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

Cooper Lecture

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


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,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...
shortens cardiac myocytes. 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-Tyr694) phosphospecific antibodies were from Upstate Biotechnology. Human cardiac myocytes were a gift of Drs Eduardo M. Mascareno and M.A.Q. Dawley rat hearts as described15 and plated at 0.35 g of cell lysate (for g) 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

**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 in a concentration-dependent manner (Figure 4g) 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 [3H]-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-
gp130, with a transgenic approach in which IL-6 and soluble

Discussion

The IL-6–related cytokine CT-1 is a potent inducer of cardiac myocyte hypertrophy and has been shown to activate the JAK/STAT pathway via the intermediate transmembrane signaling glycoprotein gp130. Continuous stimulation of gp130, with a transgenic approach in which IL-6 and soluble 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 TTTCGGAGG shares similarity with the consensus TTNCCNNAA 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 electrophoretic mobility 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. Hypertrophy was markedly attenuated by losartan, an AT1 receptor antagonist. This finding indicates that CT-1–enhanced Ang II production induced cardiac myocyte hypertrophy via an autocrine mechanism. Ang II is

Figure 4. STAT3 binds to the St-domain of the Ao gene promoter, and JAK2 is required for CT-1–induced STAT3 tyrosine phosphorylation and binding to the St-domain. Nuclear extracts were analyzed by electrophoretic mobility shift assay (a through e) for binding activity to the St-domain. (a) Time course for CT-1–induced (1 nmol/L) nuclear St-domain binding activity. (b) Concentration dependency determined at 15 minutes. (c) Specificity of binding to the St-domain verified by adding excess (100×) nonlabeled St-domain oligonucleotide (lane 5) and nonlabeled mutant St-domain (lane 6). Cells were stimulated for 15 minutes with 1 nmol/L CT-1. Results from 32P-labeled mutant St-domain (Probe M) also confirmed specificity (lanes 2 and 4). St indicates St-domain; M, mutant St-domain; and Comp., competitor. (d) Composition of CT-1–induced nuclear complex determined by a supershift assay. Nuclear extracts were prepared 15 minutes after treatment with 1 nmol/L CT-1. Only STAT3 antibody (lane 4) supershifted (S.S.) binding to the St-domain (bases −160 to −175) in the promoter of the rat Ao gene. The St-domain TTTCGGAGG shares similarity with the consensus TTNCCNNAA 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 electrophoretic mobility 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 protein synthesis, cellular protein content, and the protein-to-DNA ratio. Hypertrophy was markedly attenuated by losartan, an AT1 receptor antagonist. This finding indicates that CT-1–enhanced Ang II production induced cardiac myocyte hypertrophy via an autocrine mechanism. Ang II is
known to induce cardiac myocyte hypertrophy via the AT₁ receptor. Autocrine mechanisms have also been implicated in the hypertrophic actions of several agonists on cardiac myocytes, including Ang II and α-adrenergic. Moreover, the hypertrophic response of cardiac myocytes to uniaxial stretch, in vitro, is mediated by the release of endothelin and/or Ang II. Similarly, in vivo pressure overload results in increased STAT activity and Ao gene expression. Combined, these data suggest a critical role for autocrine mechanisms, including the JAK/STAT and cardiac RAS pathways, in the mediation of experimental and pathological cardiac hypertrophy.

Although CT-1 increased Ao mRNA levels in cardiac myocytes, and AT₁ receptor antagonist inhibited CT-1–induced cardiac myocyte hypertrophy, we were unable to detect an increase in Ang II levels (by ELISA, pmol/10 mL per 10×10⁶ cells) in medium of cells treated with CT-1 for 24 hours (0.75±0.19 control versus 0.76±0.24 CT-1 treatment) or for 48 hours (1.60±0.73 control versus 1.65±0.77 CT-1 treatment). A similar observation was made for the mechanical stretch-induced hypertrophy of neonatal rat cardiac myocytes. In this model, it was shown that an AT₁ receptor antagonist inhibited hypertrophy, although increased Ang II medium levels could not be detected by radioimmunoassay. Our inability (and others) to detect a change in Ang II levels in the medium is likely due to dilutional effects, because levels of Ang II at the plasma membrane should be substantially higher. The inhibitory effect of losartan on CT-1–induced hypertrophy strongly supports a mediating role for Ang II.

Evidence indicates that CT-1 has an important role in the process of ventricular remodelling. A significant elevation of plasma CT-1 was recently demonstrated in patients with heart failure. In addition, a heart-specific increase in CT-1 gene expression was found to precede the establishment of ventricular hypertrophy in genetically hypertensive rats. Interestingly, long-term treatment of these animals with the ACE inhibitor lisinopril prevented the development of left ventricular hypertrophy, without affecting ventricular CT-1 mRNA levels. This latter finding is consistent with our model that the hypertrophic actions of CT-1 are mediated in part through upregulation of cardiac Ao gene expression and Ang II production. Factors that upregulate cardiac CT-1 production have not been defined. Cardiac fibroblasts were recently shown to express 3.5 times more levels of CT-1 mRNA than cardiac myocytes. These investigators also found that CT-1 antibody significantly inhibited the increased gene expression and protein synthesis that is characteristic of hypertrophic growth of cardiac myocytes in coculture with cardiac fibroblasts. Release of CT-1 from cardiac fibroblasts may explain the recent observation that conditioned medium of cardiac fibroblasts elicits an increase in Ao mRNA levels of ventricular myocytes. Thus, CT-1 probably represents an important paracrine factor in the heart, affecting cardiac myocyte growth through production of cardiac Ang II.

In the present study, ~40% of the hypertrophic action of CT-1 could not be blocked by an AT₁ receptor antagonist. We would not have anticipated that all of the hypertrophic actions of CT-1 would be mediated by autocrine production of Ang II, because CT-1 and Ang II induce different patterns of hypertrophic growth 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. How the cardiac RAS interdigitates with the gp130/STAT3 survival or antiapoptotic pathway awaits to be defined.

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.

Acknowledgments

This study was supported by grants from the National Institutes of Health (HL-44883, HL-58439, and HL-60529 to K.M.B.). Dr Baker is an Established Investigator of the American Heart Association. The expert technical assistance of Anna M. Kempinski (Pennsylvania State University College of Medicine) was greatly appreciated.

References


Cardiotrophin-1 Increases Angiotensinogen mRNA in Rat Cardiac Myocytes Through STAT3: An Autocrine Loop for Hypertrophy
Jun Fukuzawa, George W. Booz, Rachel A. Hunt, Noriko Shimizu, Vijaya Karoor, Kenneth M. Baker and David E. Dostal

Hypertension. 2000;35:1191-1196
doi: 10.1161/01.HYP.35.6.1191

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/35/6/1191

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/