation of the JAK/STAT pathway couples to hypertrophic growth of cardiac myocytes through upregulation of angiotensinogen (Ao) gene expression. This hypothesis is based on the recent observation that the Ao gene promoter is activated by STAT3 and STAT6 proteins.9 The activated STAT proteins bind to a motif, denoted the St-domain, in the Ao gene promoter, and STAT3 and STAT6 proteins have recently been implicated in angiotensin (Ang) II–induced Ao gene expression in neonatal Wistar-Kyoto rats.9

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1191
rat cardiac myocytes.9 Local production of Ang II could thus contribute to CT-1–induced hypertrophic growth of cardiac myocytes. The feasibility of this hypothesis is supported by the observation that autocrine release of Ang II is involved in stretch-induced JAK/STAT activation and hypertrophy of cardiac myocytes.7,13,14

**Methods**

**Materials**

Reagents for tissue culture were obtained from Gibco BRL. STAT antibodies and protein A/G agarose were from Santa Cruz Biotechnology. STAT1 (phosphotyrosine [p-Tyr]701) and STAT3 (p-Tyr705) phosphospecific antibodies were from Quality Controlled Biochemicals. Antiphosphotyrosine (4G10) and STAT5 (p-Tyr92), phosphospecific antibodies were from Upstate Biotechnology. Human CT-1 was from PeproTech (Notwood, Mass). AG490 was from BioMol, and losartan and PD123319 were from Sigma Chemical Co. [3H]-Leucine, [32P]-ATP, chemiluminescence reagents, and nitrocellulose membranes were from NEN Life Science. Poly(dI-dC) was from Pharmacia Biotech. T4 polynucleotide kinase and reporter lysis buffer were from Promega. RNAzol B was purchased from Tel Test. BioMol, and losartan and PD123319 were from Sigma Chemical Co. [3H]-Leucine, [32P]-ATP, chemiluminescence reagents, and nitrocellulose membranes were from NEN Life Science. Poly(dI-dC) was from Pharmacia Biotech. T4 polynucleotide kinase and reporter lysis buffer were from Promega. RNAzol B was purchased from Tel Test. Effectene was from Qiagen. Plasmids that contained a wild-type or mutant St-domain of the Ao gene promoter, ligated to a luciferase reporter gene, were a gift of Drs Eduardo M. Mascareno and M.A.Q. Siddiqui (State University of New York Health Science Center at Brooklyn).

**Cardiac Myocyte Isolation**

Ventricular cardiac myocytes were isolated from neonatal Sprague-Dawley rat hearts as described15 and plated at 0.35×10⁶ cells/mm². After 24 hours, the medium was changed to serum-free DMEM/F-12 with 100 μmol/L ascorbic acid and 68 U/L insulin. Twenty-four hours before an experiment, cells were given the same medium without ascorbic acid or insulin.

**Quantification of mRNA, Reporter Plasmids, and Transient Transfection Assay**

Cardiac myocytes were treated for 4 hours with CT-1 (1 nmol/L), and RNA was extracted with RNAzol B. Ao, renin, angiotensin II type 1 (AT1) receptor, and elongation factor-1 (MSTANGLuc, Figure 2a). Pretreatment of cells for 30 minutes with a plasmid that contained a mutant St-domain increase luciferase activity in cardiac myocytes transfected with a plasmid that contained the St-domain (STANGLuc) of the Ao gene promoter linked to a luciferase reporter gene. Stimulation with CT-1 (1 nmol/L) for 1, 2, or 4 hours increased luciferase activity by 127.4%, 85.1%, and 33.6%, respectively (Figure 2a). CT-1 stimulation did not increase luciferase activity in cardiac myocytes transfected with a plasmid that contained a mutant St-domain (MSTANGLuc, Figure 2a). Pretreatment of cells for 30 minutes with AG490, a specific JAK2 inhibitor, inhibited CT-1–stimulation of promoter activity of STANGLuc in a concentration-dependent manner (Figure 2b).

**Immunoprecipitation and Immunoblotting**

Immunoprecipitation of STAT6 was performed with 500 μg of cell lysate.17 To measure STAT protein tyrosine phosphorylation, immunoprecipitated samples (for STAT6) or 5 μg of cell lysate (for STATs 1, 3, and 5) were mixed with 4× sample buffer (5% SDS, 500 mmol/L, Tris-HCl [pH 7.5], 50% glycerol, and 0.25% bromophenol blue) and boiled 5 minutes. Samples were subjected to 8% SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes, which were incubated with a pTYR-specific STAT antibody (STATs 1, 3, and 5) or an anti-pTYR antibody (STAT6). After incubation with secondary antibody, immunoreactive bands were visualized by enhanced chemiluminescence.

**Preparation of Nuclear Extracts, and Electrophoretic Mobility Shift and Supershift Assays**

Previously described methods were used to prepare nuclear extracts and perform the electrophoretic mobility shift assay.17 Sequences and end labeling of oligonucleotides have been described.7,9,17 For supershift assays, STAT antibodies (1 μg) were incubated 30 minutes with DNA-protein complexes at 4°C. Reactions were analyzed by native 4% PAGE.7 Gels were dried and visualized by autoradiography or phosphorimage analysis.7

**Measurement of Cardiac Myocyte Hypertrophy**

Cardiac myocyte hypertrophy was evaluated by [3H]-leucine incorporation (index of protein synthesis) and by total cellular protein normalized to DNA (index of cell size).15 Cardiac myocytes were labeled with 1.0 μCi/mL [3H]-leucine for 4 hours after 20 hours of treatment with CT-1 (1 nmol/L). Cells were pretreated with losartan (1 μmol/L) or PD123319 (10 μmol/L) for 30 minutes before CT-1 treatment. Protein and DNA contents were measured as described.15 In these experiments, cells were treated with CT-1, in the presence or absence of losartan (1 μmol/L), for 24, 48, or 72 hours.

**Statistics**

Results were expressed as mean±SEM. Differences among groups were assessed by 1-way ANOVA followed by the Dunnet multiple comparison test. P≤0.05 was considered statistically significant.

**Results**

**CT-1 Increases Ao mRNA Levels and Promoter Activity**

CT-1 (1 nmol/L) increased Ao mRNA at 4 hours by 220% (Figure 1a and 1c). Levels of mRNA for renin and the AT1 receptor were not increased (Figure 1a through 1c). To determine whether CT-1 increased Ao gene promoter activity through the St-domain, cardiac myocytes were transiently transfected with a plasmid that contained the St-domain (STANGLuc) of the Ao gene promoter linked to a luciferase reporter gene. Stimulation with CT-1 (1 nmol/L) for 1, 2, or 4 hours increased luciferase activity by 127.4%, 85.1%, and 33.6%, respectively (Figure 2a). CT-1 stimulation did not increase luciferase activity in cardiac myocytes transfected with a plasmid that contained a mutant St-domain (MSTANGLuc, Figure 2a). Pretreatment of cells for 30 minutes with AG490, a specific JAK2 inhibitor, inhibited CT-1–stimulation of promoter activity of STANGLuc in a concentration-dependent manner (Figure 2b).

**CT-1 Induces Tyrosine Phosphorylation of STAT Proteins in Cardiac Myocytes**

The time course for CT-1–induced tyrosine phosphorylation of various STAT proteins was determined by Western blot analysis (Figure 3). As reported by others,3 we observed that CT-1 induced tyrosine phosphorylation of STAT3 (Figure 3b). In addition, STAT1 and STAT5 were tyrosine phosphorylated (Figures 3a and 3c). Detectable phosphorylation of STATs 1, 3, and 5 occurred in 2 to 5 minutes and was maximal at 10 to 15 minutes. CT-1 did not induce STAT6 tyrosine phosphorylation (Figure 3d).

**CT-1 Enhances STAT3 Binding Activity to the St-Domain of Cardiac Myocytes**

Because CT-1 induced the tyrosine phosphorylation of STATs 1, 3, and 5, binding activity of these STAT proteins to the St-domain of the Ao promoter was evaluated. Nuclear extracts from CT-1–treated cardiac myocytes exhibited enhanced binding to the St-domain in a time- (Figure 4a) and concentration- (Figure 4b) dependent manner. Specificity of
binding to the St-domain was confirmed with a mutant probe and by adding excess a wild-type or a mutant St-domain oligonucleotide (Figure 4c). Supershift assay was used to identify the STAT proteins in nuclear extracts that exhibited enhanced binding to the St-domain (Figure 4d). Only STAT3 homodimer exhibited enhanced binding to the St-domain, and thus CT-1–induced tyrosine phosphorylation of STAT3 was further characterized. As shown in Figure 4f, CT-1 induced STAT3 tyrosine phosphorylation in a concentration-dependent manner. AG490 inhibited CT-1–induced tyrosine phosphorylation of STAT3 and inhibited CT-1–induced binding activity of the STAT3 homodimer to the St-domain (Figure 4e).

**AT1 Receptor Antagonist Inhibits CT-1–Induced Hypertrophy of Cardiac Myocytes**

CT-1 (1 nmol/L) induced hypertrophy of cardiac myocytes as indexed by [3 H]-leucine incorporation, protein content, and the protein-to-DNA ratio (Figure 5). The selective AT1 receptor antagonist losartan (1 μmol/L) significantly reduced CT-1–stimulated increases in these indices of cardiac hypertrophy (Figure 5). The Ang II type 2 receptor antagonist PD123319 (10 μmol/L) had no effect on the rate of acceler-
IL-6 receptors were overexpressed, resulted in cardiac hypertrophy. However, the downstream molecular mechanisms of CT-1– or gp130-induced cardiac myocyte hypertrophy remain to be elucidated. Our results describe a novel autocrine interaction between Ang II (G-protein–coupled receptor) and CT-1 (LIF receptor/gp 130) that affects cardiac myocyte growth. We found that CT-1 stimulated Ao gene expression by STAT3 activation and hypertrophy of neonatal rat ventricular myocytes, in part, by production of Ang II.

To the best of our knowledge, this is the first report of a cytokine-inducing Ao gene upregulation through the JAK/STAT pathway. Recently, Ang II was shown to stimulate Ao expression in cardiac myocytes by activation of STAT3 and STAT6. STAT proteins mediated this response through binding to the St-domain (bases −160 to −175) in the promoter of the rat Ao gene. The St-domain CTTCCTGGAG-5' shares similarity with the consensus TTNCNNAAA sequence that binds STAT proteins. In transient transfection experiments, we observed that CT-1 transactivated the St-domain of the Ao gene promoter. Even though STAT1, STAT3, and STAT5 were tyrosine phosphorylated by CT-1 treatment of cardiac myocytes, only STAT3 homodimer bound the St-domain of the Ao promoter, as determined with an electromobility shift assay. We found that CT-1 induced activation of STAT3 and Ao promoter activity coupled through Jak2. AG490 blocked CT-1 induced tyrosine phosphorylation of STAT3, binding activity of STAT3 homodimer to the St-domain, and Ao promoter activity in cardiac myocytes. CT-1 caused hypertrophic growth of neonatal rat cardiac myocytes, as indexed by increases in [3H]-leucine (Leu) incorporation, protein content, and protein-to-DNA ratio. AT1 receptor antagonist (losartan) inhibits CT-1–induced cardiac myocyte hypertrophy. CT-1 (1 nmol/L) inhibited CT-1–induced hypertrophy of neonatal rat ventricular myocytes, as indexed by increases in [3H]-leucine (Leu) incorporation, protein content, and protein-to-DNA ratio.
CT-1–Activated Ao Induction by JAK/STAT

Cardiac fibroblasts were recently shown to express 3.5 times more levels of CT-1 mRNA than cardiac myocytes, although increased Ang II was shown to couple to activation of phosphatidylinositol 3-kinase, which has been linked to increased protein synthesis in cardiac myocytes. In addition, gp130 was shown to couple to activation of phosphatidylinositol 3-kinase, which has been linked to increased protein synthesis in cardiac myocytes. It is also important to note that autocrine production of Ang II is unlikely to fully explain the consequences of STAT3 activation on cardiac myocytes. Recent evidence indicates that gp130, and specifically STAT3, are linked to a survival or antiapoptotic pathway in cardiac myocytes that has been postulated to be important in preventing the transition from compensatory hypertrophy to heart failure.

In summary, we demonstrate that CT-1 induces cardiac myocyte hypertrophy, in part, by upregulation of Ao mRNA expression in cardiac myocytes, via STAT3 binding to the St-domain of the Ao gene promoter. CT-1–induced STAT3 tyrosine phosphorylation was mediated by JAK2. Moreover, an AT1 receptor antagonist attenuated CT-1–induced hypertrophy of neonatal rat cardiac myocytes, suggesting that upregulation of Ao and Ang II production contribute to CT-1–induced cardiac myocyte hypertrophy. These findings emphasize the importance of an interaction between G-protein–coupled and cytokine receptors in the mediation of cardiac myocyte growth.

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